A COMPREHENSIVE EVALUATION OF STRESS IN HORSES DURING THERAPEUTIC RIDING SESSIONS

By

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Abstract

In order to conduct a comprehensive evaluation of stress in horses during therapeutic riding sessions, both observable behavioural indicators and physiological indicators of stress (heart rate variability measures and salivary cortisol concentrations) were obtained. Four Joyriders therapeutic riding horses participated in an 8-week program to provide equine therapy for riders with developmental, physical, cognitive and psychosocial disabilities. The horses also participated in 4 control sessions where they were ridden by experienced riders, without disabilities. The behavioural indicators and heart rate variability were measured across 3 activities including baseline (in their stall), mounting and dismounting. To measure salivary cortisol concentrations, a saliva sample was collected from each horse following 2 activities (baseline and dismount). In both sessions, mounting induced a significantly higher mean number of stress-related behaviours across the 3 activities, although there was no significant difference between the therapeutic riding and control sessions. Similarly, the heart rate parameter LF/HF ratio showed significant increases during the activity of mounting the horse. As well, the time domain variables MnHR and RMSSD demonstrated significant increases during the control sessions and baseline activity. There was no significant difference in cortisol levels between the sessions, however the concentrations in 2 horses' increased significantly following the dismount of the rider. The results show that mounting induces stress during riding and this stress was greater during therapeutic riding sessions. In addition, the sympathetic nervous system increased during the therapeutic riding sessions indicating physiological stress, which was a higher change than that observed in the control sessions.

Acknowledgements

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List of Abbreviations

ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of Variance
CanTRA	Canadian Therapeutic Riding Association
CRH	Corticotropin-releasing hormone
CV	Coefficient of Variation
EAT	Equine-Assisted Therapy
ELISA	Enzyme-linked Immunosorbent Assay
HRV	Heart Rate Variability
HPA-axis	Hypothalamic-pituitary-adrenal axis
IBI	Inter-beat-interval
LF/HF	Low Frequency to High Frequency Ratio
MnHR	Mean Heart Rate
RMSSD	Root Mean Square of Successive RR Interval Differences
SAM	Sympathetic-adrenal medulla
SAS®	Statistical Analysis Software
SNS	Sympathetic Nervous System
THR	Therapeutic Horseback Riding
TRS	Therapeutic Riding Sessions

INTRODUCTION

Animal-assisted therapy is an alternative type of therapy of which the treatment process involves the use of animals, and previous research has shown significant improvements in the patients' social, emotional and cognitive functioning (Johnson *et al.* 2017). In this field of therapy, the use of horses in therapeutic horseback riding (THR) programs, as a form of Equine-Assisted Therapy (EAT), has become increasingly common in recent years (Gehrke *et al.* 2011). The process of THR involves the use of horseback riding as a therapeutic or rehabilitative treatment aimed at improving the patient's coordination, balance and/or strength (Gehrke *et al.* 2011). Heightened feelings of confidence and self-esteem, as well as improvements in basic riding skills, are observed in patients as a result of the interaction between the rider and the horse (Fazio *et al.* 2013).

Although there has been substantial research conducted regarding the cognitive and physical benefits of therapeutic riding programs in patients, the documented research pertaining to the positive and negative consequences of therapeutic riding sessions (TRS) on the horses involved is limited (Gehrke *et al.* 2011). As the utilization of EAT has advanced, the expectations regarding the horse's cognitive and physical abilities have increased, leading to rising demands on the animal's ability to perform faster and more efficiently in tasks such as memorization, compared to other domestic horses (Mengoli *et al.* 2014).

In recent years, the interest in animal welfare has amplified, shifting focus towards the positive and negative impacts of TRS on the horse's behaviour, physiology and their exposure to stress (Kaiser *et al.* 2006). Equine welfare can be impacted by a variety of factors including environmental conditions such as housing, diet, interactions with other horses and therapeutic riders, and the activities experienced throughout the therapy sessions (Munsters *et al.* 2012).

The main goal of this study is to assess whether horses used in therapeutic riding programs experience stress during their sessions with patients. As the horses participating in THR are deliberately chosen based on their easygoing temperament and are specially trained by instructors to participate in the therapeutic riding activities, they may be less inclined to display obvious responses to stress (Fazio *et al.* 2013). Behaviours determined to be indicators of stress are observable through physical movements of the body. The current challenges regarding the assessment of stress in horses involves the physiological mechanisms within the neuroendocrine and cardiovascular system, which function simultaneously to produce the somatomotor behavioural stress responses. As both systems function together, pinpointing the stimulus which induced the behavioural response to stress is difficult (Rietmann *et al.* 2004).

This study monitored for changes in a number of physiological biomarkers and behavioural indicators of stress, used to obtain a more inclusive assessment of stress in horses used in therapeutic riding. Particularly, this study evaluated the horses working in the Joyriders therapeutic riding program located on Prince Edward Island. In the case that the Joyriders therapeutic riding horses experience stress during TRS, it may bring about interventions aimed at decreasing stress while improving equine welfare and allowing the horses to continue engaging in THR. As well, the methods can be replicated in horses

working in a variety of other THR programs as a comprehensive evaluation of stress.

LITERATURE REVIEW

Therapeutic Horseback Riding

Following the recent shift in focus towards equine welfare in therapeutic riding practices, there has been increased interest in the positive and negative impacts of therapeutic horseback riding (THR) on horses (Rietmann *et al.* 2004). THR programs are specialized for individuals with disabilities, as a form of therapeutic treatment, rehabilitation or education, through the acquisition of horsemanship skills. The riders acquire a broad range of abilities which incorporate social interaction with basic riding skills, ranging from mounting and dismounting the horse, to independently leading the horse through a variety of activities (Fazio *et al.* 2013). Studies have revealed a multitude of benefits resulting from THR in therapeutic riders with developmental, physical, cognitive and psychosocial disabilities (Johnson *et al.* 2017). In some cases, neuromuscular stimulation in response to the equine gait and the sensation of the horse's stride has been revealed to increase core strength, balance and coordination in therapeutic riders (Johnson *et al.* 2018). Alternative studies have also documented the benefits of THR on patients treated for autism spectrum disorder, specifically through improvements in social skills, communication and repetitive behaviours. In this case, the human-animal interaction acquired in THR has been shown to facilitate the development of social behaviours extending from eye contact to verbal communication (Malcom *et al.* 2017). The human-animal interaction gained in therapeutic horseback riding sessions has also been reported to reduce the release of stress hormones, blood pressure and heart rate, in

both the horse and therapeutic rider (Johnson *et al.* 2011).

Stress is defined as a stimulus which triggers a cascade effect as the body attempt to re-establish its original homeostatic balance, using adaptive stress-responses (Reitmann 2004). An example of a possible stressor experienced by the horses involved in TRS includes balance issues exhibited by the rider, as some riders have physical disabilities, which can challenge the physiological capabilities of the horses and cause distress (Kaiser *et al.* 2006).

Furthermore, some riders with cognitive and psychosocial disabilities may express emotions of frustration or agitations during the session, triggering stress responses in the horses (Johnson *et al.* 2012). Novice riders are also less capable of controlling the emotions they emit, such as nervousness, which can be transferred to the horse through the human-animal interaction. This concept was tested by simultaneously monitoring variations in the heart rate of both the horse and the therapeutic rider, throughout a variety of activities which were determined to be correlated (Keeling *et al.* 2009). Riders are also taught skills in how to cooperate with the horse, which is an ability that has been shown to influence the stress responses expressed by horses (Munsters *et al.* 2011).

In terms of research documenting the existence of stress in horses used in nontherapeutic horseback riding styles, a study conducted on dressage horses found that stress was present following the riding session (Christensen *et al.* 2014). Alternative studies also established that the activity of trailering induced heightened stress responses in the horses involved, through the analysis of serum cortisol concentrations (Tateo *et al.* 2012). In order to conduct a more comprehensive assessment of stress in horses during TRS, as opposed to using a single measure of stress, this study will focus on both the

physiological and behavioural indicators of stress expressed by the horses (Young *et al.* 2012).

Stress in Horses

Stress is the body's physiological response to an internal or external stimulus, triggered by the sympathetic branch of the autonomic nervous system to regain the bodies original homeostatic balance (Ishizaka *et al.* 2017). If the central nervous system deems the stimulus to be a stressor, it will invoke both behavioural and physiological reactions within the body, which can be measured and used as indicators of stress (Rietmann *et al.* 2004). An example of a behavioural stress-response observed in horses would include aggressive behaviours, muscles spasms and signs of anxiety or nervousness (Young *et al.* 2012). An example of a physiological biomarker of stress consists of increased heart rate, pupil dilation or cortisol concentrations, induced by the fight or flight response. This physiological response of the sympathetic nervous system (SNS) is primarily activated by an acute stressor. As well as the release of hormones including cortisol and catecholamine's, such as adrenaline and noradrenaline (König *et al.* 2017).

These physiological stress-responses are regulated by the activation of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic-adrenal medulla (SAM). When presented with an acute stressor, the paraventricular nucleus of the hypothalamus secretes the peptide hormone called corticotropin-releasing hormone (CRH) (Kalman and Grahn 2004). CRH functions in binding to corticotropic cells within the anterior pituitary to produce adrenocorticotropic hormone (ACTH), later released into the bloodstream (Kalman and Grahn 2004). The HPA-axis utilizes ACTH to stimulate the secretion of the cortisol, a steroid hormone, from the adrenal glands. Cortisol is then circulated

throughout the bloodstream, measurable in the blood serum concentrations, saliva and feces of horses (Pawluski *et al.* 2017). The SAM pathway works simultaneously to produce epinephrine and norepinephrine in the regulation of heart rate (Kalman and Grahn 2004). When evaluating stress, it is difficult to distinguish the origin of the stressor, as the physiological stress-responses by the HPA-axis and SAM pathway can be invoked by a variety of stimuli (König *et al.* 2017).

In horses, when exposed to chronic stress, the body's adaptive physiological stress-responses can have a negative influence on the functioning of their immune system and overall health (Keadle *et al.* 1993). As previously stated, the autonomic nervous system regulates the release of catecholamines (adrenaline and noradrenaline) in response to a stressor (Kalman and Grahn 2004). Adrenaline is a steroid hormone used to increase blood circulation and the metabolism of carbohydrates within the body. Specifically, stimulating the catabolic process of glycogenolysis in order to break down glycogen stored within the liver and skeletal muscle, generating pyruvic acid and energy (Ishizaka *et al.* 2017). Although this process provides the muscles with the energy required to cope with the stressor, the long-term elevation of cortisol concentrations and blood glucose can result in high blood sugar (König *et al.* 2017). In horses, cases of chronic stress have shown how fluctuations in blood glucose can lead to immunosuppression as well as inflammatory responses. Once immunocompromised, it increases the likelihood of the horse developing life-threatening diseases such as colic and gastric ulcers (Johnson *et al.* 2017).

Heart Rate Variability

When assessing stress in horses, there are a number of physiological and behavioural indicators that can be used to evaluate their responses. In horses, 2 of the

physiological biomarkers for stress include heart rate variability (HRV) measures and salivary cortisol concentrations (König *et al.* 2017). Heart rate variability is a noninvasive measurement of stress which monitors for variations in the time between heartbeats, also known as the inter-beat-interval (IBI) (McKinney 2015). The sinus node, or natural pacemaker, is located within the right atrium of the heart and transmits electrical impulses throughout the muscle fibres, in order to coordinate the rhythmic contractions of the heart muscle. This coordination of cardiovascular function is regulated by the parasympathetic and sympathetic branches of the autonomic nervous system (Gehrke *et al.* 2011). When exposed to an environmental stressor, the SNS increases heart rate and blood pressure through the released adrenaline and noradrenaline from the adrenal medulla. Regulated by the SAM pathway, this response utilizes adrenaline to increase the speed at which the heart muscles contract. Simultaneously, the SAM pathway uses noradrenaline to induce vasoconstriction of the blood vessels (König *et al.* 2017). This process results in an increased vascular resistance in an attempt to circulate a sufficient volume of blood throughout the body, causing higher blood pressure and heart rate. However, as heart rate increases in response to the stressor, heart rate variability decreases due to the fluctuations in the sympathetic activity (Ishizaka *et al.* 2017).

Through the evaluation of heart rate variability measures, assessments can be conducted regarding the stress-related responses displayed by horses' during TRS (König *et al.* 2017). Although studies have demonstrated the reliability and repeatability of heart rate variability measures when evaluating stress in horses, the results can be easily influenced by a variety of factors. These influential factors can include the duration and intensity of exercise, changes in temperature, arousal, diet, exposure to stressors and other environmental changes (König *et al.* 2017). Regardless, heart rate variability measures

remain the most precise physiological parameter used in the evaluation of stress in horses, specifically as a biomarker for increased sympathetic activity or reduced vagal activity (McKinney 2015). The Polar® Equine H7 Heart Rate Sensor Belt used to collect HRV data, can be worn comfortably under the saddle and does not induce stress in the horses (Grehrke 2011) (Figure 1). A previous study conducted using heart rate variability as a measure of stress in horses also used a Polar® Equine Heart Rate Sensor Belt, which was applied around the horse's girth, directly underneath the saddle. The results of this study were successful in obtaining reliable HRV measures, using the Polar® Equine Hear Rate Belt, without influencing the stress responses of the horses (Keeling *et al.* 2009).

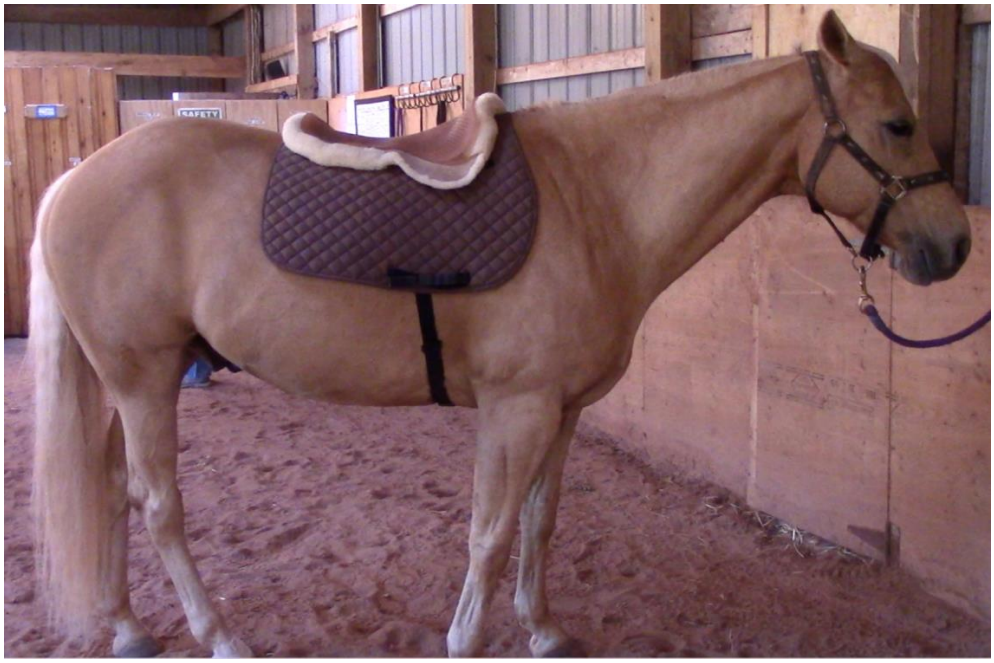

Figure 1. Photograph showing placement of the Polar® Equine H7 Heart Rate Sensor Belt, applied around the girth of the horse, directly under the saddle.

The heart rate variability measures chosen for analysis using the Kubios HRV Standard® software included both time and frequency domain variables. Time domain variables are used to quantify the measure of variability in the time between heartbeats or inter-beat-interval (Shaffer and Ginsberg 2017). The IBI is measured as the time between the two contractions of the lower cardiac ventricles, reflected as the distance between the successive R peaks of the QRS complex (Gehrke *et al.* 2011).

Frequency domain variables are used in estimating the distribution of a signal within 4 frequency bands including ultra-low frequency (ULF), very-low-frequency (VLF), low-frequency (LF), and high-frequency (HF) (Shaffer and Ginsberg 2017). Heart rate variability parameters in the time domain analysis include mean heart rate (MnHR) and the root mean square of successive differences (RMSSD) (Shaffer and Ginsberg 2017). Firstly, the MnHR reflects the average difference between the highest and lowest heart rate measures recorded during a specific respiratory cycle, measured in beats per minute (bpm). Evaluating the MnHR is a beneficial HRV measure as it reflects the respiratory sinus arrhythmia (RSA), defined as variations in heart rate that occur during the breathing cycle, and not simply the vagal tone (Shaffer and Ginsberg 2017). The second heart rate parameter, RMSSD, is the primary time-domain variable used to detect changes in heart rate in response to the vagal stimulation of the parasympathetic nervous system (PNS) mediated by the vagus nerve (Gehrke *et al.* 2011). RMSSD is measured in milliseconds and reflects the variation in the hearts beat-to-beat variances (IBI's) (Shaffer and Ginsberg 2017).

The heart rate variability parameters in the frequency domain analysis includes the low frequency (LF) to high frequency (HF) ratio. The LF/HF ratio is reflective of the neural regulation of the cardiovascular system in response to an external stimulus,

through the balance of the sympathetic and parasympathetic nervous system (Rietmann *et al.* 2004). As short-term heart-rate variability can be influenced by the balance between the two branches of the autonomic nervous system, as well as the regulatory mechanisms within the heart, the LF/HF ratio allows for an estimate of the ratio between the sympathetic and parasympathetic responses (Shaffer and Ginsberg 2017). The low frequency (LF) band (0.04-0.15 Hz) is produced primarily by the parasympathetic nervous system (PNS) and represents the baroreceptor activity, while at rest. Baroreceptors are mechanoreceptors located within the aortic arch and carotid sinus, that regulate the tension of the atrial wall in response to changes in blood pressure (Shaffer and Ginsberg 2017). In addition, the high frequency (HF) band (0.15-0.40 Hz) is called the respiratory band and represents parasympathetic activity through variations in heart rate, which correlate with the respiratory cycle (inhalation and exhalation) (Shaffer and Ginsberg 2017). Specifically, a low LF/HF ratio demonstrates higher PNS activity, while a high LF/HF ratio indicates the influence of the SNS (Shaffer and Ginsberg 2017).

The Kubios HRV Standard® software was set to a medium artifact correction, in which the segments were accepted when the artifact correction was below 10%, as the editing can influence the results of the heart rate variability analysis. An artifact, in heart rate variability measures, is defined as the abnormal beats (errors) within the R-R interval time series (Peltola 2012).

Salivary Cortisol

The second biomarker for stress in horses includes the secretion of glucocorticoids, which has been documented as an effective physiological indicator of stress in horses as the concentrations of cortisol released by the adrenal gland fluctuates in

response to stress (König *et al.* 2017). Cortisol is found within the blood plasma as both protein bound and free cortisol, while the saliva only contains free cortisol due to passive diffusion into the salivary glands (Nuchprayoon *et al.* 2017). However, in cases of acute stress, salivary cortisol can increase within 10 minutes of exposure to the stressor and is considered a reliable estimate of total serum cortisol within the blood. This increases its reliability as an efficient biomarker for stress in conditions involving acute stress (Ishizaka *et al.* 2017). Nevertheless, due to the relationship between the release of cortisol and physical exercise, cortisol concentrations are the least reliable when used as a biomarker for stress in horses, compared to heart rate variability and behaviour scoring (König *et al.* 2017).

Within blood plasma and saliva, cortisol concentrations can increase due to circadian rhythm as well as environmental stressors which activate the SNS (Kalman and Grahn 2004). The circadian rhythm of cortisol presents higher concentrations within the blood in the morning hours, with a maximum value at 10 am and a minimum value at approximately 10 pm (Bohák *et al*. 2013). However, previous studies have documented significantly higher concentrations in the evening with exposure to stressful stimuli (Kalman and Grahn 2004).

When cortisol is analyzed as a physiological biomarker for stress, the cortisol levels are measured from saliva samples collected prior to and following each horses' exposure to stress (Nuchprayoon *et al.* 2017). In this study, the two activities included the horses' baseline (in their stalls) and following the last dismount of the session. In order to compensate for circadian fluctuations in cortisol concentration, measurements of the horses' salivary cortisol concentrations were obtained during morning and evening therapeutic riding and control sessions. The collection of saliva samples is a non-invasive

procedure, which does not induce stress in horses and will not influence the cortisol levels measured within the saliva. The samples can also be frozen and stored easily in a freezer without affecting the cortisol concentrations within the samples (Nuchprayoon *et al.* 2017).

Behavioural Indicators of Stress in Horses

Behavioural scoring is a non-invasive method of assessing the stress and welfare of animals, such as horses, through the observation of behavioural reactions to stressful stimuli (Young *et al.* 2012). Behaviour scoring has been used as an indicator for stress along with physiological biomarkers, in order to gather a more comprehensive evaluation of stress in horses. However, the only behaviour scale currently available involving domestic horses is concentrated on horse and rider combinations, derived from the ethogram used by Visser et al. (2010) which was directed towards sports horses (Munsters *et al.* 2012).

For this study, 15 stress-related behaviours were identified as behavioural indicators of stress in horses, based on the behaviours observed during the Joyrider TRS and those observed throughout previous research studies (Young *et al.* 2012). Movements of the head and neck, such as the pinning back of the ears or the tossing of the head, are thought to indicate stress as they have been shown to indicate a mild form of aggression. Also, these behaviours are commonly one of the first observable indicators of stress in horses (Kaiser *et al.* 2006). These behaviours of the head and neck are typically accompanied by other stress-related behaviours displayed through movements of the body directed at the leader, sidewalkers or rider. These movements of the body include the kicking of the legs, biting or flicking motions of the tail (Kaiser *et al.* 2006). Oral

behaviours are also thought to indicate stress in horses as they are believed to relieve stress through the manipulation of oral cavity, including yawning or licking the bit (Kaiser *et al.* 2006). In some circumstances, the oral behaviour of the horse chomping on their bit has been investigated as a behavioural indicator of stress. However, in this study, not all horses wore a bit during the TRS and as a result this stress-behaviour was not considered in the behaviour scoring (Kaiser *et al.* 2006).

Other behavioural indicators of stress include periodic spontaneous erection and movements of the penis, which were observed in the 2 male Joyriders therapeutic riding horses and were considered behaviours indicative of stress. This behaviour presents as an erection of the penis from the prepuce, followed by the rhythmic dorsoflexion of the penis against the abdomen, and is indicative of an aroused state or excessive sexual energy (McDonnell and Hinze 2005). The compulsive oral behaviour of cribbing was also observed throughout the study, as a repetitive behaviour which can indicate a horse easily affected by stress (Mazzola 2016). The behaviour of cribbing is a stereotypy, defined by the repetitive behaviour pattern identified as an obsessive-compulsive disorder (OCD). Cribbing is considered a behavioural indicator of stress as the behaviour is presented through genetic or environmental factors including stress, lethargy or reduced welfare and can induce stress if the horse is deprived of engaging in the cribbing compulsion (Nurnberg and Paxton 1997). The behaviour of cribbing is defined as the placement of the top incisors against a hard surface, followed by the influx of air through the esophagus noticeable by the audible “grunt” and tightening of the ventral neck muscles (Albright *et al.* 2017).

In order to increase the reliability of the behavioural scores obtained, using the 15 behavioural indicators of stress, the results were compared to the two physiological

biomarkers used. This will increase accuracy as the physiological indicators selected, including heart rate variability and salivary cortisol, have been established as reliable indicators of stress through previous research (Young *et al.* 2012).

METHODS

Horses and the Therapeutic Riding Program

The data collection took place over an 8-week period at the Joyriders Facility in Hunter River, which is a Canadian Therapeutic Riding Association certified program (CanTRA) with each instructor being CanTRA-certified. The Joyriders facility consists of an indoor and outdoor arena, where the sessions took place, as well as individual stalls for each horse between sessions. The data collected included heart rate variability measures, saliva samples and video recordings. Four horses (N=4) were selected to participate in the study including a 20-year-old female Morgan Quarter Horse (ID-2), a 22-year-old female Quarter Horse (ID-3), and two male Quarter Horses being 8 and 15 years old (ID-4 and ID-1). The horses each participated in sessions 3 times per week, over an 8-week period, beginning in May of 2018. Each horse was ridden for a maximum of 3 sessions in a day, lasting 40 minutes each, by up to 3 riders registered in the Joyriders therapeutic riding program. The therapeutic riding sessions were conducted on Monday and Tuesday evenings (over 3hrs), as well as Saturday mornings (over 5hrs). The Monday and Tuesday sessions involved one to 2 rounds of activities, where the horses were ridden a maximum of 2 times. While the Saturday sessions involved 3 rounds of activities, in which some horses were ridden a maximum of 3 times throughout the entirety of the session. The Joyriders therapeutic riding program had 12 registered therapeutic riders, with one rider participating in the Monday sessions, 4 riders in the Tuesday sessions and 7 riders in the Saturday morning sessions. Each rider was assigned to a weekly session, as well as a

specific therapeutic riding horse. When the horses were not participating in therapeutic riding sessions, they were allotted 12 hours per day in their paddock. Data was also collected during 4 control sessions, which were replicated using the same 4 therapeutic riding horses throughout the same 3 activities. However, the therapeutic riders were replaced with experienced staff working at Joyriders to eliminate any influence the rider may have on the horse's stress responses. As well, each horse was ridden once during each control session. The control sessions were also conducted on Friday mornings, in order to ensure there would be no TRS held on that day.

Research Design

Before the data collection of heart rate variability, saliva samples and video recordings commenced, general information was collected regarding each of the horses participating in the study. This information included their age, breed, the length of time they have worked in the Joyriders therapeutic riding program, their diet and lastly their medical history including any medications they receive (Table 1). Owners consent forms were signed regarding each horse, by the owner or agent. As well, before the collection of data began, the research design was approved by the Animal Care Committee (ACC). Throughout the sessions, notes were taken in a laboratory notebook to log any possible stressors observed during the sessions such as weather conditions and the temperament or attitude of the riders and horses. These field notes were used to explain certain behavioural or physiological indicators of stress observed during the sessions, which were uncharacteristic of the horses involved. Furthermore, the time of each video recording and the activity taking place was logged in the lab notebook. The dependent variable within the study was the observed behavioural indicators of stress, which were compared across

each activity and between both sessions. This also includes comparisons between the heart rate variability measures and salivary cortisol concentrations, in which statistical analysis software (SAS®) was used to conduct a 2 by 3 analysis of variance (ANOVA), in order to obtain an inclusive evaluation of stress.

Table 1. Documented background information on the 4 therapeutic riding horses used in the Joyriders therapeutic riding program.

Horse ID	Age	Years of Experience (Years)	Diet (am/pm)	Medical History
1	20	7	1/2L of elevate, only dry hay	Abscess on front left hoof, thrush, foundered slightly.
2	15	3	1/2L of beet pulp, 1/2L of fat/fiber	Low selenium (give dose daily), 15g dexamethasone (Mon, Wed, Fri), surgery for cribbing.
3	22	1.5	1/3 of elevate	Abscess on the front left hoof, arthritis in hocks.
4	8	5	40mL of omega oil, hay	-

Heart Rate Data Collection

When recording heart rate, each horse was equipped with a Polar® Equine H7 Heart Rate Sensor Belt. The belt was applied around the girth, with a heart rate sensor attached, to send the recorded R-R interval data to the Polar® Equine V800 Heart Rate Monitor. However, before the heart rate sensor belts were placed on the horses, a stethoscope was used to manually determine the horse's heart rate. This allowed us to ensure the heart rate monitors were collecting data correctly and increased reliability. Using a sponge, the girth was wet using water in order for the sensor to detect the heart rate transmission through the skin. The heart rate sensor belt was worn under the horse's saddle during the sessions without causing additional agitation. The data collection for heart rate variability and behaviour scoring was obtained during 3 specific activities which occurred during the therapeutic riding and control sessions including 1) the baseline (in their stall before the session began); 2) while the rider was mounting the horse; and 3) while the rider was dismounting. The horses' baselines were measured in their stalls as this activity is consistent with their day to day routines. The heart rate sensors recorded the beat-to-beat R-R interval data which was retrievable using the online Polar Flow system. This data collected for each horse was then imported into the Kubios HRV Standard® software, a heart rate variability analysis software, where it was organized into 2 to 5-minute segments for each sessions activity, as the length of time varied based on the activity. A medium artifact correction was applied in order to correct errors in the R-R interval data collected, and each segment was adjusted to meet the required artifact correction (below 10%) before being accepted. Each segment was analyzed through Kubios® for the selected heart rate variability measures, including the time domain variables (MnHR, RMSSD) and a frequency domain variable (LF/HF ratio).

The time and frequency domain variable data were then inputted into statistical analysis software (SAS®) to determine whether there was a significant difference among the mean heart rate data collected among the sessions, and throughout the 8-week period. The data collected from the 2 to 5-minute HRV segments, obtained through the Kubios® HRV software, also matched the 2-minute video recordings used in the process of behaviour scoring. This allowed for comparisons between the horses' behaviour and their heart rate variability measures, throughout specific segments, as the observable behaviours were reflective of the heart rate variability measures obtained during the segment.

Video Recording of Activities

The video recordings were obtained during the same 3 activities as the heart rate variability segments, including baseline (in their stall), mounting and dismounting. Each horse was recorded, using a single video recorder, for 2 minutes in their stall before the session began, to acquire their baseline. However, the mounting and dismounting videos were obtained using 2 Canon VIXIA HF (R800 and R700) video recorders in order to obtain a clear view of the front and back of the horse.

Behaviour Scoring

Each video recording ranged from 2 to 5 minutes in length and included video clips of each horse during their baseline, while the rider mounted and dismounted. To conduct the behaviour scoring, each video was watched and each individual behaviour was scored according to the ethogram containing the 15 selected stress-related behaviours expressed by the Joyriders therapeutic riding horses (*Appendix*). Each horse was assigned 1 point for each stress-related behaviour observed during the therapeutic riding activities,

in both sessions. These values were combined for each activity, and divided by the total number of video segments obtained for that particular activity, producing a mean number of stress-related behaviours for each activity during both the therapeutic riding and control sessions.

Saliva Sample Collection

To measure changes in the release of cortisol following the therapeutic riding sessions, a saliva sample was collected during the horses' baseline prior to the session and following the horses' last session of the day, after the last rider had dismounted. The baseline saliva sample was collected in their stall following their breakfast and they were given a treat before the second sample was collected, following the dismount of the rider, to induce salivation. The treats used included sliced apple, carrot, grain and/or sugar cubes. The method of saliva collection included the use of a hemostat holding a piece of gauze that was inserted into the side of the horse's mouth and placed over their tongue, while gloved. The gauze was held in their mouth for approximately 20 seconds to absorb the saliva and was inserted into a syringe in order for the sample to be extracted into a centrifuge tube. The centrifuge tubes containing the saliva samples were stored in a portable cooler with ice packs, for transportation to the laboratory at the Atlantic Veterinary College.

Enzyme-linked Immunosorbent Assay

The centrifuge tubes containing the saliva samples were first stored in a cooler and were later transferred to a freezer, regulated at approximately -20°C , located in a

laboratory at the Atlantic Veterinary College. This method of saliva collection was proven effective through previous studies, as that it does not cause an increase in cortisol levels and therefore, the action of collecting a saliva sample is not considered a stressor to the horses (Kalman and Grahn 2004). The samples were tested using Salimetrics® Salivary Cortisol Enzyme Immunoassay (ELISA) kits designed for the quantitative analysis for salivary cortisol, in a laboratory at the Atlantic Veterinary College.

The immunoassay kit used standards (saliva-like matrix), samples and controls (high, low, in a saliva-like matrix) which were conjugated to horseradish peroxidase on a microtiter place, to compete for the binding site (Salimetrics® 2016). According to the manufacturer's instructions, the samples were first thawed to room temperature and centrifuged at 1500 x g for 15 minutes, in order to eliminate any particulate which may have developed while the samples were frozen (Salimetrics® 2016). Using a pipette, 25 µL of the standards, controls and saliva samples were inserted into the designated wells on the 96-well microtiter plate, directly preceding the assay diluent. As well, the saliva samples were conducted in duplicate. The enzyme conjugate was diluted through the addition of 15 µL of the conjugate, and 200 µL was added to each well with a multichannel pipette. The plates were placed on a plate rotator for 5 minutes at 500 rpm and incubated at room temperature for 1 hour. Using a pipette, the plates were washed 4 times using 400 µL a 1X wash buffer, following the incubation period, to remove any unbound particles (Salimetrics® 2016). The appearance of a blue colour was used to measure the bound cortisol enzyme conjugate, indicative of a reaction between the enzyme and substrate. A micropipette was used to add 200 µL of TMB Substrate Solution to the plate, where it was mixed on a plate rotator for 5 minutes at 500 rpm. The plate was then incubated in the dark for 25 minutes, followed by the addition of 50 µL of a stop

solution to each well before being mixed at 500 rpm until the green colour changed to yellow. Once removed from the plate rotator, the samples were quickly read using a standard plate reader (Salimetrics® 2016).

The mean cortisol concentration for each sample, acquired from the ELISA as each sample was tested twice using 2 wells on the microtiter plate, were accepted if the coefficient of variation (CV) was below 20%.

Statistical Analysis

All of the data collected was organized using spreadsheets and allowed for the important variables from the HRV measures, salivary cortisol measures and behaviour scores to be identified. The data collected for each heart rate parameter including the MnHR, RMSSD and the LF/HF ratio, were analyzed using SAS®. Using the SAS®, a 2 by 3 analysis of variance (ANOVA) was conducted for each horse, between both sessions and across each activity. A 2 by 3 ANOVA was selected for use in the statistical analysis and comparison involved the use of 2 or more treatments, in which an ANOVA would allow for the comparison of variance within each sample group. An ANOVA test also determined if the differences between the estimates of variance were due to random sampling errors or due to systemic treatment effects that caused one group to differ from the other (Mazzola 2016). The statistical analysis of the behaviour scores was also conducted using a 2 by 3 ANOVA, in comparing variance in the mean number of stress-related behaviours calculated for each horse during the 3 activities, throughout both sessions. A 2-way ANOVA was used when comparing the variance within each group of salivary cortisol samples, as they were collected from each horse following 2 treatments (baseline and dismount), for each session. A Pearson correlation test was conducted using

the behavioural indicators of stress and salivary cortisol concentrations in order to evaluate for a correlation between the presence of these two indicators of stress.

RESULTS

Behavioural Indicators of Stress

A total of 302 videos were recorded throughout the activities of the therapeutic riding sessions, amounting to 604 minutes of video footage. The control sessions included a total of 45 video recordings, which was equivalent to 90 minutes of footage (Table 2).

During the therapeutic riding sessions, a total of 768 stress-related behaviours were observed for all 4 horses, throughout the 3 activities (Table 3). Comparably, there was a total of 100 behavioural indicators observed from the 4 horses during the control sessions (Table 4). Of the 15 stress-related behaviours observed throughout the therapeutic riding and control sessions, the most frequently observed behavioural indicator was having the ears pinned back (ID-A). This behaviour was observed 240 times during the TRS, accounting for 31.2% of the total behaviours scored during the sessions. This stress-related behaviour was demonstrated most frequently throughout the control sessions, as it was scored 44 times, which reflects 44% of the total behaviours observed during the session.

The second most frequently observed stress-related behaviour involved moving the tail (ID-K), which represented 19.8% of total behaviours observed during the TRS (scored 152 times). While this behaviour accounted for 28% of the total behavioural indicators observed during the control sessions (scored 28 times). The behaviour scores were also used in calculating the total mean number of stress-related behaviours observed for each activity in both sessions, reported in Table 5.

Table 2. The number of 2-minute video recordings acquired for each horse collected throughout the 3 activities experienced during the therapeutic riding and control sessions.

	Baseline		Mounting		Dismounting	
Horse ID	TRS	Control	TRS	Control	TRS	Control
1	13	2	42	4	42	4
2	15	2	40	4	40	4
3	5	2	14	4	14	4
4	13	3	32	6	32	6
Sum	46	9	128	18	128	18
Time (min)	92	18	256	36	256	36

Table 3. The observed frequency for each of the 15 behavioural indicators of stress among the 4 therapeutic riding horses, during the 3 activities of the therapeutic riding sessions.

	Baseline				Mounting				Dismounting			
Horse ID	1	2	3	4	1	2	3	4	1	2	3	4
A	26	48	-	10	52	28	-	27	29	4	2	14
B	-	-	-	1	8	8	7	8	2	3	13	7
C	-	-	-	-	-	-	-	-	-	-	1	-
D	-	-	-	-	27	6	11	1	5	1	5	2
E	1	1	-	-	9	3	1	4	7	-	9	1
F	-	1	-	-	4	2	1	1	1	3	1	-
G	-	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	6	-	1	-	-	-	-
I	-	1	-	-	4	2	-	-	-	-	-	-
J	-	-	-	-	-	10	-	1	-	5	-	8
K	8	7	-	4	51	11	4	26	12	3	27	7
L	-	47	-	-	-	-	-	-	-	-	-	-
M	-	-	-	10	0	-	-	-	-	2	2	12
N	-	-	-	-	5	-	-	-	2	-	-	-
O	-	-	-	-	33	14	2	4	15	4	7	5
Sum	35	105	0	25	193	90	26	73	73	25	67	56

Behaviour Codes (A-O): (A) Ears pinned back, (B) Raising the head, (C) Turning the head from left to right independent of the rider, (D) Tossing the head, (E) Shaking the head, (F) Holding the head down, (G) Defecating, (H) Biting at the leader or rider, (I) Kicking at the leader or rider, (J) Penile erection in stress, (K) Moving the tail, (L) Cribbing, (M) Yawning, (N) Swinging hindquarters, (O) Licking the bit.

Table 4. The observed frequency for each of the 15 behavioural indicators of stress among the 4 therapeutic riding horses, during the 3 activities of the control sessions.

	Baseline				Mounting				Dismounting			
Horse ID	1	2	3	4	1	2	3	4	1	2	3	4
A	5	11	1	1	4	3	-	6	5	2	2	4
B	-	-	-	-	1	1	1	-	-	-	1	-
C	1	-	-	-	-	-	-	-	-	-	-	-
D	-	-	-	-	-	-	5	-	-	-	1	-
E	-	-	-	-	-	-	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	1	-
G	-	-	-	-	-	-	-	-	-	-	-	-
H	1	-	-	-	-	-	-	-	-	-	-	-
I	-	-	-	-	-	-	-	-	-	-	-	-
J	-	-	-	-	-	1	-	-	-	-	-	1
K	-	11	-	-	10	-	-	-	3	-	3	1
L	-	-	-	-	-	-	-	-	-	-	-	-
M	-	-	-	-	-	1	-	-	-	4	-	1
N	-	-	-	-	-	-	-	-	-	1	-	-
O	-	-	-	-	1	-	2	-	-	2	1	-
Sum	7	22	1	1	16	6	8	6	8	9	9	7

Behaviour Codes (A-O): (A) Ears pinned back, (B) Raising the head, (C) Turning the head from left to right independent of the rider, (D) Tossing the head, (E) Shaking the head, (F) Holding the head down, (G) Defecating, (H) Biting at the leader or rider, (I) Kicking at the leader or rider, (J) Penile erection in stress, (K) Moving the tail, (L) Cribbing, (M) Yawning, (N) Swinging hindquarters, (O) Licking the bit.

Table 5. The mean number of observed behavioural indicators of stress in the four horses during therapeutic riding and control sessions.

Session	Activity	Sum of Behaviours	Number of Video Segments	Range	Mean ± Standard Deviation
TRS	Baseline	164	46	0-14 (14)	3.565 ± 3.902
Control	Baseline	31	9	0-12 (12)	3.444 ± 4.531
TRS	Mounting	383	64	0-26 (26)	5.984 ± 5.287
Control	Mounting	36	9	1-8 (7)	4.0 ± 2.5
TRS	Dismounting	221	64	0-22 (22)	3.453 ± 4.588
Control	Dismounting	33	9	1-6 (5)	3.666 ± 1.581

Abbreviation: TRS, Therapeutic Riding Session.

There was no significant difference in the number of stress-related behaviours for each horse during the therapeutic riding and control sessions ($p<0.05$). However, a comparison between the 3 activities showed that 2 of the horses' experienced significant changes in the number of behavioural indicators expressed. One horse (ID-2) demonstrated a significantly higher number of stress-related behaviours during mounting, with an increase of 3.63 in the mean number of behaviours compared to the dismount ($p<0.0001$). This horse also displayed a significant increase of 5.86 in the number of stress-related behaviours expressed during the baseline measure, compared to the rider's dismount ($p<0.0001$). A second horse (ID-1) expressed the highest number of stress-related behaviours during the activity of being mounted, with an increase of 7.95 in the mean number of stress behaviours, compared to the baseline ($p<0.0001$). Also, the activity of mounting produced an increase of 7.32 in the mean number of stress-related behaviours, compared to dismounting ($p<0.0001$). Furthermore, a third horse (ID-3) demonstrated a significant change throughout the activities and experienced an increase in their stress-related behaviours of 7.53 during dismounting, compared to their baseline measure ($p=0.0222$). The fourth horse (ID-4) examined demonstrated no significant changes in the number of behavioural indicators expressed during the 3 activities ($p=0.2355$). Figure 2 illustrates the significant increase in behaviour scores during the mounting of the rider, followed by a slight decrease during the dismount. However, both activities promote higher stress behaviours than compared to the horses' baseline.

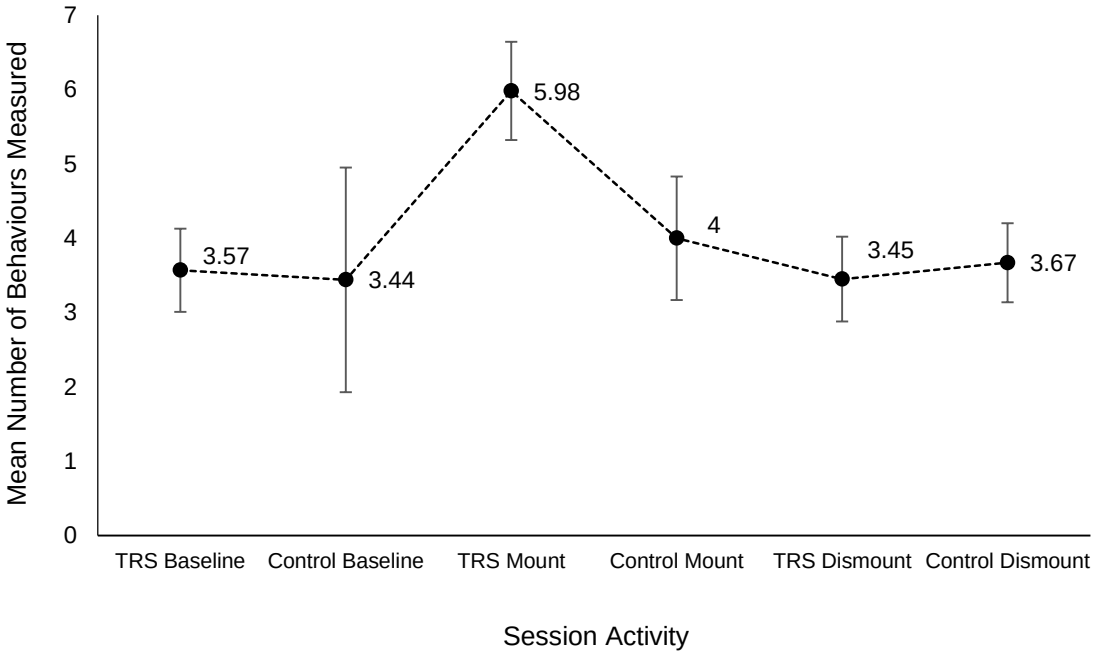

Figure 2. Comparison of the mean behavioural indicators of stress observed by the 4 horses during each activity of the therapeutic riding and control sessions. The error bars represent standard error of the mean.

Heart Rate Variability Measures

The mean time and frequency domain variables compiled for all 4 horses across each session activity showed increased values during the mounting of the rider, followed by the dismount of the rider (Table 6). The MnHR was significantly higher in 2 of the horse's during the control sessions. One horse (ID-2) experienced a significantly higher MnHR of 50.62 beats/min during the control sessions, compared to 40.84 beats/min when ridden during therapeutic riding sessions ($p=0.0026$). Similarly, the second horse (ID-1) measured with an MnHR of 45.6 beats/min during the control, compared to 37.93 during the therapy session ($p=0073$).

The analysis of the RMSSD showed no significant difference in any of the activities or between sessions in 3 of the horses, with the exception of one horse which expressed a significant difference across the 3 activities ($p<0.001$). The horse (ID-2) experienced a significant increase in the RMSSD during their baseline measure, which was calculated to be 32.83 ms higher than the activity of mounting and 36.61 ms higher than dismounting.

The low frequency (LF) to high frequency (HF) ratio resulted in no significant differences between the session activities or sessions, with the exception of one horse which demonstrated a significant difference in their baseline measure ($p=0.038$). This horse (ID-3) experienced an LF/HF ratio that was significantly higher by 1.33 during their baseline measure, compared to their dismount. An ANOVA comparing the variability of the three activities, using the mean LF/HF ratio for all four horses on both therapeutic riding and control sessions, demonstrates a significant increase in the LF/HR ratio during the mounting of the rider, followed by the riders' dismount Figure 3).

Table 6. The calculated means (±SD) for the heart rate variability time and frequency domain variables, collected from all 4 horses during the therapeutic riding and control sessions, during the 3 activities.

Session	Activity	N	MnHR ± Standard Deviation	RMSSD ± Standard Deviation	LF/HR Ratio ± Standard Deviation
TRS	Baseline	46	41.69 ± 5.11	80.37 ± 26.75	2.22 ± 1.53
Control	Baseline	9	45.40 ± 6.15	75.35 ± 25.04	1.9 ± 1.09
TRS	Mounting	64	41.69 ± 41.69	72.85 ± 5.9	2.37 ± 1.61
Control	Mounting	9	54.33 ± 3.57	56.57 ± 21.78	3.03 ± 1.5
TRS	Dismounting	64	40.64 ± 6.6	63.54 ± 20.83	2.84 ± 2.53
Control	Dismounting	9	48.46 ± 5.75	65.57 ± 21.58	2.4 ± 1.26

Abbreviation: TRS, Therapeutic Riding Session.

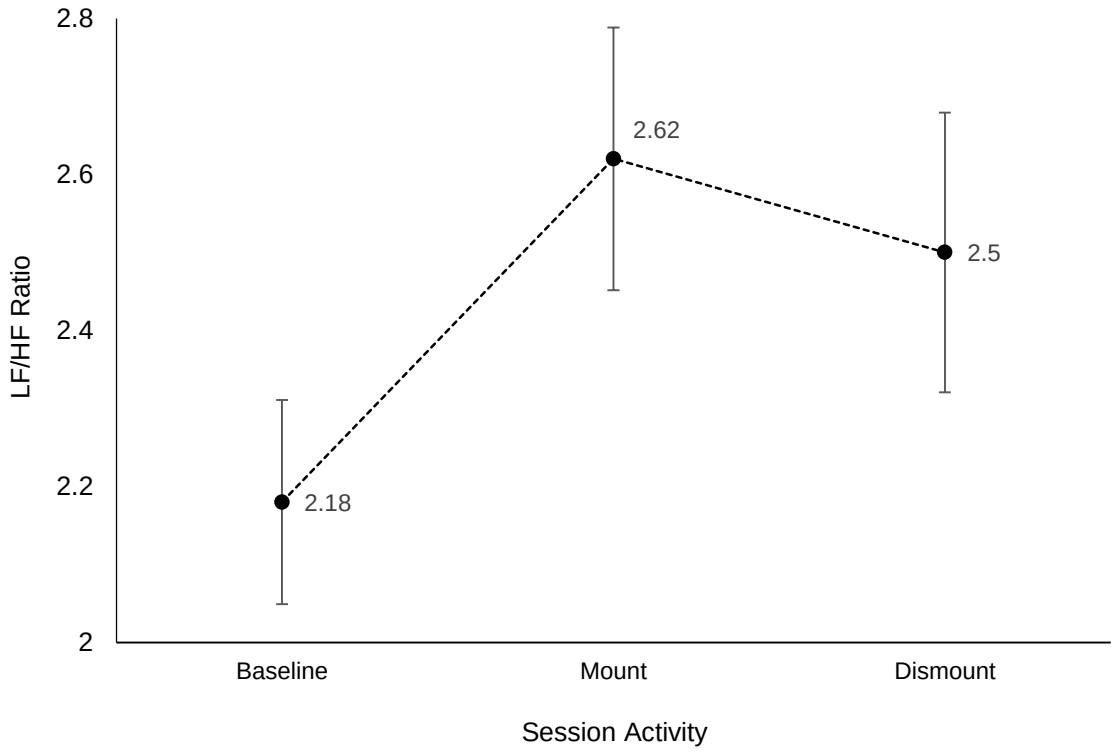

Figure 3. Comparison of the LF/HF ratio, a heart rate parameter measured for the 4 horses during each activity, including both therapeutic riding and control sessions. The error bars represent standard error of the mean.

Salivary Cortisol Concentrations

A total of 93 saliva samples collected from the 4 horses, were analyzed for the therapeutic riding sessions including 46 baseline samples and 47 dismount samples. For the control sessions, a total of 17 samples were analyzed including 9 baseline samples and 8 dismount samples. Using the Salimetrics® Salivary Cortisol Enzyme Immunoassay kits in a lab at the Atlantic Veterinary College, the mean cortisol concentrations for the horses (during their baseline and following the last dismount) were obtained for both therapeutic riding and control sessions. There was no significant difference, in any of the horses, between the concentrations measured during the therapeutic riding and control sessions. As a result, the concentrations from the TRS and control sessions were compiled in order to compare the concentrations measured before and after the sessions (baseline and dismount). The results showed a significant difference in 2 of the horses' (ID-1, ID-4) when their baseline salivary cortisol concentrations were compared to the concentrations following the riders dismount. This demonstrated a significant increase in the release of cortisol following the riders dismount in 2 of the horses (Table 7). A Pearson correlation test was also conducted using the behaviour scores and salivary cortisol concentrations, which resulted in a significantly positive correlation between the behavioural indicators observed and the secretion of cortisol.

Table 7. A comparison of mean salivary cortisol concentrations for each horse before and after the sessions, acquired from all samples obtained during the therapeutic riding and control sessions.

Horse ID	N	Baseline ± Standard Deviation	N	Dismount ± Standard Deviation	p-value
1	15	0.2 ± 0.33	14	1.26 ± 0.91	0.0002 *
2	17	0.27 ± 0.46	18	0.36 ± 0.37	0.5344
3	7	0.14 ± 0.06	6	1.08 ± 1.49	0.0602
4	16	0.11 ± 0.07	15	0.96 ± 1.59	0.0492 *

If $p<0.05$ then the difference is significant.

DISCUSSION

The aim of this study was to evaluate whether the horses used in therapeutic riding programs experience stress during their sessions with the therapeutic riders, through the use of a more comprehensive assessment. Particularly focusing on both the physiological and behavioural indicators of stress. The majority of research conducted on therapeutic horseback riding involves the cognitive and physical benefits to the rider, with limited research performed on the possible impacts to the horses themselves (Fazio *et al.* 2013). As a result, it was hypothesized that the horses in the Joyriders therapeutic riding program do experience stress during their sessions with the patients. The analysis of the physiological and behavioural indicators of stress demonstrated that the Joyriders therapeutic riding horses do experience stress throughout the session. This was evident through significant changes in heart rate variability measures, salivary cortisol concentrations and behavioural indicators of stress. However, the presence of these physiological and behavioural indicators of stress in both therapeutic riding and control sessions indicates that the stress exhibited by the horses was not a direct result of the therapy sessions, but could be attributed to the activities experienced throughout the sessions.

The evaluation of the mean behavioural indicators of stress for each horse displayed a significant change in the number of behaviours expressed by 3 of the horses (ID-1, ID-2, ID-3) during the session's activities. Particularly, during the activity of mounting where the highest number of stress related behaviours were observed. This

could be due to the mounting ramp used to assist the rider in mounting the horse, as well as the number of people surrounding and restraining the horse during this activity. There was also one horse who demonstrated a significantly higher number of stress behaviours during their baseline (ID-2), compared to their dismount. This can be justified by the compulsive cribbing behaviour exhibited by this horse. When presented with an opportunity to crib, this horse would continue to express this compulsive behaviour regardless of the activity or session (Nurnberg and Paxton 1997). This behaviour could be indicative of stress or anxiety being expressed by the horse, as this behaviour is compulsive in that it relieves stress. During their baseline, the horses are in their individual stalls, which gave ample opportunity to repeatedly engage in this compulsive behaviour, thus reflecting in a significantly higher number of stress-related behaviours during this activity. This evaluation also demonstrated no significant change in the horses' behaviour during the TRS when compared to the control sessions and indicates that the experience of stress is not limited to the TRS.

It was also evident through the analysis of the horses' physiological indicators of stress, that the horses were experiencing stress in both the therapeutic riding and control sessions. This was visible through the ANOVA conducting using the heart rate variability measures across both sessions. This supports the conclusion that stress is not directly associated with the therapeutic riding sessions, involving the patient, as the expression of stress-responses were not isolated to the TRS. This was first demonstrated in the MnHR which was significantly higher during the control sessions, for 2 of the horses (ID-1, ID-2), compared to the TRS. Possibly because the control sessions were completed in approximately 1 hr, while the therapeutic riding sessions which were conducted over a 2 to 4 hr period, however the horses were given rest periods in-between activities.

The RMSSD parameter also displayed a significant increase in one horse (ID-2) during their baseline measure when compared to the other activities. This also correlated with the behaviour scores obtained for this horse (ID-2), as there was a significantly larger number of stress behaviours observed during their baseline (ID-2). This increase in RMSSD could be explained by the cribbing behaviours observed by this horse throughout their baseline, as cribbing involves the repetitive manipulation of the oral cavity, neck and respiratory system. As the behaviour of cribbing is obsessive-compulsive behaviour, with physiological effects mediated by the parasympathetic nervous system, this could have influenced the results obtained from this heart rate parameter (Albright *et al.* 2017).

Lastly, the LF/HF ratio showed a significant increase in the baseline measure for one of the horses (ID-3) when compared to their dismount measures. This could be explained as the horse (ID-3) was only used in the last 4-weeks of the therapeutic riding program due to an abscess on the front left hoof. As a result, the pain experience in response to the injury could have influenced the heart rate variability measures obtained. However, in order to obtain reliable LF/HF ratio data, the segments used for analysis should exceed 4 minutes in length, which was difficult to obtain as some segments were as short as 2 minutes in length (Shaffer and Ginsberg 2017). One horse (ID-4) did not show any significant changes in their heart rate variability measures throughout the activities, which was also observed in the results of the ANOVA conducted using their behaviour scores. This could have been influenced by age and years of experience in the program, as this horse was the youngest at 8 years old and had the least amount of experience in the Joyriders therapeutic riding program with only 5 years. This could explain why the horses above 10 years of age and who have spent over 10 years in the

program, display a larger number of stress-related behaviours, which increased consistently, throughout the progression of the 8-week program.

The analysis of the salivary cortisol concentrations, obtained through an ELISA, further supported the results demonstrating no significant differences in the release of cortisol between sessions. However, the results did show a significant increase in the salivary cortisol concentrations in 2 of the horses (ID-1, ID-4) when the baseline concentrations were compared those following the dismount. This indicates that both horses produced significantly higher levels of cortisol in their saliva following the riders dismount, illustrating that somewhere during the session the horses' experienced a stressor, which triggered a physiological stress response indicated through the secretion of cortisol (Ishizaka *et al.* 2017). Since serum-free cortisol can increase in response to an acute stressor as quickly as 10 min following exposure, it cannot be determined which stimuli within the session were stressful to the horses specifically (Johnson *et al.* 2017). However, the results obtained from the saliva samples can conclude that the 2 horses did experience a stressful event throughout the session, reflected in their salivary cortisol concentrations following the riders dismount.

The Pearson correlation test which was conducted using the behavioural indicators and salivary cortisol concentrations demonstrates that as the number of stress-related behaviours observed by the horse's increases, so does their salivary cortisol concentrations and vice-versa.

A possible sources of error could have presented in the construction of the research study, as there was a significantly larger number of TRS conducted in relation to the number of control sessions. This could pose an issue in regards to the amount of physiological and behavioural data collected for each of the sessions and could reflect in

the results of the statistical analysis. As well, the horses were ridden a larger number of times during the TRS, and there was up to 7 riders participating in a single day and some horses were ridden up to 3 times, while they were only ridden once during each control session. This could have induced a higher level of exhaustion or stress in the horses during the TRS when compared to the controls.

Other possible sources of error could have incurred during the collection of the data. For instance, the reliability of the Polar® Equine V800 Canada Heart Rate monitors® could have impacted the heart rate variability measures obtained. In some cases, the Polar® Equine V800 Heart Rate Monitor would lose connection with the Polar® Equine H7 Heart Rate Sensor Belt, resulting in a loss of R-R interval data. Also, during the sessions, the water applied to the horses' hair would dry causing the sensor to lose contact with the skin, resulting in a loss of data. Also, in some cases, once the data was imported into the Kubios HRV Standard® software we were unable to get the percent artifact correction below 10% for some segments, indicating abnormal beats in the R-R interval time series and deeming the segment unusable.

Throughout the process of behaviour scoring, some behavioural indicators expressed by the horses throughout the activities could have been missed due to the angle of the camera and the length of the video recording, as the video recordings were a maximum of 2-minutes in length. In terms of the salivary cortisol concentrations, a possible source of error could have incurred in the collection of the saliva samples. As this was performed in the field, and the samples were kept in a cooler before transport to a freezer at the Atlantic Veterinary College, the samples could have been contaminated or affected by environmental factors, such as temperature, during both transport and storage. Indicated as the Salimetrics manual suggests that samples be frozen (below -20°C) as

quickly as 4 hours following sample collection (Salimetrics® 2016). The analysis of the saliva samples in the lab could have also been a source of error, as an ELISA was used to measure the cortisol concentrations. Some saliva samples required repeated ELISA tests due to the high coefficient of variation percentages (CV%), indicative of a larger dispersion around the mean (Calvi 2017). The treats given to the horses to induce salivation could have also influenced the salivary cortisol concentrations measured as acidic foods and sugar can compromise the reliability of the assay through the reduction of pH and bacterial growth (Salimetrics® 2016). Some of the horses participating in the study were also on medications for medical conditions unrelated to the study. Although we documented their prescription and dosing schedule, this could have impacted the salivary cortisol concentrations measures, as once horse (ID-2) was taking dexamethasone. Dexamethasone is 25 times more potent and functions longer than cortisol, acting as a stimulant on the horse's mood and behavioural activity (Soma 2005).

The results obtained using behaviour scoring indicates that stress can be measured using an ethogram constructed from the 15 observed stress-related behaviours, with the highest number of stress-related behaviours observed during the mounting of the rider and the most frequently observed behavioural indicator of stress being the "ears pinned back". The heart rate variability measures also demonstrated that the activities endured by the therapeutic riding horses, specifically mounting and dismounting, induced physiological stress in the horses involved through an increase in the sympathetic nervous system. These results were further supported by the salivary cortisol concentrations obtained through an ELISA, in which 2 of the horses' experienced a stressor during the session, indicated by a significant increase in salivary cortisol. The use of salivary cortisol as a

physiological biomarker for stress was also shown to be a reliable indicator of stress in horses throughout this study.

However, due to the small sample size of 4 horses used in this study, we cannot generalize the stress observed in the Joyriders horses to all therapeutic riding horses. However, we have successfully developed a more comprehensive method of assessing stress in horses used in therapeutic riding sessions and across a variety of activities. This is because this study incorporated both physiological and behavioural indicators of stress, which were directly reflective of each other through comparisons of the heart rate variability measures to the behavioural indicators. As well and the positive correlation observed between the behavioural indicators and salivary cortisol concentrations. the Unlike previous studies, we have shown that stress in therapeutic riding horses can be evaluated using both physiological and behavioural indicators of stress, with further supportive evidence including salivary cortisol concentrations. This method uses and ethogram of behavioural indicators of stress, and physiological indicators which could be replicated on horses participating in other therapeutic riding programs. This is an important finding as the horses participating in the activities involved in therapeutic riding at Joyriders do experience an increase in their sympathetic stress responses, which could be modified to improve the welfare of the horses involved. This would shift the focus in this area of research towards animal welfare, and the effects of therapeutic riding on the horses involved in this method of equine-assisted therapy.

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APPENDIX

Ethogram of the 15 stress-related behaviours observed in the horses being ridden in the Joyriders therapeutic riding program.

Behaviour	Behaviour Description
Ears pinned back *	Ears held caudally against the head and neck
Raising the head *	Head raised above the withers, with the nose extended upward and neck stretched.
Turning head from left to right independent of the rider *	The movement of the head to the left or right, independent of the rider and their use of the reins.
Tossing the head *	Head lowered below the withers, with ears back, followed by a sharp raise of the head.
Shaking the head *	Repeated movement of the head from left to right, flipping the head.
Holding the head down *	Head held below the withers, with nose extended downward and neck stretched.
Defecating *	Expelling of feces with tail raised.
Biting at the leader or rider **	Bite movement directed at the rider, leader or sidewalker.
Kicking at the leader or rider **	Thrusting motion of one or both hind legs towards the side or back, directed toward the leader or sidewalker.
Penile erection in stress	Erection of the penis.
Moving the tail *	Excessive movements of the tail, characterized by a swinging motion from the left to the right.
Cribbing **	Placement of upper incisors on a hard surface, followed by the quick influx of air into the esophagus and the flexion of the ventral neck muscles.
Yawning ***	Deep inhalation with open mouth.
Swinging Hindquarters	Swinging motion of the hindquarters.
Licking the bit *	Manipulation of the bit using the tongue, independent of the rider and their use of the reigns.

Descriptions derived from the stress-related behaviours compiled by:
* (Kaiser *et al.* 2006)
** (Young *et al.* 2012)