NONLINEAR ANALYSIS OF HEART RATE VARIABILITY FOR MEASURING PAIN IN DAIRY CALVES AND PIGLETS, HEAT STRESS IN GROWING PIGS, AND THE GROWING PIG SICKNESS RESPONSE TO A LIPOPOLYSACCHARIDE CHALLENGE

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For Kathryn, Ginny, Llewyn and Darla

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LIST OF ABBREVIATIONS

ABBREVIATION	DEFINITION
cm	centimeter
CV	coefficient of variation
d	day
F	F-distribution
g	gram
h	hour
Hz	hertz
m	meter
min	minute
mL	milliliter
mo	month
n	treatment sample size
Adj. R^2	adjusted coefficient of
s	determination
SE	second
t	standard error
wk	t-distribution
	week

ABSTRACT

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Heart rate variability (**HRV**), or the variation in time between adjacent heart beats over time, is a non-invasive proxy measure of autonomic nervous system (**ANS**) function that has been used regularly in studies focused on evaluating livestock stress and welfare. The autonomic nervous system regulates involuntary physiological processes (*e.g.* respiration and heart rate) and consists of two main components, the parasympathetic (**PNS**), and sympathetic (**SNS**) branches, which act to maintain bodily homeostasis (PNS) or stimulate the "fight-or-flight" response after exposure to a stressor (SNS). Traditional linear HRV measures provide an estimation of overall autonomic activity or changes to the balance between the PNS and SNS branches by evaluating changes to the mean, variance, or frequency spectra of the R-R intervals.

To interpret HRV data obtained via linear HRV measures, particularly spectral HRV analysis, a linear assumption has to be assumed where SNS and PNS activity act in a purely antagonistic manner. However, this assumption is not always met. In many cases, ANS activity is altered in a nonlinear manner, which is reflected to some degree in the variability of heart rate output. Therefore, HRV measures that evaluate nonlinear changes to organizational or structural aspects of the R-R interval variability may be a useful compliment to traditional linear HRV measures for distinguishing between stressed and non-stressed states. The purpose of this dissertation was to evaluate the use of nonlinear HRV measures for evaluating dairy calf

disbudding pain, piglet castration pain, growing pig heat stress, and as potential indicators of the subsequent immune response to a lipopolysaccharide (**LPS**) challenge in growing pigs.

Chapter 1 provides a knowledge base for understanding HRV and its use as a measure of autonomic stress in studies with livestock species. A brief explanation of animal welfare science, measures used to evaluate an animal's welfare, and a demonstration of need for non-invasive physiological measures is provided before discussing the physiological basis of HRV. Relevant linear and nonlinear HRV measures are explained and examples of their use in livestock stress research are provided. Finally, a rationale for the studies conducted in this dissertation is presented.

Chapter 2 evaluates the use of HRV as an indicator of castration pain in 9-d-old piglets over a 3-d experimental period. Compared to sham castrated piglets, surgically castrated piglets exhibited greater low frequency to high frequency ratios (**LF/HF**), reduced sample entropy (**SampEn**), and greater percent determinism (**%DET**) during the post-castration period. However, postural behavior was not different between treatments and serum cortisol concentrations only tended to differ between treatments at 1 and 24 h post-castration treatment, with surgically castrated pigs having numerically greater serum cortisol concentrations at both timepoints. These results demonstrate the ability of nonlinear HRV measures (SampEn and %DET) to complement the physiological interpretation of linear HRV measures (LF/HF) in response to castration. Specifically, pigs who were surgically castrated exhibited more regularity (SampEn) and periodicity (%DET) in their HRV data, and potentially more sympathetic activity (LF/HF) compared to sham castrated piglets, indicating greater pain-related stress. Additionally, HRV was a more sensitive measure of the stress response to castration than readily identifiable behaviors such as posture and the serum cortisol response.

Chapter 3 evaluates the use of HRV as an indicator of disbudding pain in dairy heifer calves (4 to 7-wk of age) over a 5-d experimental period. Calves who were given lidocaine and meloxicam prior to disbudding exhibited lower mean R-R interval (**RR**) values and a greater short-term detrended fluctuation analysis scaling exponent (**DFAα_1**) than sham disbudded calves. Together, these results indicate that calves who received pain mitigation exhibited greater pain-related stress (RR) and reduced physiological complexity in their heart rate signal (DFAα_1). Calves who were disbudded without pain mitigation had an intermediate response compared to sham disbudded calves and calves provided lidocaine and meloxicam. However, their numerical values closely followed those of calves provided lidocaine and meloxicam. These results demonstrate the usefulness of nonlinear HRV measures (DFAα_1) for evaluating nonlinear and correlational aspects of physiological complexity in response to disbudding. Additionally, the HRV results suggest that the provision of meloxicam does not reduce the amount of pain-related stress experienced by calves following disbudding.

Chapter 4 evaluates the use of HRV as an indicator of heat stress in growing pigs exposed to an acute heat episode. Heat stressed pigs exhibited greater body temperatures and spent less time in an active position compared to thermoneutral control pigs. Additionally, heat stressed pigs displayed an altered nonlinear HRV response to the acute heat phase compared to non-heat stressed control pigs. Specifically, heat stressed pigs exhibited lower SampEn and tended to exhibit greater %DET, but no alterations to linear measures were observed in response to the acute heat episode. The low frequency to high frequency ratio was higher in heat stressed pigs during the period following the acute heat phase. Therefore, nonlinear HRV measures (particularly SampEn) may be more sensitive to the immediate physiological stress response to increased environmental temperature than traditional linear HRV measures.

Chapter 5 evaluates the use of baseline HRV as a potential indicator of the subsequent cortisol and pro-inflammatory cytokine response to an LPS challenge in growing pigs. The time for a pig to approach a human (**approach time)** prior to LPS administration was inversely related to baseline standard deviation of the R-R intervals (**SDNN**), and directly related to RR and the mean length of diagonal lines in a recurrence plot (**Lmean**). This result may have implications for the use of HRV as a measure of temperament in livestock species, since pigs with lower baseline SDNN (*i.e.* greater stress) and greater baseline Lmean (*i.e.* increased periodicity length in HRV data; greater stress) values took longer to approach a human observer before LPS administration (which occurred 1 d after HRV measurement). Area under the curve values for approach time following LPS administration were inversely related to high frequency spectral power (**HF**) and directly related to body weight, where pigs with low baseline HF values (*i.e.* lower parasympathetic activity) and higher body weights were slower to approach a human observer following LPS administration. Additionally, pigs with greater Lmean values had a greater change in body temperature following LPS administration. In conclusion, while baseline HRV measures were not directly representative of the cortisol or cytokine response following an LPS challenge, HF and Lmean may be useful indicators for evaluating certain aspects (sickness behavior and fever) of the innate immune response to an LPS challenge.

In conclusion, these studies demonstrate the usefulness of nonlinear HRV measures for evaluating livestock stress. Measures such as sample entropy and those derived from recurrence quantification analysis (%DET, Lmean) seem to be particularly useful for complementing traditional linear HRV measures and, in some cases, are more sensitive measures of the physiological stress response (see chapter 4). Therefore, their inclusion in future studies on

livestock HRV is warranted. However, further work is needed to fully elucidate the physiological significance of nonlinear HRV measures and their response to stress.

CHAPTER 1. INTRODUCTION

1.1 The Science of Animal Welfare

1.1.1 History and Conceptualization

In 1965, the Brambell committee, headed by Professor Rogers Brambell, published their seminal report on the welfare of animals raised in production. Meant as an investigation into the claims made by Ruth Harrison's 1964 book *Animal Machines*, the report outlined the issues that different livestock species faced and provided input for what needed to be considered to ensure their welfare. As a result, the five freedoms were born (Webster, 1994). Specifically, animals should be free from: 1) hunger and thirst, 2) discomfort, 3) pain, injury and disease, 4) fear and distress, and 5) they should be able to express normal behaviors. The five freedoms have been widely recognized by production companies, scientists, veterinarians, and legislators since their introduction, however, there has been little tangible input for how they can be achieved as livestock numbers have continued to grow (McCulloch, 2013). Consequently, societal concerns for the care of livestock species continue, with more than 58% of a recent survey's respondents claiming that they are "more concerned with animal welfare now than they were a few years ago" (Packaged Facts, 2017).

The science of animal welfare aims to bridge the gap between those societal concerns and how livestock species are cared for in production by utilizing empirical data to draw conclusions about how an animal is coping with its environment (Fraser et al., 1997). However, exactly which attributes of an animal's welfare are most important are less clear since societal norms and personal ethics largely determine the level to which animal welfare can be deemed "acceptable" (Sandøe et al., 2003; Fisher 2009). Therefore, arriving at a single definition or conception of animal welfare has been difficult. Animal welfare is most commonly conceptualized using a three-domain

approach, where ethical concerns for biological functioning, natural behavior, and affective states overlap with each other to encompass an animal's welfare (Duncan and Fraser, 1997). Some scientists place more importance on one domain over the others (see Kiley-Worthington, 1989; McGlone, 1993; Duncan and Petherick, 1991; Broom, 1991) and recent work evaluating consumer perceptions has attempted to expand the conception of ethical concerns regarding animal welfare (Ventura et al., 2016). Nevertheless, while animal welfare as a concept is still unclear, it is generally agreed upon by scientists, veterinarians, and the public that livestock species are sentient animals who are able to subjectively experience positive and negative affective states (Duncan 2006). Accordingly, the environment in which animals are raised should be optimized to suit their welfare needs.

1.1.2 Measuring Welfare

One obvious hurdle to accurately evaluating how an animal copes with its environment is the inability to directly communicate with the animal on matters related to its welfare. As a result, a multi-measure approach is often taken to infer the welfare state as it relates to the three ethical domains previously described (biological functioning, natural behavior, or affective states). Production measures (*e.g.* feed intake, litter size in piglets, milk production in dairy cattle, egg production in laying hens) have been used extensively in the literature as indicators of animal welfare. However, production parameters largely focus on the biological functioning of the animal, where alteration does not necessarily provide insight into the animal's affective state or natural behavior when evaluating production measures alone (Fraser, 2003; Bracke, 2007). Additionally, production measures may not be sensitive enough in certain cases to accurately characterize a change in the animal's welfare state (Broom, 1986).

Ethology, or the study of animal behavior, is frequently thought to go hand-in-hand with the study of animal welfare and is inextricably linked to ethical concerns regarding an animal's ability to perform natural behaviors (Broom, 2011). Animal behavior can also give insight into biological functioning, as animals express specific behavioral adaptations to stressors such as illness and infection (Koolhaas et al., 1999). Therefore, animal behavior is an important tool since it can be used to evaluate animal welfare concerns in multiple ethical domains, however, not without its own caveats. Similar to production parameters, behaviors of interest are not always sensitive enough to characterize the animal's welfare state, so selection of relevant behaviors that can be objectively and accurately measured is of great importance (Martin and Bateson, 1993). The data collection procedure can also be time consuming, particularly when studying infrequent behaviors that require continuous monitoring for long periods of time (CAST, 2018).

Technical drawbacks aside, meaningful interpretation of animal behavior can be difficult in the absence of underlying physiological indicators, so the inclusion of physiological measures in studies focused on animal welfare is essential. However, physiological indicators that are not at least mildly invasive are sometimes difficult to obtain, so an animal's response may be altered as a result of restraint or interaction with personnel during a study. For example, animal welfare scientists frequently measure cortisol in the blood as measures of stress (Möstl and Palme, 2002). Cortisol (or corticosterone in rodents and birds) is stimulated by the hypothalamic-pituitary-adrenal (HPA) axis and secreted into circulation by the adrenal glands during times of physiological stress, where it acts to induce gluconeogenesis, increase protein and fat catabolism, and suppress certain aspects of the inflammatory immune response in an effort to maintain bodily homeostasis (Buckingham, 2006). As a result, cortisol is secreted at greater levels during times of stress and has become one of the most commonly used measures of the physiological stress

response in studies on animal welfare. However, factors such as handling and restraint are often confounding when attempting to accurately interpret the cortisol response to a stressor of interest (Rushen, 1991; Hopster et al., 1999; Rault et al., 2011). Therefore, non-invasive methodologies for collecting accurate, interpretable physiological data are essential for understanding and improving animal welfare.

Interest in the use of non-invasive methodologies for studying animal welfare science is not a new concept. Measures such as average heart rate have been recorded non-invasively as a measure of stress in livestock for a number of years (White et al., 1995; Grøndahl-Nielsen et al., 1999). However, over time, advances in technology and a better understanding of animal physiology have provided animal researchers with opportunities to obtain more meaningful, non-invasive physiological data (Lane, 2006; Stewart et al., 2005). One good example, and the focus of this dissertation, is the use of heart rate variability (HRV) as a measure of autonomic function and stress in livestock species. Heart rate variability is non-invasive, relatively easy to collect using widely available equipment, and has been shown to be useful in addressing ethical concerns regarding both physiological and psychological aspects of animal welfare (von Borrel et al., 2007). Additionally, HRV builds upon what we can infer from measuring average heart rate alone by providing increasingly specific information regarding autonomic function in response to a stressor. Accordingly, the use of HRV for evaluating livestock stress and welfare has greatly increased over the previous two decades.

The purpose of this chapter is to introduce the concept of HRV and its use in swine and dairy welfare studies as a measure of stress. Explanations of common HRV measures are provided, along with best-practice methodologies for their use. Finally, a large portion of this chapter will

focus on nonlinear HRV measures, which are currently under-utilized in the animal literature and serve as the basis for the studies contained in this dissertation.

1.2 The Autonomic Nervous System

The autonomic nervous system (**ANS**) consists of two major components, the parasympathetic (**PNS**) and sympathetic (**SNS**) autonomic branches, which modulate involuntary physiological processes (*e.g.* heart rate, blood pressure, digestion) and play an active role in maintaining bodily homeostasis (PNS) or preparing the body for interaction with a physiological stressor ("fight-or-flight"; SNS). Through a series of pre- and post- ganglionic efferent ANS nerve fibers originating in the midbrain, pons, medulla oblongata, and spinal cord, ANS branch activity exerts a physiological effect on effector organs throughout the body. In contrast, afferent sensory neurons associated with the ANS illicit involuntary reflex responses by relaying neural activity through the dorsal root ganglion to the brain (Gabella, 2012).

1.2.1 Sympathetic Nerve Fiber Organization and Control of The Heart

The sympathetic nervous system is responsible for the "fight-or-flight" response and supplies the body with sympathetic nerve fibers that directly innervate cardiac muscle, glands, and smooth muscle. When activated, pre-ganglionic sympathetic nerve fibers secrete acetylcholine at the level of the sympathetic ganglia chain to illicit an electrical impulse down the post-ganglionic axon body (Gordan et al., 2015). As a result, the post-ganglionic sympathetic nerve fibers secrete norepinephrine at the level of the effector organ, which exerts a physiological effect on multiple organs throughout the body (*e.g.* adrenal glands, heart and vasculature, lungs, intestines and stomach; Gordan et al., 2015). In the heart, the norepinephrine secreted by post-ganglionic SNS nerve fibers generally acts to increase heart rate, conduction velocity and contractile force by

binding to β_1 adrenergic receptors located on the sinoatrial node (**SN)**, atrioventricular node (**AV**) and myocytes of the heart atria and ventricles. Other adrenergic receptors (β_2, α_1, α_2) are also present in small quantities on the heart or within the vascular soft tissue and play a role in vasodilation and vasoconstriction (Gordan et al., 2015).

1.2.2 Parasympathetic Nerve Fiber Organization and Control of The Heart

The PNS is largely involved in maintaining bodily homeostasis and is sometimes called the "rest and digest" branch (Rea, 2016). Like the SNS, the nerve fibers associated with the PNS consist of a pre- and post-ganglionic component, where secretion of acetylcholine by the pre-ganglionic nerve fibers activates the post-ganglionic fibers at the level of the parasympathetic ganglia. However, most of the ganglia associated with the PNS lie closer to the effector organ than the SNS ganglion, making the post-ganglionic PNS fibers much shorter than post-ganglionic SNS fibers (Gordan et al., 2015). Additionally, the post-ganglionic PNS nerve fibers secrete acetylcholine instead of norepinephrine to exert a physiological effect on the effector organ. Parasympathetic innervation of the heart is largely conducted through the paired vagus nerve, which supplies approximately 75% of the PNS nerve fibers to the lungs, heart, stomach, pancreas, and small intestine. Both branches of the vagus nerve innervate the heart and play a role in decreasing HR through the activation of M_2 muscarinic receptors located on the SA, AV, and atrial tissue (von Borrell et al., 2007; Gordan et al., 2015).

1.3 Heart Rate Variability as a Measure of Stress in Swine and Dairy Cattle

In many cases, the branches of the ANS act in an antagonistic manner, where an increase in SNS activity coincides with a decrease in PNS activity, which is reflected by a subsequent decrease to the interval length between adjacent heart beats over time. As a result, changes to the interval

between adjacent heart beats can provide insight into the state of the autonomic system in response to a stressor. Heart rate variability has traditionally attempted to quantify these changes through a number of linear HRV measures that can be further classified into “time domain” or “frequency domain” measures. Measures in the time domain describe quantitative properties of the heart rate signal such as mean value and standard deviation, while measures in the frequency domain utilize spectral analysis of the HRV data to approximate the balance between the PNS and SNS branches.

1.3.1 Time Domain HRV Measures

Time domain measures are commonly used in HRV analysis and are also the easiest to implement and interpret. Commonly used time domain HRV measures include mean R-R interval (**RR)**, standard deviation of the R-R intervals (**SDNN**), and the root mean square of successive differences (**RMSSD**), all of which have been recommended for inclusion in studies focused on heart rate variability (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996).

1.3.1.1 Mean R-R Interval (**RR**)

Mean R-R interval is simply the average distance (measured in ms) between adjacent heart beats over a period of time. It is a reciprocal function of average heart rate and is the least informative HRV measure in terms of providing information regarding autonomic activity (von Borell et al., 2007). However, RR can be a valuable measure of overall ANS function, where lower RR values are most commonly interpreted as indicators of greater stress compared to higher RR values. Mean R-R interval should be measured with data sets of at least 5 min for consistent comparison between studies and reliability, however, experimental design may dictate data length (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; Bourdillon et al., 2017).

1.3.1.2 Standard Deviation of the R-R Intervals (**SDNN**)

Standard deviation of the R-R intervals measures the standard deviation of all R-R intervals (measured in ms) in an HRV data set. Like RR, long-term SDNN provides a measure of overall ANS function but little extra information regarding the interaction between the SNS and PNS branches (Shaffer et al., 2014). Lower values of SDNN indicate lower variability in the HRV data set and, in general, greater stress. Analysis of SDNN requires stationarity, so care should be taken to ensure the data are detrended, if needed. Additionally, longer datasets (*e.g.* 24 h) are recommended (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). Although shorter data sets (5 min or less) are commonly used for SDNN analysis, short term changes to SDNN tend to reflect vagally mediated changes to HRV instead of overall autonomic function (Shaffer and Ginsberg, 2017). Therefore, the information obtained from RMSSD (see below) and SDNN in the short term is often redundant.

1.3.1.3 Root Mean Square of Successive Differences (**RMSSD**)

The root mean square of successive differences is a measure of variation between adjacent heart beats and is commonly referred to as a "short term" standard deviation measure (Shaffer and Ginsberg, 2017). Specifically, the differences between adjacent R-R intervals are calculated and squared for the entire data set before calculating the average squared difference value. Finally, the RMSSD is determined by taking the square root of the average squared difference value. Root mean square of successive differences is highly correlated with HF power spectra, an indicator of parasympathetic activity. Five minutes of stationary data are recommended for analysis (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996), however, several studies investigating the use of short and ultra-short

HRV measurements have found RMSSD to be fairly reliable over a range of shorter data lengths (Lin et al., 2005; Baek et al., 2015)

1.3.2 Frequency Domain HRV Measures

Frequency domain HRV measures attempt to provide information regarding the balance between the ANS branches by applying a fast Fourier transformation (**FFT**) to the HRV data to decompose the signal into separate frequency components that correlate with SNS and PNS activity. Fast Fourier transformation assumes data stationarity and equidistant sampling, so data containing a non-constant rate of data acquisition are often re-sampled at a lower frequency (*e.g.* 4Hz) prior to transformation. A minimum of 512 re-sampled data points has been recommended for HRV analysis in the frequency domain, so care should be taken to make sure the sampling frequency meets this requirement (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). Additionally, a window function should be applied to the data to reduce spectral leakage during FFT. Recommendations on how to apply the window function vary depending on data and window type (Harris, 1978).

The most common frequency domain measure used in HRV analysis is the low frequency to high frequency ratio (**LF/HF**), which requires previously determined spectral limits for high frequency spectral power (**HF**; ms^2) and low frequency spectral power (**LF**; ms^2) to be defined prior to obtaining the frequency spectra (von Borell et al., 2007; Poletto et al., 2011). Some studies that use frequency spectra for HRV analysis also include a "very low" frequency component and "ultra-low" frequency component, however, the physiological interpretation of these components is not fully established (Shaffer and Ginsberg, 2017). For this reason, neither component is usually considered in studies on livestock stress or welfare and will not be discussed here.

1.3.2.1 High Frequency Spectral Power (**HF**)

High frequency spectral power, sometimes called the respiratory band, is a measure of the high frequency component within the HRV spectra generated by FFT. It is used as an indicator of parasympathetic function because it reflects vagally mediated changes during respiration (also known as respiratory sinus arrhythmia), where vagal activity decreases during inspiration and increases during expiration (Akselrod et al., 1985; Hansson and Jönsson, 2006). Additionally, the administration of antimuscarinic agents, such as atropine, inhibit the HF spectral band, indicating a loss of parasympathetic tone (Pomeranz et al., 1985; Poletto et al., 2011). Therefore, lower values of HF are typically considered to indicate lower parasympathetic activity.

1.3.2.2 Low Frequency Spectral Power (**LF**)

Low frequency spectral power represents the low frequency portion of the HRV spectrum generated by FFT. The physiological interpretation of the LF band is less clear than HF since it likely represents both branches of the ANS or baroreflex mediated autonomic activity (Pomeranz et al., 1985; Goldstein et al., 2011; Rahman et al., 2011). As a result, LF is not typically used as a stand-alone measure.

1.3.2.3 LF/HF Ratio (**LF/HF**)

The LF/HF ratio is simply the ratio between low frequency and high frequency spectral power obtained through FFT. An increase in LF/HF is typically interpreted as an increase in SNS activity, however this assertion has been thoroughly challenged (Billman et al., 2013). In order to interpret LF/HF, several inconsistent assumptions need to be made, including a constant linear relationship between the ANS branches (Billman et al., 2013). Changes to respiration can also dramatically alter the measure's interpretation, so controlling for respiration and the inclusion of several additional HRV measures has been recommended for more accurate interpretation of

LF/HF (Brown et al., 1993; Koh et al., 1995). Additionally, further investigation into the role of baroreflexes on autonomic outflow is necessary for understanding the underlying physiology driving changes to the LF band. (Goldstein et al., 2011; Rahman et al., 2011).

1.3.3 Linear HRV Analysis in Swine

The majority of studies focused on swine HRV exclusively utilize linear measures such as RR, SDNN, RMSSD, and LF/HF for evaluating new HRV methodology (Marchant Forde et al., 2004; Poletto et al., 2011; Krause et al., 2015) and measuring autonomic stress as an indicator of an animal's welfare state (von Borrell et al., 2007). This is likely due to their relatively straightforward implementation using available HRV analysis software (*e.g.* Kubios HRV Standard; Kubios Oy, Kuopio, Finland), and ease of interpretation compared to nonlinear HRV measures, which require additional inputs to implement and, in some cases, lack clear physiological interpretation (Sassi et al., 2015).

Linear HRV measures have been evaluated as a physiological measure of swine stress in response to a several potential on-farm stressors, including social stress and mixing (de Jong et al., 2000; Sun et al., 2017), interaction with a handler (Tallet et al., 2014); gestation (Marchant-Forde et al., 2004); loading and simulated transport (Peeters et al., 2005; Lay et al., 2017), heat stress (Parois et al., 2018), housing and novelty (Jaskulke and Mantueffel, 2011; Zupan et al., 2016), and euthanasia (Rault et al., 2015). Additionally, several studies have also used linear HRV measures to investigate swine affective states and individual coping styles in response to several on-farm situations. These include exposure to handlers and conspecifics (Ruis et al., 2002; Düpjan et al., 2011; Reimert et al., 2014; Leliveld et al., 2017), environmental enrichment (Zebunke et al., 2013); restraint (Geverink et al., 2002; Goumon and Špinka, 2016), novel environments and occurrences (Lelived et al., 2016), anticipation of positive and negative stimuli (Imfeld-Mueller et al., 2011;

Zebunke et al., 2011; Krause et al., 2017; Henzen and Gygax, 2018), and isolation (Leliveld et al., 2017). It is worth noting that some authors have reported changes to linear HRV measures that are somewhat indicative of emotional valence or affective state (Zebunke et al., 2011; 2013; Leliveld et al., 2017), while others have shown linear HRV measures to be largely indicative of arousal only (Düpjan et al., 2011; Goumon and Špinka, 2016; Reimert et al., 2014; Henzen and Gygax, 2018). Therefore, more research is needed in this area. However, linear HRV measures, alone, are likely not consistently sufficient for evaluating emotional valence in swine.

1.3.4 Linear HRV Analysis in Dairy Cattle

Similar to the body of literature on swine, HRV studies with dairy cattle mainly consist of linear HRV measures such as RR, SDNN, RMSSD, and LF/HF. However, in contrast with swine HRV research, very little work evaluating affective state, individual coping styles, and emotional valence has been conducted with dairy cattle (but see Sutherland et al., 2012 and Frondelius et al., 2015 for investigation into individual temperament). Instead, most studies focus on the physiological response to on-farm stressors such as disease (Mohr et al., 2002; Pomfrett et al., 2004; Konold et al., 2011;Yoshida et al., 2015), housing (Hopster et al., 1998; Hagen et al., 2005; Gygax et al., 2008; Kovács et al., 2013), heat stress (Mohr et al., 2002; Bun et al., 2017), disbudding and castration pain (Stewart et al., 2008; 2009; 2010), lameness (Kovács et al., 2015a), issues related to reproduction and gestation (Kovács et al., 2014; 2015b; Stojkov et al., 2015 Trenk et al., 2015), euthanasia (Lambooij et al., 2012), environmental enrichment (Westerath et al., 2014), and metabolic stress (Mohr et al., 2002; Erdmann et al., 2017).

1.3.5 Drawbacks of Linear HRV Measures

Linear measures are commonly used to evaluate HRV in swine and dairy cattle, however, a lack of study repetition, differences in experimental design, and conflicting results make

comparisons between studies and interpretation difficult. One potential reason for this difficulty may have to do with the assumptions required for interpreting linear HRV measures. For example, interpretation of spectral data requires a linear and consistently antagonistic interaction between the SNS and PNS, which may result in a conclusion that is inaccurate and contradictory (Billman, 2013). Additionally, linear HRV measures may be limited in their ability to distinguish between stressed and non-stressed animals, given the likelihood that heart rate variability, to some degree, is nonlinear. Therefore, qualitative nonlinear aspects (structure and organization) of HRV data sets may be drastically different from each other, but linear aspects of the data (mean, standard deviation, etc.) may be statistically similar (Fig. 1).

1.3.6 Nonlinear HRV Analysis

While linear measures attempt to provide an indication of autonomic function using quantitative descriptors (mean, variance, spectral analysis) of the variability present in R-R interval data, nonlinear analyses based on complexity, regularity, and self-affinity have been used in the HRV literature to measure qualitative aspects (structure and organization) of HRV by evaluating patterns and correlations present within the data. Output from physiological systems, including the heart, is often nonlinear in nature and may, therefore, be better explained by nonlinear analyses (Goldberger et al., 2002b). Additionally, this nonlinearity is an integral component in the theory of physiological complexity, where nonlinear variability begins to break down in response to stress and disease (Goldberger et al., 2002b). Therefore, nonlinear HRV measures may be particularly useful for evaluating stress, health, and future disease risk (Sassi et al., 2015)

Multiple nonlinear measures have to been used to evaluate HRV with varying success. Therefore, this chapter will only discuss measures that were analyzed in future chapters, including sample entropy (**SampEn**), short-term detrended fluctuation analysis (**DFAα_1**), and those obtained

via recurrence quantification analysis [percent recurrence (**%REC**), percent determinism (**%DET**), and mean length of diagonal lines within a recurrence plot (**Lmean**)].

1.3.6.1 Sample Entropy

Sample entropy is a method used in time-series analysis for measuring the unpredictability of fluctuations present within a data set and is an improvement upon the previously utilized approximate entropy (Pincus, 1995). Specifically, it is the negative log of the conditional probably that a vector of length *m* data points with a tolerance of *r* will remain similar if that vector is extended to *m* + 1 data points (Richman and Moorman, 2000), where, unlike approximate entropy, values are not influenced by self-matches. Lower SampEn values indicate higher stress (Lake et al., 2002). Previous research has indicated that SampEn should be conducted with stationary data sets that contain greater than 200 data points in order to obtain a reliable measurement (Turukalo et al., 2010; Yentes et al., 2013). Additionally, a data vector length (*m*) of 2 and a tolerance (*r*) between 0.1 and 0.25 times the standard deviation of the dataset is recommended (Pincus, 1995; Yentes et al., 2013; Zhao et al., 2015).

1.3.6.2 Detrended Fluctuation Analysis

Detrended fluctuation analysis is a measure of self-similarity that attempts to evaluate fractal-like properties in a time series by measuring long-range correlations in the data. Fractals describe particular geometric properties in natural phenomena (including physiological properties), where if a single sample is resampled at a smaller scale, the structure of the two samples will be statistically similar (Mandelbrot, 1982; Sharma, 2009). Fractal theory can be used to investigate physiological systems because of their inherent complexity, where the variability present in healthy physiological signals displays long-term correlations, nonlinear behavior, and a

dependency on initial conditions (Goldberger, 2002a). However, these conditions begin to deteriorate under stress, disease, and aging (Goldberger 2002a, 2002b).

Detrended fluctuation analysis measures fractal-like properties in HRV data by evaluating the relationship between HRV data fluctuations and the scale of measurement over several different scales (Peng et al., 1994). Specifically, the HRV tachogram is first integrated by subtracting the overall mean interval value from each data point and divided into a series of boxes, each containing a length of *n* heart beats. Within each box, the data is fit with a least square line and detrended before calculating the root mean square deviation (**RMSD**). The RMSD of each box is then used to calculate an overall average value for the box length *n*. The procedure is then repeated over several different box lengths. Once the average RMSD values have been obtained for each box length, the points are plotted on a log-log plot and a best-fit line is fit to the data. The slope of the best-fit line gives the scaling exponent (**α**) which is used to indicate the correlational properties of the data with the following generalizations:

1. $\alpha < 0.5$ = negatively correlated
2. $\alpha = 0.5$, White noise, data are uncorrelated.
3. $0.5 < \alpha < 1$ = positive correlation in the data
4. $\alpha = 1$, Pink noise, data show correlated properties within a power law slope.
5. $\alpha = 1.5$, Brown noise or a random walk, where neighboring data points are correlated but do not follow a power law slope.

In general, a scaling exponent (α) around 1 is indicative of long-range correlation in the data and is considered to be a hallmark of health in humans (Goldberger 2002b). However, stress and disease may cause the scaling exponent to increase or decrease. For example, physiological changes, such as pregnancy, result in scaling exponents that are higher than 1, where correlation

in the variability is present but does not follow a power law slope (Baumert et al., 2012). Individuals with heart disease, on the other hand, may exhibit uncorrelated variation in HR (α = 0.5) or short-term correlation with a scaling exponent above 1, depending on data length.

Detrended fluctuation analysis can be conducted with at least 256 data points to obtain a reliable result, however data sets with 512 or more data points are recommended (Delignieres et al., 2006). Recommendations for range of box lengths is less clear, however, many studies on HRV have set a range of 4-11 beats for short-term analysis of DFA (Beckers et al., 2006; Šikner et al., 2015).

1.3.6.3 Recurrence Quantification Analysis

Several physiological components and properties exert an effect on HR, however, their individual contribution to HRV cannot be visualized by a one-dimensional representation of the data. Therefore, HRV data can be "unfolded" into multi-dimensional state-space, where different physiological components and properties each occupy one dimension, allowing for evaluation of different properties and patterns present in the multi-dimensional data that would otherwise go unobserved (Wallott, 2017). Multi-dimensional analysis can be conducted using recurrence quantification analysis (RQA), which allows a one-dimensional data set to be unfolded in multi-dimensional state-space and then visualized on a 2-dimensional graph known as a recurrence plot (Eckmann et al., 1987). Specifically, after selecting an appropriate time lag (Liebert and Schuster, 1989; Wallott, 2017), embedding dimension (Kennel et al., 1992; Wallott, 2017), and an appropriate radius (Wallott, 2017), the data set is plotted against itself on the recurrence plot. Individual recurring points within the specified radius are indicated by a single point placed on the plot and recurring sequences, or periodicites, in the data appear on the plot in diagonal lines of adjacent recurring points. Using this information, several measures can be utilized to gain a better

understanding of the variability's multi-dimensional structure (Webber and Zbilut, 1994). Recurrence quantification analysis is particularly useful for evaluating nonlinear aspects of HRV since it does not require data stationarity and can be used with a range of data lengths.

1.3.6.3.1 Percent Recurrence Within a Recurrence Plot (**%REC**)

Percent recurrence quantifies the number of individual points on the recurrence plot in relation to the total number of data points evaluated in the recurrence plot. Previous studies evaluating the effect of stress on %REC from HRV data have indicated that greater %REC values are indicative of greater stress (Mohr et al., 2002; Hagen et al., 2005; Frondelius et al., 2015).

1.3.6.3.2 Percent Determinism Within a Recurrence Plot (**%DET**)

Percent determinism is related to percent recurrence but instead of a measure of recurring individual data points, it evaluates the number of individual points that make up diagonal lines within the recurrence plot. Therefore, it's used to quantify the number of recurring sequences present within the data. Similar to %REC, greater %DET values seem to be an indicator of greater stress (Mohr et al., 2002; Hagen et al., 2005).

1.3.6.3.3 Mean Length of Diagonal Lines Within a Recurrence Plot (**Lmean**)

Mean length of diagonal lines within a recurrence plot builds upon %DET by quantifying the average length of diagonal lines in the recurrence plot. In other words, Lmean quantifies the average length of recurring sequences in the data, where greater values are generally indicative of greater stress (Dua et al., 2012).

1.3.7 Nonlinear HRV Analysis in Swine

Investigation of nonlinear HRV measures in swine have typically been conducted in biomedical research studies, where pigs are used as human models for understanding the

development of HR dynamics over time or the effect of disease and injury on HR complexity (Lipsitz et al., 1997; Batchinsky et al., 2007; 2010). However, one recent study investigating the impact of floor cooling on heat stress in late lactation sows found that SampEn was lower in sows that were not provided with a floor cooling apparatus compared to sows that were exposed to floor cooling (Parois et al., 2018). Therefore, sows not provided floor cooling had more regularity present in their HR signal, generally indicating increased stress. The same study also investigated %REC and Shannon entropy (via RQA analysis), but no differences between treatments were found for either measure.

1.3.8 Nonlinear HRV Analysis in Dairy Cattle

Slightly more interest has been paid to nonlinear aspects of HRV in dairy cattle when compared with swine (Mohr et al., 2002; Hagen et al., 2005; Kovács et al., 2013; Frondelius et al., 2015; Erdmann et al., 2017). The limited use of nonlinear HRV measures in studies on dairy cattle has shown %DET and %REC to be greater in cattle exposed to automated milking systems compared to conventional herringbone systems (Hagen et al., 2005), and greater in calves who were subjected to heat stress or suffered from diarrhea (Mohr et al., 2002). Surprisingly, however, a recent study evaluating temperament in late-lactation dairy cows found that %DET collected prior to an avoidance test was negatively correlated with the subsequent performance of avoidance behaviors (Frondelius et al., 2015), indicating that animals who were under lower stress prior to the avoidance test performed more severe avoidance behaviors during the test. Therefore, while general assumptions can be made regarding the effect of stress on nonlinear HRV measures, additional research is needed to fully understand how nonlinear HRV measures are affected by on-farm factors, including individual animal temperament (Frondelius et al., 2015).

In addition to %DET and %REC, the longest diagonal line segment in a recurrence quantification plot (Lmax) obtained from RQA has been used in place of Lmean, where a single value for the longest line present in the recurrence plot is used to evaluate periodicity within the data set. Previous studies have shown Lmax to be altered in response to milking parlor type, heat stress, diarrhea, and metabolic state, however, with slightly conflicting results depending on the issue being researched (Mohr et al., 2002; Hagen et al., 2005; Erdmann et al., 2017).

1.4 Rationale for Proposed Research

Given the nonlinear nature of heart rate output, it's possible that nonlinear HRV measures may be sensitive indicators of physiological stress in livestock species and can be used to complement traditional linear HRV measures. However, too few studies evaluating nonlinear HRV measures in livestock exist to make a sound conclusion. Therefore, the experiments in this dissertation were conducted to evaluate the suitability of nonlinear HRV measures for measuring stress in response to various on-farm stressors. These include piglet castration pain, dairy calf disbudding pain, heat stress in growing pigs, and the growing pig sickness response to a lipopolysaccharide challenge.

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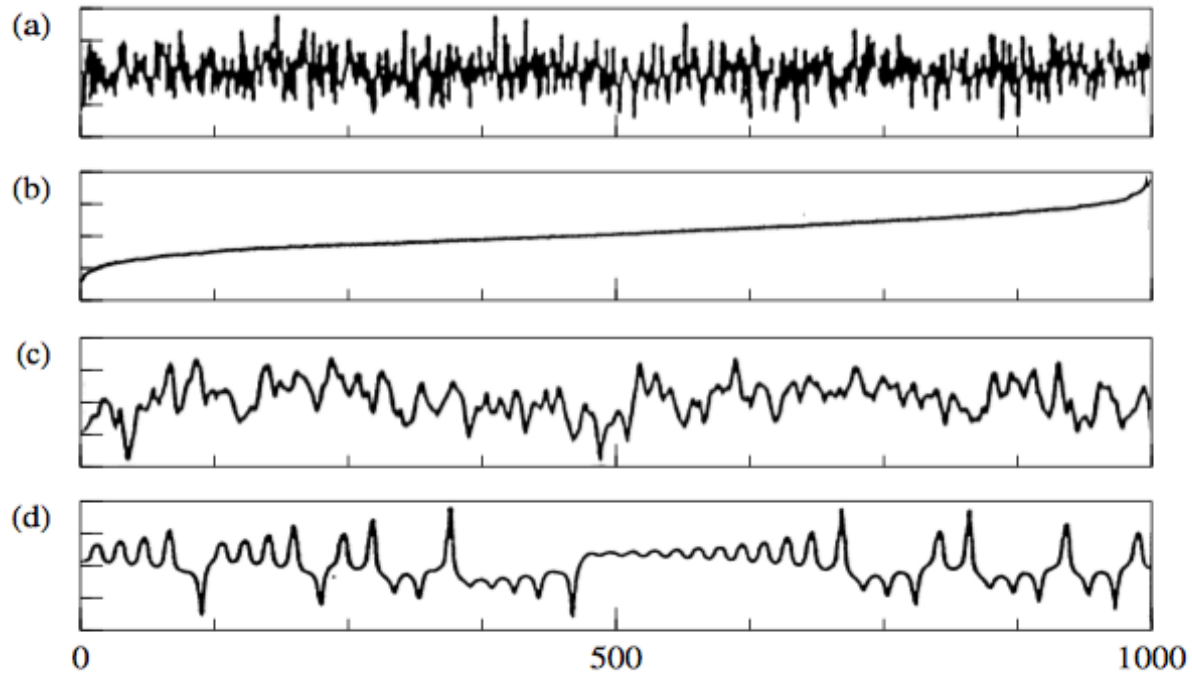

Figure 1.1 Synthesized time series demonstrate the limitations of linear measures for detecting differences between data sets. Despite gross differences in structure, (a), (b), (c), and (d) have identical means, ranges, and standard deviations. Additionally, (c) and (d) have identical power spectra. Originally published by Kaplan (1994).

CHAPTER 2. MEASURING CASTRATION PAIN IN PIGLETS USING NONLINEAR MEASURES OF HEART RATE VARIABILITY

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2.1 Abstract

The purpose of this study was to evaluate the usefulness of linear and nonlinear heart rate variability (**HRV**) measures for evaluating castration pain in piglets over a 3-d experimental period. Thirty individually selected piglets were randomly allocated to 1 of 4 treatments: 1) sham castrated HRV (**SHRV**; n = 8), 2) surgically castrated HRV (**CHRV**; n = 7), 3) sham castrated blood collection (**SBC**; n = 7), or 4) surgically castrated blood collection (**CBC**; n = 8). Piglets in the SHRV and CHRV treatment groups underwent a 1 h heart rate variability and postural behavior evaluation on d -1 (baseline), d 0 (castration treatment), d 1, and d 3 of the experimental procedure. Piglets in the SBC and CBC groups underwent blood collection for serum cortisol analysis at -0.5, 1, 2, 3, 24, 48, and 72 h relative to castration treatment. No effect of treatment was found for amount of time spent lying ($P > 0.05$), however, there was a tendency for serum cortisol to be greater in CBC pigs at 1 h (52.1 ± 8.9 ng/mL; $t_{52.4} = 2.60$; $P = 0.08$) and 24 h (31.4 ± 6.9 ng/mL;

$t_{51.52}$ = 2.60; *P* = 0.08) post-castration compared to SBC piglets (1h: 23.1 ± 6.5 ng/mL; 24 h: 11.3 ± 3.9 ng/mL). Castrated piglets exhibited greater low frequency to high frequency ratio (26.8 ± 3.2 vs. 9.0 ± 1.1 arbitrary units; $F_{10.42}$ = 7.53; *P* = 0.02), lower sample entropy (1.47 ± 0.15 vs. 1.97 ± 0.16 bits; $F_{10.4}$ = 5.33; *P* = 0.04) and greater percent determinism (54.0 ± 2.1 vs. 38.8 ± 2.0%; $F_{12.07}$ = 4.93; *P* = 0.05) compared to SHRV piglets, indicating greater pain-related stress due to the surgical castration procedure. Therefore, the inclusion of nonlinear HRV measures may be valuable for assessing pain-related stress in future studies investigating swine welfare.

Keywords: castration, heart rate variability, pain, piglet, stress

2.2 Introduction

In the United States, male piglets not intended for the breeding herd are surgically castrated to reduce the performance of aggressive behavior and improve meat quality (AVMA, 2013). Previous studies evaluating the behavioral and physiological response to castration have shown that surgically castrated piglets exhibit greater plasma cortisol concentrations (Sutherland et al., 2012), slowed growth prior to weaning (Kielly et al., 1999; but see Carroll et al., 2006), altered behavior (Weary et al., 1998; Taylor et al., 2001; Davis et al., 2017), and, if castration is conducted at an older age, an altered response to an immune challenge (Lessard et al., 2002) compared to their sham castrated counterparts. Therefore, while castration is commonly carried out early in life to minimize any lasting negative effects, it is evident that the procedure causes considerable pain and stress to the piglet.

In the past, non-invasive behavioral indicators of castration pain have typically been the most beneficial for distinguishing between castrated and non-castrated piglets (Hay et al., 2003; Moya et al., 2008; Sutherland et al., 2012), whereas physiological measures (*e.g.* cortisol or catecholamines) may be affected by a number of additional factors including handling (Carroll et

al., 2006; Rault et al., 2011). Accordingly, there is a need for additional non-invasive methodologies to evaluate the physiological response to castration pain. Heart rate variability (**HRV**) is a non-invasive proxy measure of autonomic function that has been used as an indicator of the autonomic stress response in livestock species, however, little work has evaluated its use as an indicator of pain-related stress (von Borell et al., 2007). The autonomic nervous system (**ANS**) consists of 2 main components, the parasympathetic (**PNS**) and sympathetic (**SNS**) branches, which regulate essential involuntary physiological processes (*e.g.* breathing, digestion, heart rate) and are altered in times of stress (Gordan et al., 2015). Both branches directly innervate the sinoatrial and atrioventricular nodes of the heart, as well as the cardiomyocytes, and play a large role in changes to heart rate (**HR**) over time (Gordan et al., 2015). As a result, traditional linear HRV measures that measure mean change or the extent of variation in the time between adjacent heart beats have been used as a "snapshot" of autonomic function in response to a stressor.

While the PNS and SNS branches act in a mostly antagonistic manner (in general the PNS decreases HR while the SNS increases HR), the interaction between the ANS branches is not strictly linear (Uijtdehaage and Thayer, 2000; Billman, 2013). Physiological influences (*e.g.* respiration and blood pressure) exert a nonlinear effect on the heart, leading to HR changes that are not representative of direct antagonism between the ANS branches and cannot always be determined using a linear approach to measurement (Uijtdehaage and Thayer, 2000). Therefore, the introduction of nonlinear HRV measures for evaluating nonlinear changes to the structure of HRV data may strengthen HRV methodology for measuring castration pain-related stress in piglets.

The purpose of this study was to evaluate the usefulness of linear and nonlinear HRV measures for measuring castration pain in piglets over a 3-d experimental period. We hypothesized that castrated piglets would exhibit altered autonomic activity and increased stress compared to

sham-castrated piglets. Specifically, we predicted that castrated piglets would exhibit a greater cortisol response, lower average R-R interval (**RR**; Table 2.1), lower standard deviation of the R-R intervals (**SDNN**; Table 2.1), lower root mean of successive squared differences (**RMSSD**; Table 2.1), and greater **LF/HF** ratios than sham-castrated piglets. Additionally, we predicted that nonlinear HRV analysis would result in castrated piglets having lower sample entropy (**SampEn**; Table 2.1), lower HR signal self-affinity (as measured by detrended fluctuation analysis; **DFAα_1**; Table 2.1), greater percent determinism (**%DET**; Table 2.1) and recurrence (**%REC**; Table 2.1), and greater average diagonal line length in a recurrence quantification plot (**Lmean**; Table 2.1) compared to sham-castrated piglets.

2.3 Materials and Methods

All experimental procedures were approved by the Purdue University Animal Care and Use Committee (protocol #1703001554).

2.3.1 Animals and Housing

Thirty-two individually selected piglets from 32 litters (1 piglet/ litter) were selected for enrollment in the study and housed with their littermates and sow in similar sized farrowing stalls (0.61 m x 2.29 m). All piglets were kept under environmentally controlled conditions (24.6 ± 0.1℃; 52.8 ± 0.1% relative humidity) with supplemental heat provided by heat lamps in each farrowing stall. Artificial lighting was provided from approximately 0700 h until 1500 h each day; however, windows in the room provided natural lighting when artificial lighting was not in use. All piglets remained in their home farrowing stall for the entirety of the experimental procedure.

2.3.2 Treatments and Experimental Design

All piglets were randomly allocated to 1 of 4 treatments: 1) sham castrated HRV (**SHRV**; n = 8), 2) castrated HRV (**CHRV**; n = 8), 3) sham castrated blood collection (**SBC**; n = 8), or 4) castrated blood collection (**CBC**; n = 8). Pigs in the SHRV and CHRV groups were either sham castrated or castrated and underwent HRV measurement but did not undergo blood collection for cortisol analysis, since repeated handling during the procedure would have likely influenced HRV results. Therefore, each piglet in the SHRV and CHRV groups was "matched" with a male piglet of similar body weight in a separate, previously unutilized litter. These matched piglets were allocated to the SBC or CBC treatment groups (based on castration treatment of their matched HRV piglet) and underwent blood collection for cortisol analysis only.

The experimental procedure was conducted over 2 repetitions during July and December 2017 at Purdue University's Animal Sciences Research and Education Center. Twelve piglets (n = 3 SHRV, 3 CHRV, 3 SBC, 3 CBC) underwent treatment during the first repetition. Twenty piglets (n = 5 SHRV, 5 CHRV, 5 SBC, 5 CBC) underwent treatment during the second repetition.

2.3.3 Acclimation to Personnel and Heart Rate Monitors

All piglets were tail docked and had needle teeth clipped at 2 d of age. To reduce the likelihood that these procedures would influence subsequent experimental results, the experimental procedure was not initiated until 7 d of age, where all piglets enrolled in the study began a 2-d acclimation period to the handlers and HRV equipment (SBC and CBC piglets were only exposed to handlers). Specifically, piglets in the SHRV and CHRV groups were removed from the farrowing stall, weighed, and placed in a 0.61 m^2 wooden crate for approximately 5 min. A heart rate monitor (Polar H10; Polar Electro Oy, Kempele, Finland) was then fit immediately behind the piglet's forelegs with flexible veterinary wrap (VetWrap; 3M, Maplewood, MN). To

reduce targeting of the HR monitor on experimental piglets by its littermates not on study, long black socks were altered to make body socks that fit over the body of all piglets in litters containing a SHRV or CHRV piglet (Fig. 2.1). All SHRV and CHRV piglets were then weighed and returned to their farrowing stall for a period of 1 h. After 1 h, study personnel entered the crates and removed all body socks and HR monitors from the piglets. This procedure was repeated a second time the next day. Piglets in the SBC and CBC groups were also handled for approximately 30 s each d and weighed, however, no further acclimation was conducted.

2.3.4 Baseline HRV Measurement

Following the acclimation procedure, at 9 d of age, experimental piglets in the SHRV and CHRV treatment groups underwent a baseline HRV measurement (d -1 of the experimental procedure). The baseline HRV measurement process was similar to the acclimation procedure, where all piglets in litters containing SHRV or CHRV experimental piglets were removed and fit with body socks. Piglets in the SHRV and CHRV treatment groups were also fit with HR monitors. All piglets were then returned to their respective farrowing stalls for 1 h of HRV measurement. Following the 1 h measurement period, experimental personnel entered the crates and removed all body socks and HR monitors.

2.3.5 Experimental Procedure

All piglets underwent treatment the following day (d 0). First, all piglets in litters containing a SHRV or CHRV piglet were removed from their pens, fit with body socks, and placed in a 0.61 m^2 wooden crate to await castration (if a boar). Gilts were returned to their home farrowing stall after fitting the body sock. Piglets allocated to the SHRV or CHRV treatment groups were treated similarly to their male littermates, however, they were also fit with HR monitors underneath their body socks prior to castration treatment. Male piglets in litters

containing SBC and CBC piglets were also removed from their pen and placed in a 0.61 m^2 wooden crate to await castration, however, no body socks or HR monitors were used.

The surgical castration procedure was carried out on CHRV and CBC piglets (and their male littermates) by 2 study personnel, where 1 study personnel suspended the piglet upside down by the rear legs with the piglet's scrotum facing outward. The second study personnel cleaned the area with isopropyl alcohol and made a small, slightly left of middle, vertical scrotal incision. The left testicle was removed, the spermatic cord pulled taut, and then cut with a sharp scalpel. After removal of the left testicle, the tunica vaginalis around the right testicle was cut and the right testicle and spermatic cord were removed in similar fashion. Male piglets undergoing sham-castration (SHRV, SBC, and their male littermates) were handled similarly, however, no incision or actual castration took place. Instead, the blunt end of a plastic pen was used in place of a scalpel to simulate scrotal incision.

Following castration treatment, all piglets were returned to their home farrowing stalls. Heart rate variability data were collected for a period of 1 h following the procedure and for 1 h at similar times on d 1 and d 3 post-castration. Piglets in the SBC and CBC treatment groups underwent blood collection at -0.5, 1, 2, 3, 24, 48, and 72 h relative to castration treatment. Briefly, piglets were carefully removed from the farrowing stall and placed on their backs in a wooden v-trough covered with absorbent paper. One personnel held the piglet while the second experimental personnel cleaned the jugular area with 70% isopropyl alcohol and made a jugular puncture. One and a half mL of blood was collected into a 3 mL serum collection tube (Vacutainer Plastic Serum Collection Tube; Becton-Dickinson, Franklin Lakes, NJ) using a 2.54 cm 22-gauge hypodermic needle (Vacutainer; Becton-Dickinson; Franklin Lakes, NJ) at each time point. Piglets were returned to their home farrowing stall immediately following blood collection.

2.3.6 Behavioral Analysis

Postural behavior (lying, standing) of piglets in the SHRV and CHRV treatment groups was recorded continuously during HRV collection (1 h), so HRV data sets could be selected from periods of inactivity. These data were also quantified (% time spent lying) and used to evaluate whether differences were observed between SHRV and CHRV treatment groups. Two cameras (KPC-N502NUB; KT&C USA, Fairfield, NJ) were placed behind the gestation stall, on the right and left side, to reduce the number of blind spots created by the sow. Video data were transmitted and stored on a digital video system (Geo Vision VMS Software; Geo Vision Inc., Taipei, Taiwan).

2.3.7 Heart Rate Variability Analysis

Heart rate variability data collected by the HR monitor were transmitted telemetrically to a data recorder (Polar V800 Sports Watch; Polar Electro Oy, Kempele, Finland) and subsequently downloaded for screening and editing using previously published HRV correction guidelines (Marchant-Forde et al., 2004). A single 256-beat data set with less than 5% corrected erroneous beats was selected at each time point (d -1, 0, 1, and 3) for each piglet in the SHRV and CHRV treatment groups. The data sets were chosen during the first available period of inactivity that contained a sufficient amount of useable data. Chosen data sets had to have occurred at least 10 min after interaction with study personnel.

Linear HRV measures (RR, SDNN, RMSSD, LF/HF), as well as SampEN and DFAα_1 were obtained using available HRV analysis software (Kubios HRV Standard; Kubios Oy, Kuopio, Finland). All data were detrended (first-order differencing) prior to analysis of SDNN, RMSSD, LF/HF, SampEn, and DFAα_1. As is recommended, data used for spectral analysis of LF/HF were re-sampled at 4 Hz to obtain at least 512 equidistant data points before undergoing fast Fourier transformation to obtain a HRV spectrum (Task Force of the European Society of Cardiology and

the North American Society of Pacing and Electrophysiology, 1996). High frequency (**HF**) and low frequency (**LF**) spectral limits were set according to previously published guidelines (HF: 0.33-0.83; LF: 0.0-0.33; von Borell et al., 2007). A spectral window length of 50% was set to reduce spectral leakage in the signal. Measurement of sample entropy was standardized using an embedding dimension of 2 heart beats with a threshold of 0.15 x SD. Short-term detrended fluctuation analysis ($DFA\alpha_1$) was conducted using a window range of 4 to 16 heart beats.

The remaining nonlinear HRV measures (%DET, %REC, Lmean) were determined using recurrence quantification analysis (**RQA**; RHRV package in R 3.3.3; R Foundation for Statistical Computing; Vienna, Austria), which quantifies recurring data points or periodicities present in multi-dimensional state-space (Eckmann et al., 1987). The time delay was determined using the average mutual information method ("mutual" command in the 'tserieschaos' package) and set to 4 by taking an average time delay value from all data sets. A single embedding dimension of 5 was selected similarly using the nearest false neighbor method ("FNN" command in the 'fractal' package; Parameters: dimension = 15, lag = determined from AMI for each data set, R_{tol} = 15, A_{tol} = 2). The radius required for RQA was set to 4 beats so the majority of the HRV datasets had a %REC between 1 and 5% (Wallott, 2017).

2.3.8 Blood Analysis

After each collection, blood samples were allowed to clot at room temperature for approximately 2 h before undergoing centrifugation (1,900 x g for 15 min) at 4℃. Samples were then collected, aliquoted, and stored at -80℃ until subsequent cortisol analysis. A commercially available cortisol RIA kit (ImmuChem Cortisol Coated Tube Kit; MP Biomedicals, Inc., Santa Ana, CA) was used to determine serum cortisol concentrations. Intra- and inter-assay CV were 5.2 and 13.5%, respectively.

2.3.9 Statistical Analysis

Data were analyzed in SAS 9.4 (SAS Institute Inc., Cary, NC) using a generalized linear mixed model procedure (Proc GLIMMIX) with repeated measures (experimental unit: piglet nested within treatment). Percent time spent lying, cortisol concentration, and each HRV measure were used as independent variables in individual models. Treatment (HRV analysis: SHRV or CHRV; cortisol analysis: SBC or CBC), time (HRV analysis: d 0, d 1, d 3 relative to treatment; cortisol analysis: 1, 2, 3, 24, 48, 72 h relative to treatment), and the interaction between treatment and time served as independent fixed factors. Baseline measurement (for all independent variables), piglet weight, and repetition (1 or 2) were also included as covariates. Prior to analysis, data were transformed as needed ($\log_{10}$ transform: SDNN, RMSSD, LF/HF, %DET, %REC; square root transform: cortisol concentration, RR, Lmean) in order to meet the residual normality and homogeneity of variance assumptions of the model. A Kenward-Roger degrees of freedom approximation was applied during all analyses and pre-determined multiple comparisons were evaluated using a Bonferroni correction. Data are presented as least squares means ± SE. Data that were transformed prior to analysis are presented as back-transformed least squares means ± approximated SE determined using the delta method. A significant result was defined as $P \leq 0.05$. A tendency in the data was defined as $0.05 \leq P \leq 0.1$. Data from 1 CHRV piglet and 1 SBC piglet were removed from statistical analysis due to highly erroneous HRV data and a case of illness, respectively. Therefore, statistical analyses of HRV data were carried out with 8 SHRV and 7 CHRV piglets. Statistical analyses of serum cortisol concentrations were conducted with 7 SBC and 8 CBC piglets.

2.4 Results

2.4.1 Postural Behavior

Piglets spent more time lying on d 0 (71.1 ± 3.5%; $t_{23.67} = 3.09$; $P = 0.01$) and tended to spend more time lying on d 1 (66.1 ± 3.5%; $t_{23.67} = 2.22$; $P = 0.08$) compared to d 3 (53.0 ± 3.8%). This result was likely due to a large numerical decline in time spent lying exhibited by SHRV piglets on d 3 (Fig. 2.2). There was no effect of treatment, the interaction between treatment and time, or weight on time spent in an inactive position ($P > 0.05$).

2.4.2 Cortisol

After adjustment for multiple comparisons, castrated piglets in the blood collection group tended to exhibit greater serum cortisol concentrations than SBC piglets at 1 h (52.1 ± 8.9 vs. 23.1 ± 6.5 ng/mL; $t_{52.4} = 2.6$; $P = 0.08$; Fig. 2.3) and 24 h post-castration treatment (31.4 ± 6.9 vs. 11.3 ± 3.9 ng/mL; $t_{51.52} = 2.6$; $P = 0.08$; Fig. 2.3). No effects of weight or repetition on serum cortisol concentration were detected ($P > 0.05$).

2.4.3 Linear HRV Measures

No effect of treatment, time, the interaction between treatment and time, weight, or repetition were detected for RR (Fig. 2.4), SDNN (Fig. 2.5), or RMSSD (Fig. 2.6; $P > 0.05$).

Castrated piglets exhibited greater LF/HF compared to SHRV piglets (26.8 ± 3.2 vs. 9.0 ± 1.1 arbitrary units; $F_{1,10.42} = 7.53$; $P = 0.02$; Fig. 2.7). No additional effects of time, the interaction between treatment and time, weight, or repetition were detected for LF/HF ($P > 0.05$).

2.4.4 Nonlinear HRV Measures

Castrated HRV piglets exhibited lower SampEn compared to SHRV piglets (1.47 ± 0.15 vs. 1.97 ± 0.16 bits; $F_{1,10.4} = 5.33$; $P = 0.04$; Fig. 2.8). No additional effects of time, the interaction between treatment and time, weight, or repetition were detected for SampEn. ($P > 0.05$).

No effect of treatment, time, the interaction between treatment and time, weight, or repetition were detected for DFAα_1 (Fig. 2.9; $P > 0.05$).

Castrated piglets exhibited greater %DET compared to SHRV piglets (54.0 ± 2.1 vs. 38.8 ± 2.0%; $F_{1,12.07} = 4.93$; $P = 0.05$; Fig. 2.10). No additional effects of time, the interaction between treatment and time, weight, or repetition were detected for SampEn. ($P > 0.05$).

No effect of treatment, time, the interaction between treatment and time, weight, or repetition were detected for %REC (Fig. 2.11) or Lmean (Fig. 2.12; $P > 0.05$).

2.5 Discussion

The purpose of the current study was to evaluate HRV as a potential non-invasive indicator of pain-related stress over a 3-d period following surgical castration in piglets. Surgical castration is typically conducted without pain mitigation and results in altered piglet behavior and physiology. For example, there is some evidence that castrated piglets lay less and stand more than sham-castrated piglets (Taylor et al., 2001). In the current study, however, there was no difference in the amount of time spent lying by CHRV and SHRV piglets, a result that is in agreement with other previously published studies on castration pain behavior that found little influence of castration on posture alone (Hay et al., 2003; Moya et al., 2008; Sutherland et al., 2012). One study found that surgically castrated piglets prostrated more often, exhibited greater levels of stiffness, trembling, scratching, and tail wagging, and were more inactive regardless of posture than their sham-castrated counterparts (Hay et al., 2003). This indicates that postural behavior is not a consistently reliable measure of pain and a more detailed behavioral observation is likely needed

In addition to the likelihood that posture is not a consistently reliable measure of pain, it is also possible that the performance of postural behaviors was affected by the HR monitor that was worn by CHRV and SHRV piglets during the study. Although light in weight by human standards

(60 g) and despite the acclimation process, the HR monitor and electrode strap may have led to altered behavior for both treatments, making them indistinguishable from one another. This concern has been raised previously in animal studies that utilize automated data collection equipment attached to the experimental animal (Buijs et al., 2018).

No significant differences in serum cortisol concentration were observed between treatments following the castration procedure, however, CBC piglets tended to exhibit greater cortisol concentrations than SBC piglets at 1 and 24 h post-castration. Cortisol is secreted by the adrenal glands during times of stress in an attempt to maintain homeostasis by suppressing the immune response and inducing gluconeogenesis, protein catabolism, and lipid catabolism (Buckingham, 2006). Previous work evaluating the cortisol response of surgically castrated piglets has indicated that serum cortisol reaches maximal concentration approximately 0.5 to 1 h post-castration (Carroll et al., 2006; Sutherland et al., 2012) before declining. However, the ability of cortisol to distinguish between surgically castrated piglets and sham castrated piglets varies (Hay et al., 2003; Prunier et al, 2005; Moya et al. 2008; Marchant Forde et al., 2009). Additionally, interpretation of the cortisol response to castration can be difficult given the likelihood that cortisol secretion is affected by additional factors such as general arousal and handling (Rault et al., 2011).

Linear measures of HRV were largely unchanged in CHRV piglets compared to SHRV piglets. One particularly interesting finding was that there was very little variability present in the HRV data sets, as measured by RR, SDNN, and RMSSD. This may have been due to limitations associated with the HR monitor, which exhibited a clear upper HR limit of measurement (approximately 240 beats per min). As a result, all HRV measures in the study were derived from clean HR data (less than 5 % erroneous beats prior to correction) that occurred during times of rest and did not go above the upper HR limit of measurement.

There was an effect of castration on LF/HF, which potentially indicates increased sympathetic activity in response to the procedure. The low frequency to high frequency ratio is obtained via spectral analysis of the HRV data that has undergone a fast Fourier transformation. Previous studies have indicated that HF spectra can be removed by the administration of anti-cholinergic drugs (*e.g.* atropine), and the LF spectra can be reduced (but not completely removed) by beta-adrenergic receptor antagonists such as propranolol (Poletto et al., 2011). Therefore, the LF/HF ratio is commonly interpreted to represent the balance between the SNS and PNS branches, where an increase in LF/HF indicates increased sympathetic activity. There is some debate on the physiological interpretation of LF/HF since the interaction between the SNS and PNS is not exclusively linear and LF spectra likely represent sympathetic and parasympathetic activity together (Billman, 2013). Additionally, both LF and HF can be highly affected by respiration, which was not monitored during this study (Brown et al., 1993). Therefore, it's possible that sympathetic activity was increased in CHRV piglets compared to SHRV piglets, however, this interpretation lacks confidence without additional changes to the remaining linear HRV measures.

An additional focal point of the current study was the inclusion of nonlinear HRV measures that complement traditional linear HRV measures and may improve HRV methodology for distinguishing between treatment groups. Heart rate is affected by a number of physiological processes that interact and exert a nonlinear influence on the sinoatrial node of the heart, leading to HRV that cannot be described as strictly linear (von Borell et al., 2007). However, little work evaluating nonlinear HRV measures in swine (outside of biomedical research; *e.g.* Batchinsky et al., 2007) has been conducted. In previous research with dairy cattle, nonlinear HRV measures such as %DET, %REC, and maximum diagonal line length within a recurrence quantification plot

have been used to evaluate various stressors such as milking system types, heat stress, illness, and metabolic status (Mohr et al., 2002; Hagen et al., 2005; Erdmann et al., 2017)

In the current study, piglets in the CHRV group exhibited lower overall SampEn and greater %DET than SHRV pigs, indicating that they experienced more pain-related stress in the days following castration. Sample entropy is defined as "the negative natural logarithm of the conditional probability that a dataset of length *N*, having repeated itself within a tolerance *r* for *m* data points, will also repeat itself for *m* + 1 points, without allowing self-matches (Lake et al., 2002)." In other words, sample entropy evaluates the likelihood of data fluctuations over time, where lower SampEn values are indicative of less HRV fluctuation unpredictability and greater stress (Batchinsky et al., 2007; Sassi et al., 2015). While there was no treatment by time interaction, SampEn was numerically lower for CHRV pigs on each day of the 3-d post castration period. This result is particularly evident on d 3, where SampEn continued to decrease for CHRV piglets compared to SHRV piglets. Therefore, it's possible that the pain-related stress resulting from castration did not completely diminish prior to the end of 3-d experimental period. This is in agreement with previous studies evaluating piglet behavior, where castrated piglets exhibited altered scratching and tail wagging behaviors for 3 and 5 d post-castration, respectively (Hay et al., 2003).

Percent determinism is obtained using RQA, which allows multi-dimensional data to be represented in a 2-dimensional plot for quantification of recurring data points, patterns, and trajectories that, in many cases, go undetected by other nonlinear measures (Eckmann et al., 1987). Specifically, a HRV data set that has been unfolded into multi-dimensional state-space is graphed against itself on the x and y axes of the RQA plot, where any recurring points (within a certain radius, *r*) are marked with a single point on the plot (Wallott, 2017). Periodicity, or recurring

patterns, within a data set are detected due to diagonal lines that are formed by recurring points that occur in similar order (Wallott, 2017). Percent determinism uses the number of recurring points that make up diagonal lines in the recurrence plot as an indicator of periodicity present within the data (Wallot, 2017). Piglets in the CHRV treatment had greater %DET compared to SHRV piglets, indicating more HR periodicity and further demonstrating greater pain-related stress following the castration procedure (Hagen et al., 2005).

2.6 Conclusion

Following surgical castration, CHRV piglets exhibited lower SampEn and greater %DET than SHRV piglets, indicating greater pain-related stress. However, postural behavior, serum cortisol, and most of the linear HRV measures (other than LF/HF) were unable to distinguish between castration and sham-castration treatments. Therefore, the inclusion of nonlinear HRV measures may be valuable for evaluating surgical castration pain in future studies. Additional work should evaluate new HR monitor technologies and any potential effect they may have on behavior and physiology when worn by the piglet.

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Table 2.1. Definitions of Heart Rate Variability Parameters.

Parameter	Practical Definition
Linear Measures	
Time Domain	
Average RR Interval (RR), ms	Average interval between adjacent heart beats over a period of time.
Standard deviation of RR intervals (SDNN), ms	The standard deviation of all RR intervals over a period of time.
Root Mean Square of Successive Differences (RMSSD), ms	The root mean square of successive RR intervals over a period of time. Greater levels indicate increased parasympathetic input.
Frequency Domain	
Low frequency to high frequency ratio (LF/HF)	The ratio between low and high frequency spectra after fast Fourier transformation of RR interval data. Greater values indicate increased sympathetic input.
Nonlinear Measures	
Sample Entropy (SampEn)	Measures the likelihood that runs of data patterns (vector length of *m* data points) that are close to each other will remain close if the vector length is increased by one ($m + 1$; Pincus 1995). Lower values indicate increased regularity in the HRV data.
Short-term detrended fluctuation analysis (DFAα_1)	A short-term measure of RR fluctuations at various time lengths to evaluate HR signal self-similarity. $\alpha_1 > 0.5$: Data are negatively-correlated. $\alpha_1 = 0.5$: Data are random, no long-range correlations. $0.5 < \alpha_1 < 1$: Data have long-range correlations. $1 < \alpha_1 < 2$: Data are correlated but do not have long-range correlations. Long-range correlations indicate increased self-similarity of the HRV data at different time lengths.
Recurrence rate (%REC), %	Determined using recurrence quantification analysis (RQA), the percentage of recurrent points (within some radius, *r*) in the recurrence plot. Greater values indicate increased HR regularity.
Determinism rate (%DET), %	Determined using recurrence quantification analysis (RQA), the percentage of recurrent points that form a diagonal line in the recurrence plot. Larger values indicate greater incidence of periodicity in the HRV data.
Mean line length of diagonal lines (Lmean), beats	Determined using recurrence quantification analysis (RQA), the mean length of diagonal lines in the recurrence plot. Greater values indicate periodicities with longer durations in the HRV data.

Figure 2.1. Body socks placed on all piglets in a litter to reduce “targeting” of piglets wearing heart rate monitors by littermates. Heart rate monitors were worn underneath the body sock.

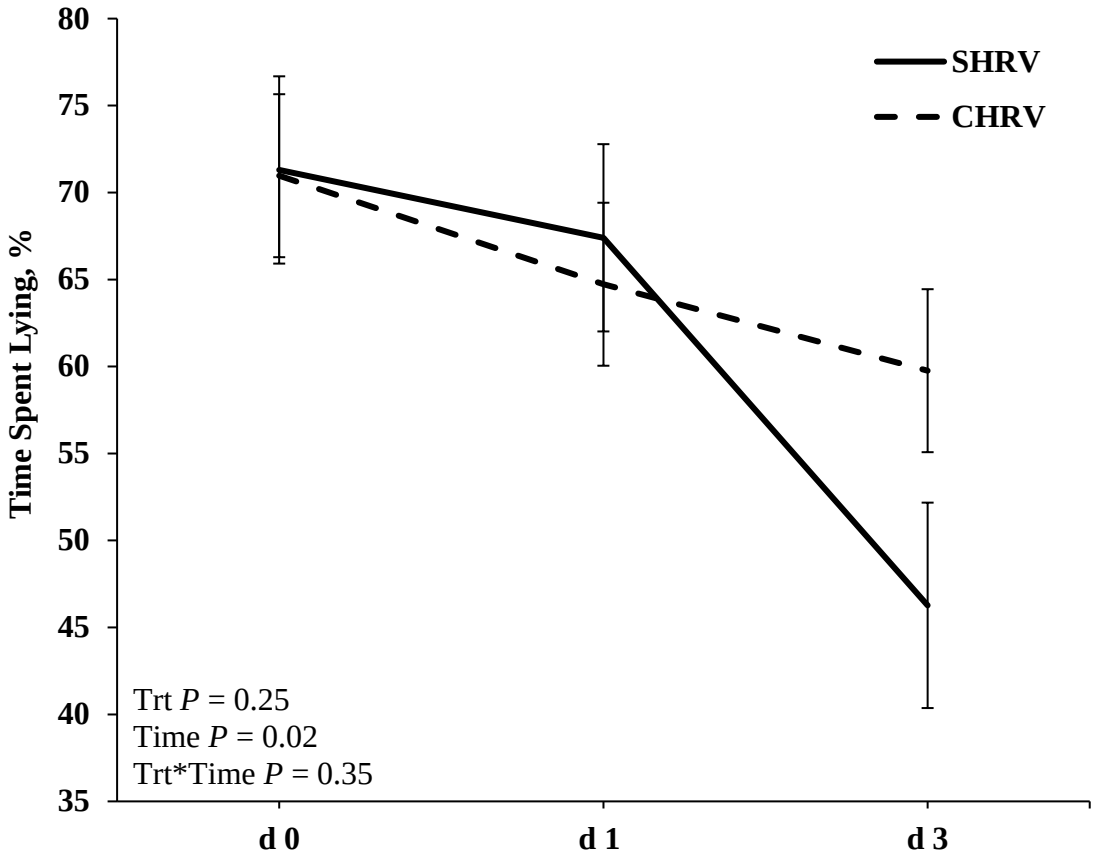

Figure 2.2. Least squares means ± SE of time spent lying (%) for the 3-d experimental period organized by treatment. Piglets in the CHRV treatment group were castrated on d 0. Piglets in the SHRV treatment group underwent simulated castration on d 0 but were not castrated until after the experiment ended.

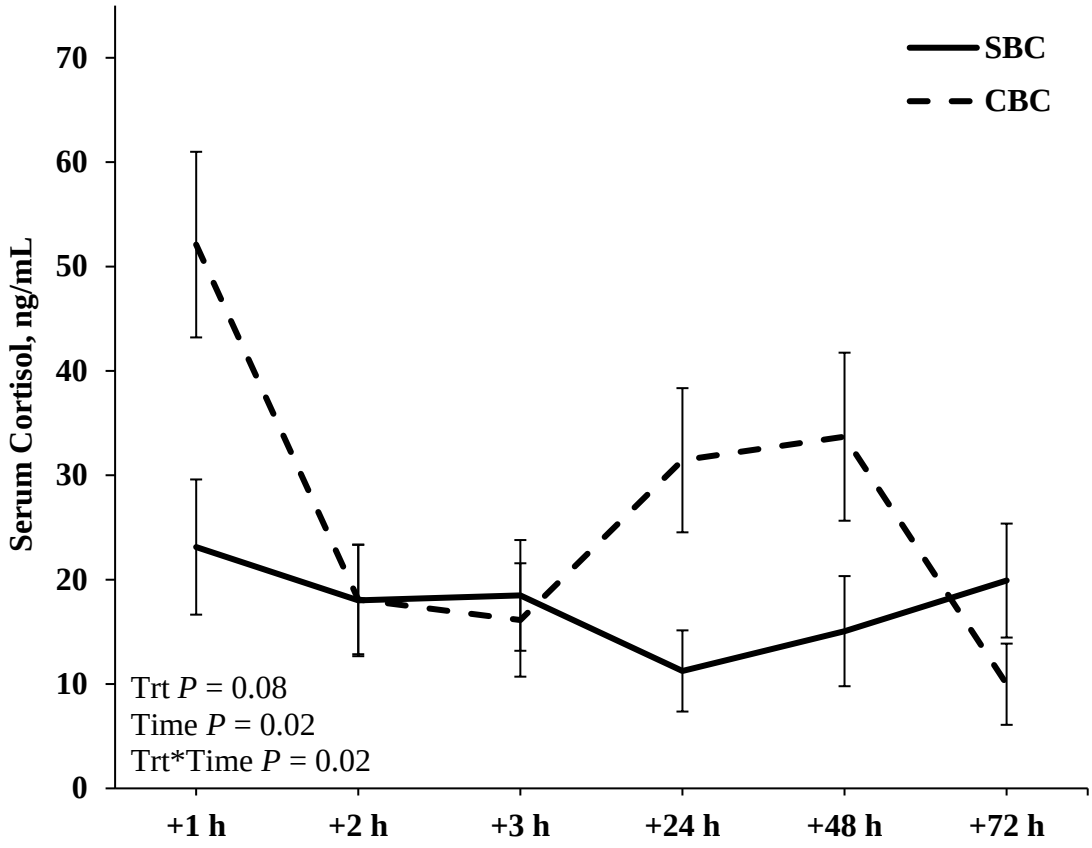

Figure 2.3. Back-transformed least squares means ± approximated SE of piglet serum cortisol concentrations for the 3-d experimental period organized by treatment. Piglets in the CBC treatment group were castrated on d 0. Piglets in the SBC treatment group underwent simulated castration on d 0 but were not castrated until after the experiment ended. After adjustment for multiple comparisons, there was no effect of time on cortisol concentration ($P > 0.10$) and cortisol concentrations only tended to differ between treatments at 1 and 24 h post-castration. No other differences were detected.

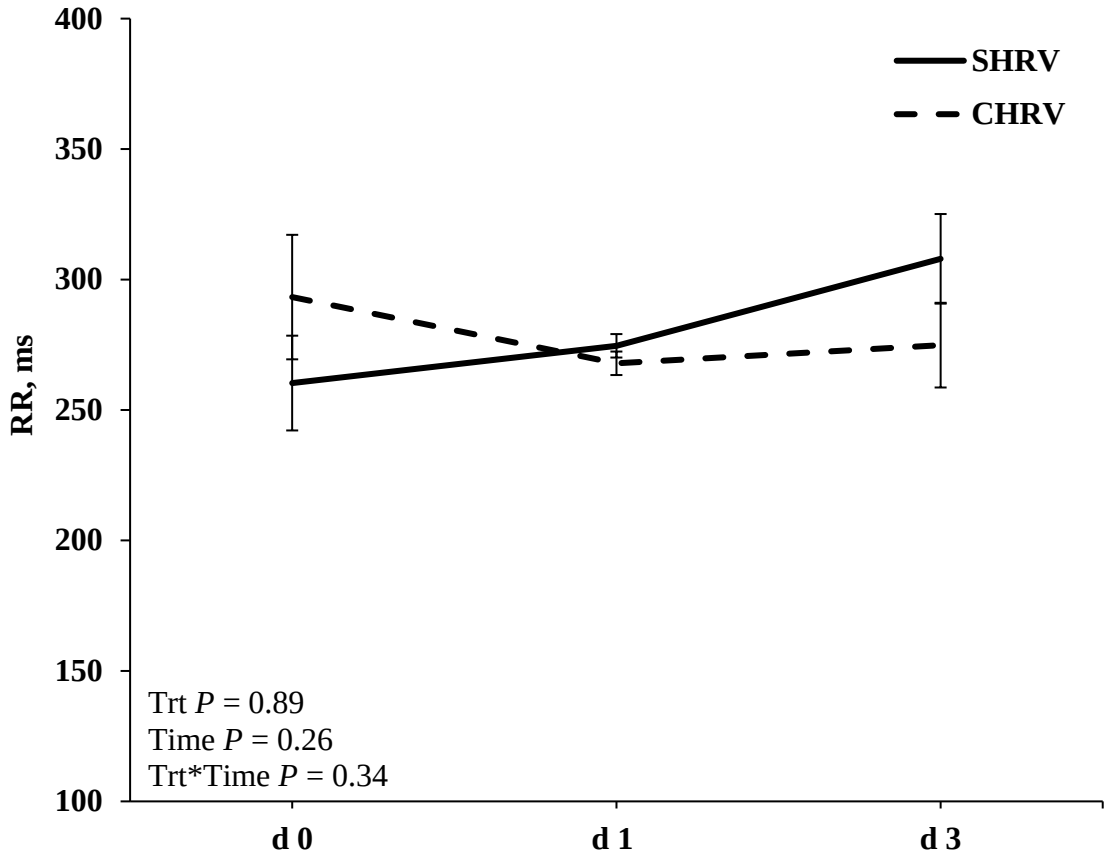

Figure 2.4. Back-transformed least squares means ± approximated SE of mean R-R interval (RR) over the 3-d experimental period organized by treatment. Piglets in the CHRV treatment group were castrated on d 0. Piglets in the SHRV treatment group underwent simulated castration on d 0 but were not castrated until after the experiment ended.

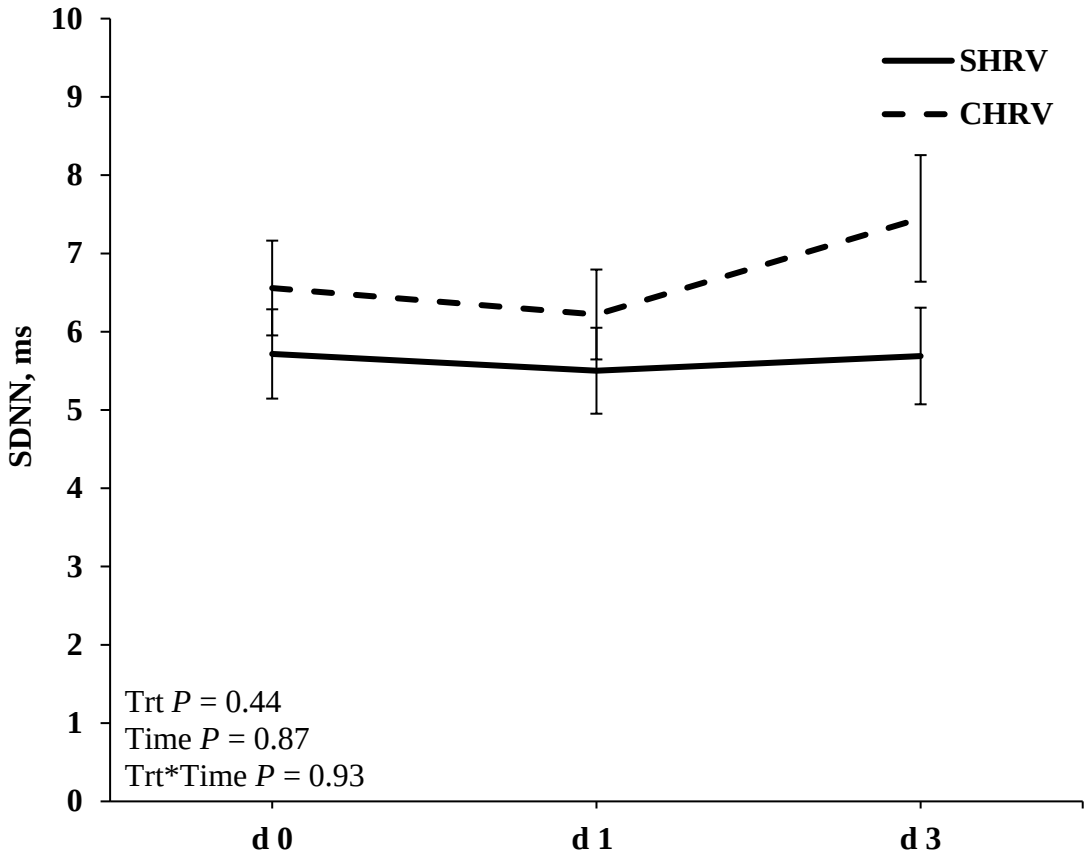

Figure 2.5. Back-transformed least squares means ± approximated SE of standard deviation of the R-R intervals (SDNN) over the 3-d experimental period organized by treatment. Piglets in the CHRV treatment group were castrated on d 0. Piglets in the SHRV treatment group underwent simulated castration on d 0 but were not castrated until after the experiment ended

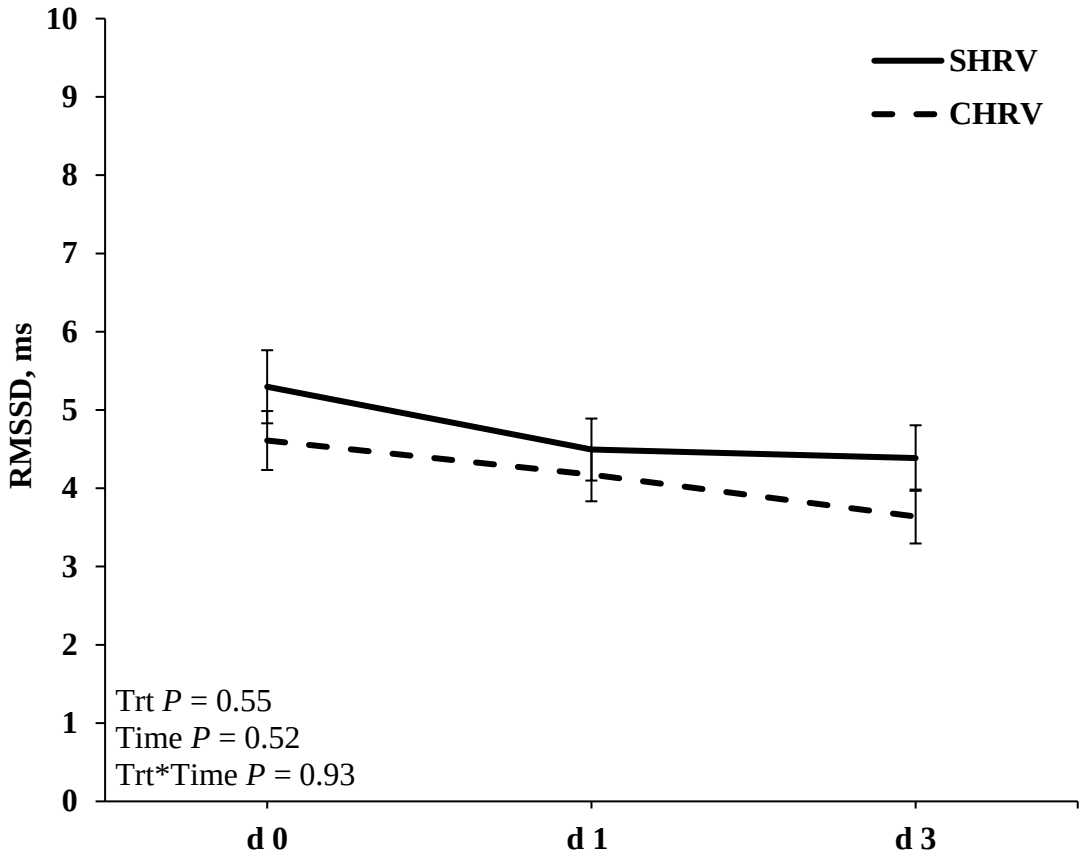

Figure 2.6. Back-transformed least squares means ± approximated SE of root mean of successive squared differences (RMSSD) over the 3-d experimental period organized by treatment. Piglets in the CHRV treatment group were castrated on d 0. Piglets in the SHRV treatment group underwent simulated castration on d 0 but were not castrated until after the experiment ended.

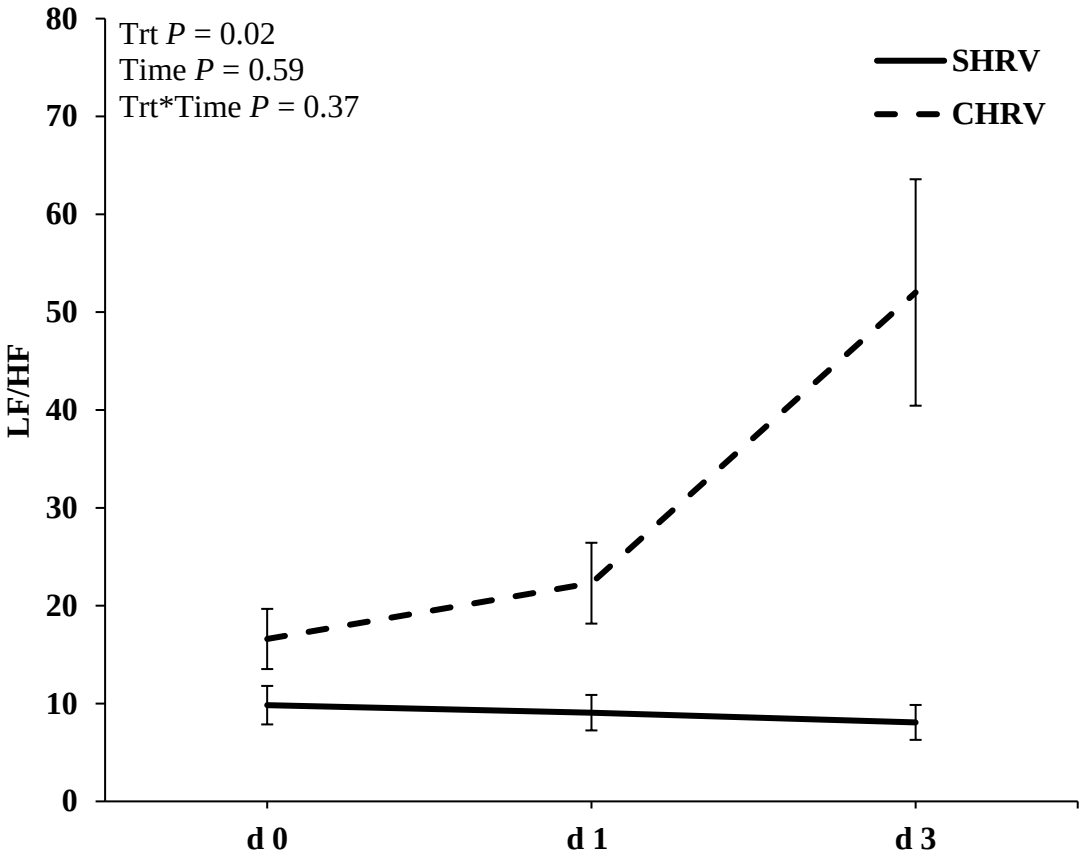

Figure 2.7. Back-transformed least squares means ± approximated SE of low frequency to high frequency ratio (LF/HF) over the 3-d experimental period organized by treatment. Piglets in the CHRV treatment group were castrated on d 0. Piglets in the SHRV treatment group underwent simulated castration on d 0 but were not castrated until after the experiment ended.

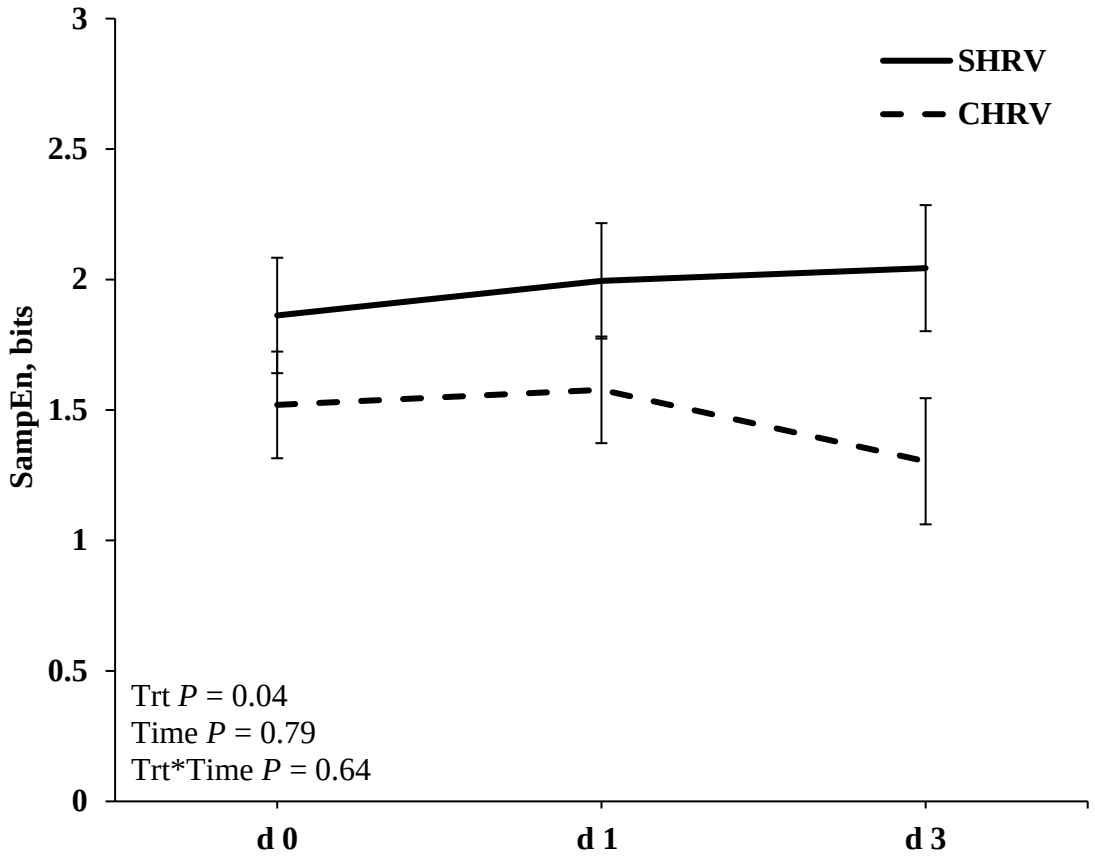

Figure 2.8. Least squares means ± SE of sample entropy (SampEn) over the 3-d experimental period organized by treatment. Piglets in the CHRV treatment group were castrated on d 0. Piglets in the SHRV treatment group underwent simulated castration on d 0 but were not castrated until after the experiment ended.

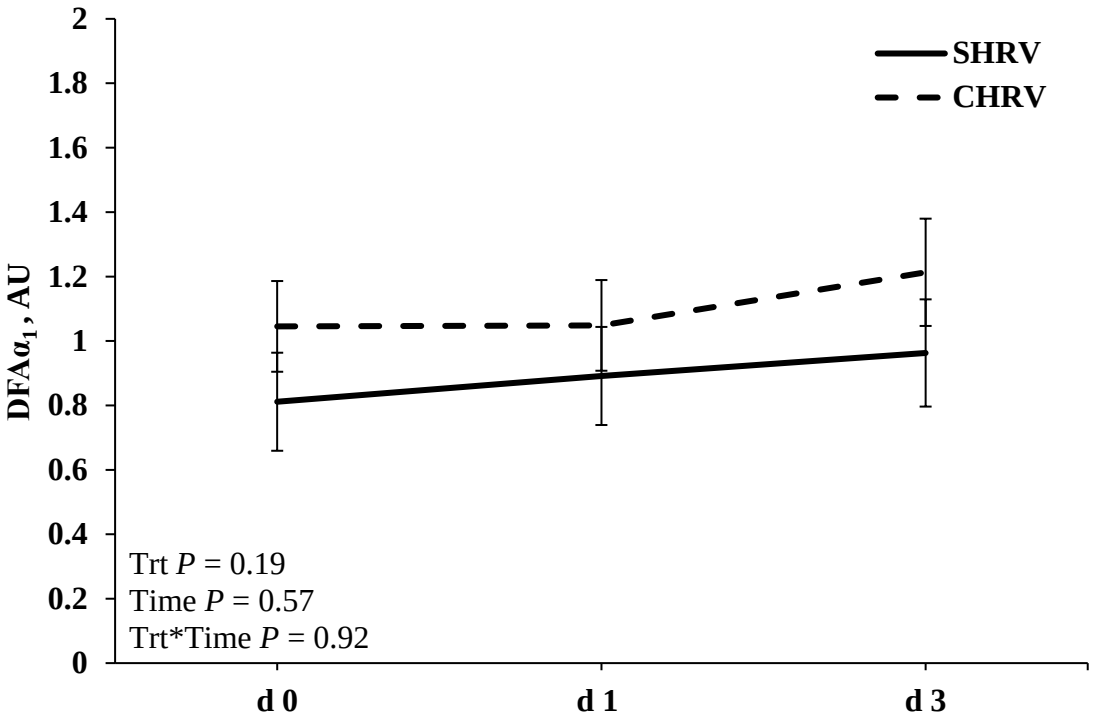

Figure 2.9. Least squares means ± SE of detrended fluctuation analysis (DFAα_1) over the 3-d experimental period organized by treatment. Piglets in the CHRV treatment group were castrated on d 0. Piglets in the SHRV treatment group underwent simulated castration on d 0 but were not castrated until after the experiment ended.

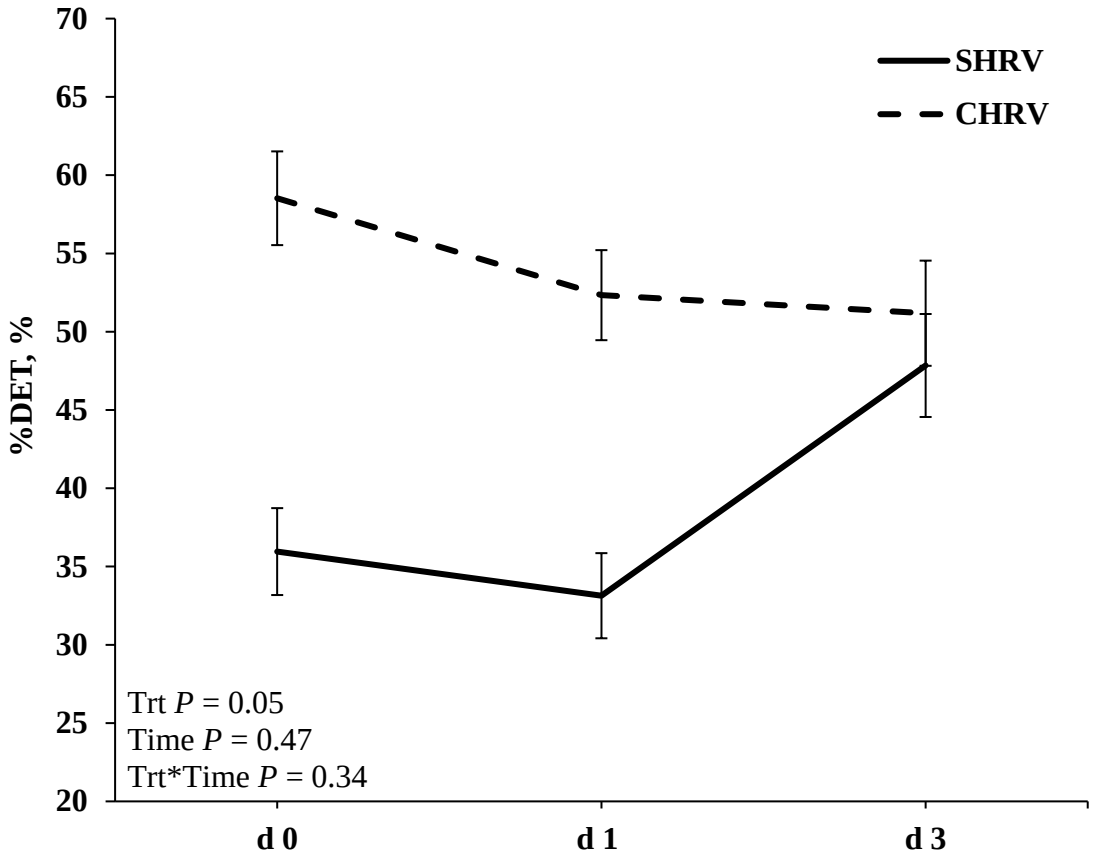

Figure 2.10. Back-transformed least squares means ± approximated SE of percent determinism (%DET) over the 3-d experimental period organized by treatment. Piglets in the CHRV treatment group were castrated on d 0. Piglets in the SHRV treatment group underwent simulated castration on d 0 but were not castrated until after the experiment ended.

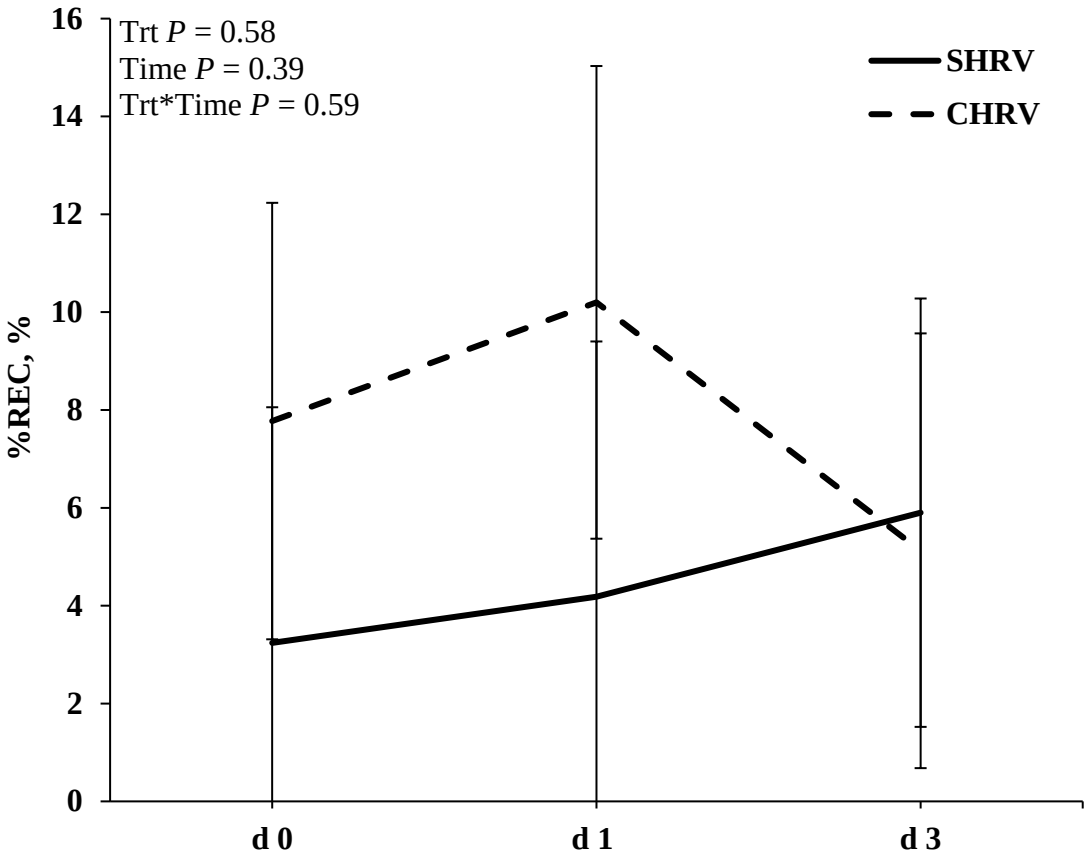

Figure 2.11. Back-transformed least squares means ± approximated SE of percent recurrence (%REC) over the 3-d experimental period organized by treatment. Piglets in the CHRV treatment group were castrated on d 0. Piglets in the SHRV treatment group underwent simulated castration on d 0 but were not castrated until after the experiment ended.

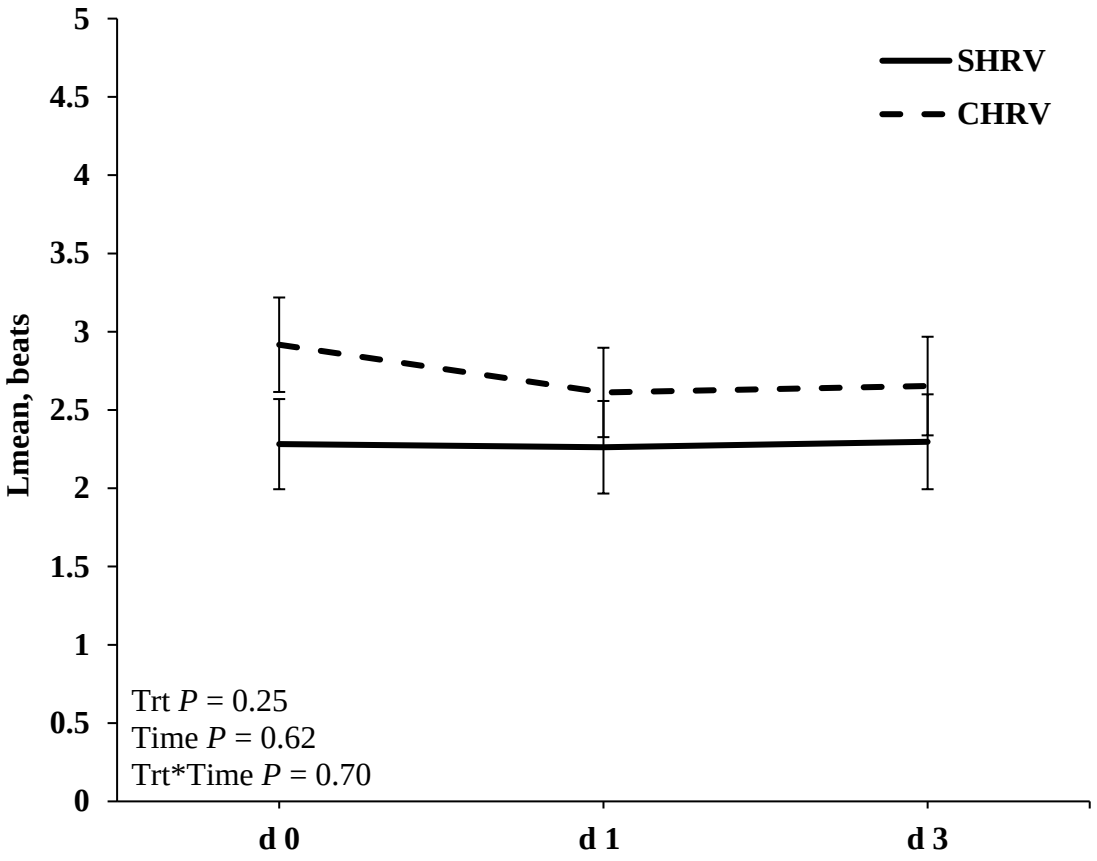

Figure 2.12. Back-transformed least squares means ± approximated SE of mean length of diagonal lines in a recurrence plot (Lmean) over the 3-d experimental period organized by treatment. Piglets in the CHRV treatment group were castrated on d 0. Piglets in the SHRV treatment group underwent simulated castration on d 0 but were not castrated until after the experiment ended.

CHAPTER 3. MEASURING DISBUDDING PAIN IN DAIRY CALVES USING NONLINEAR MEASURES OF HEART RATE VARIABILITY

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3.1 Abstract

Hot-iron disbudding is a common, but painful, procedure for dairy calves in the United States and has been shown to alter autonomic function in the period immediately following the disbudding procedure. The purpose of this study was to evaluate whether pain-related stress resulting from disbudding was long-lasting and could be measured using heart rate variability, a proxy measure of autonomic nervous system (**ANS**) function. Twenty-five female Holstein calves (4 to 7 wk of age) were randomly assigned to 1 of 3 treatments: 1) sham disbud (**SHAM**; n = 9), 2) disbud with lidocaine/meloxicam pain mitigation (**MED**; n = 8), or 3) disbud without pain mitigation (**NoMED**; n = 8). Heart rate variability was collected on d -1, 0, 1, 3, and 5 of the experimental procedure, with disbudding taking place on d 0. Additionally, blood was collected via jugular catheter at –0.5, 0.5, 1, 2, 4, 8 and 24 h relative to disbudding for cortisol analysis. Plasma cortisol concentrations for NoMED calves were greater (45.9 ± 7.6 ng/mL) at 0.5 h post-disbudding compared to both MED (11.4 ± 4.8 ng/mL; $P = 0.03$) and SHAM calves (10.5 ± 3.7

ng/mL; P = 0.003). Calves in the SHAM treatment group spent more time in an active posture (32.7 ± 2.6 %) than MED (21.2 ± 2.8 %; P = 0.02) and NoMED (22.1 ± 2.8 %; P = 0.03) calves. Average R-R interval (**RR**) was lower (P = 0.004) and the short-term detrended fluctuation analysis scaling exponent (**DFAα_1**) was greater (P = 0.04) in MED calves compared to SHAM calves. In conclusion, RR and DFAα_1 were altered in MED calves compared to SHAM calves, indicating increased stress as a result of the disbudding procedure. Calves in the NoMED group displayed an intermediate HRV response when compared to MED and SHAM calves, however numeric temporal changes to RR and DFAα_1 in NoMED calves clearly mirrored MED calves instead of SHAM calves. These results may indicate that calves in the MED group also experienced pain-related stress as a result of the disbudding procedure. Future research on this topic should address additional potential confounding factors, such as the influence of the ANS's role in wound healing, that may prohibit HRV measurement as an indicator of disbudding pain severity.

Keywords: dairy calf, disbudding, heart rate variability, pain, stress

3.2 Introduction

Removal of the horn buds is a common, but painful procedure for dairy calves in the United States, where 94% of dairies perform some type of disbudding or dehorning procedure (USDA, 2018). Approximately 50% of calves born annually are disbudded with a hot iron dehorner, and of those, approximately 70% go without analgesic or anesthetic treatment for management of pain (USDA, 2018). The reluctance to provide disbudding pain management in dairy calves is often cited as a matter of cost to the producer, but recent stakeholder surveys have also reported a belief that young calves exhibit decreased sensitivity to pain due to an immature central nervous system and that disbudding pain is relatively short lasting (Robbins et al., 2015); although, previously

published evidence has shown that alterations to physiology and behavior can be long-lasting (Heinrich et al., 2009; 2010).

Disbudding or dehorning without pain management increases plasma cortisol (Petrie et al., 2006; Heinrich 2009), increases sensitivity to pressure applied to the horn bud area (Heinrich et al., 2010), increases heart and respiration rates (Stewart et al., 2008; Heinrich et al., 2009), and alters behavior for 12 to 44 h (Faulkner and Weary, 2000; Heinrich 2010). Additionally, there is evidence that changes to heart rate variability (**HRV**) and eye temperature are altered immediately post-disbudding, indicating alterations to the autonomic nervous system as a result of disbudding pain (Stewart et al., 2008). However, little work has been done to evaluate whether HRV changes resulting from disbudding or dehorning are long-lasting.

Heart rate variability is a non-invasive proxy measure of autonomic function and has been used in studies focused on evaluating the stress response of dairy cattle to common on-farm stressors (Kovács et al., 2014). The sympathetic (**SNS**) and parasympathetic (**PNS**) branches of the ANS directly innervate the heart, and in many cases, act in an antagonistic manner in response to a stressor (generally, the SNS increases heart rate while the PNS decreases heart rate). As a result, changes to the interval between adjacent heart beats over a period of time can be used as an indicator of autonomic function.

The objective of this study was to evaluate temporal changes to HRV for 5 d post-disbudding using heart rate variability. Typically, linear HRV measures are used to evaluate changes to mean, variance, and the frequency spectra of the HRV data. However, changes to both autonomic activity and HRV over time often exhibit nonlinear properties that may be better measured using nonlinear analyses capable of evaluating correlational, structural, and organizational aspects of the data (Billman, 2013; Goldberger et al., 2002a,b). Therefore, this study

differs from previous research by including nonlinear HRV measures as a complementary method for detecting disbudding pain-related stress.

We hypothesized that calves disbudded without pain mitigation would exhibit altered autonomic function and greater stress compared to calves that were disbudded with pain mitigation or sham disbudded. Specifically, we predicted that calves disbudded without pain mitigation would have lower average RR interval (**RR**; Table 3.1), lower standard deviation of the RR intervals (**SDNN**; Table 3.1), lower root mean squared of successive differences (**RMSSD**; Table 3.1), and greater low frequency to high frequency ratio (**LF/HF**; Table 3.1) than sham disbudded calves and calves that received pain mitigation. In the nonlinear HRV domain, we predicted that calves disbudded without pain mitigation would have lower sample entropy (**SampEn**; Table 3.1), greater percent determinism (**%DET**; Table 3.1), percent recurrence (**%REC**; Table 3.1), mean length of diagonal lines in a recurrence plot (**Lmean**; Table 3.1), as well as lower signal self-affinity (as measured by short term detrended fluctuation analysis; **DFAα_1**; Table 3.1) compared to calves that received pain mitigation prior to disbudding or were sham disbudded.

3.3 Materials and Methods

All experimental procedures were approved by the Purdue University Animal Care and Use Committee (#1705001576).

3.3.1 Animals and Housing

Thirty Holstein heifer calves (4 to 7 wk of age) were housed outdoors in individual calf hutches (2.13 m L x 1.17 m W x 1.35 m H) with outside runs (1.83 m L x 1.22 m W) and were managed by Purdue University Dairy Research and Education Center staff according to standard farm protocols. Approximately 2 L of milk were provided to calves twice daily at approximately

0600 h and 1600 h. Supplemental starter feed and water were provided in buckets placed inside each calf hutch and refilled as needed twice daily at similar times. Pine shavings were provided as bedding and replaced in the morning every 2 to 3 d, unless inclement weather dictated a bedding change to ensure a dry surface for the calf. All aspects of the current study occurred between July and October 2017.

3.3.2 Experimental Design and Treatment Allocation

Each calf was randomly allocated to 1 of 3 treatments: 1) sham disbud (**SHAM**; n = 10), 2) disbud with pain mitigation (**MED**; n = 10), or 3) disbud without pain mitigation (**NoMED**; n = 10). Calves that underwent hot-iron disbudding on d 0 of the experimental procedure were either given no pain mitigation (NoMED), or a cornual nerve block under each horn bud (5 mL of injectable 20 mg/mL 2% lidocaine HCl for each horn bud; VEDCO Inc., St. Joseph, MO) and 1 mg/kg BW meloxicam (15 mg oral meloxicam tablets; Patterson Veterinary Generics; Patterson Veterinary, Inc., Greely, CO, USA) approximately 15 min prior to the disbudding procedure (MED). The calves in the SHAM treatment group were treated similarly on d 0 of the experimental procedure (see Experimental Procedures below), however, they were not disbudded until after they were taken off study. The experimental procedure was conducted with groups of 3 to 4 calves over 8 repetitions. At least one calf per disbudding treatment was included in each repetition.

3.3.3 Acclimation and Baseline HRV Measurement

All calves underwent a period of acclimation to study personnel, halter, and heart rate equipment prior to the start of the experimental procedure. Once a day for 2 days, study personnel entered the outdoor portion of the hutch area and restrained the calf for approximately 5 min using a halter. A heart rate monitor (Polar H10; Polar Electro Oy, Kempele, Finland) was then fit to the calf for a period of 60 min. Following the acclimation period, the calf was once again restrained

using a halter and the HR monitor was removed. The hair immediately behind the forelegs of each calf was clipped prior the first acclimation period to ensure direct contact between the HR monitor and the calf's skin for the remainder of the study.

Following the acclimation period, each calf underwent a 2-h baseline HRV measurement period (d -1 of the experimental procedure; between 0700 h and 1000 h). Immediately prior to data collection, calves were restrained with a halter and the calf's heart rate was quickly checked via hand palpation before placing the heart rate monitor over the palpation area. Electrocardiograph gel (Spectra ECG Gel; Parker Laboratories, Inc., Fairfield, NJ) was applied to the HR monitor to improve signal conduction before securing the HR monitor and telemetric data recorder (Polar V800 Sport Watch; Polar Electro Oy, Kempele, Finland) to the calf with flexible veterinary wrap (VetWrap; 3M, Maplewood, MN). Following the 2-h baseline HRV measurement, the HR monitor and data recorder were removed from the calf.

3.3.4 Catheterization

Three hours after baseline HRV collection, an intravenous catheter (90 cm Long Line Catheter; MILA International, Inc., Florence, KY) was inserted into the right jugular vein of each on-study calf. Specifically, each calf was restrained using a halter while one study personnel restricted neck and body movement. The jugular area was then clipped and cleaned with 2% chlorhexidine/ 70% isopropyl alcohol before the personnel performing the placement procedure inserted a 19-gauge hypodermic catheter needle into the jugular vein. The catheter tubing was then threaded through the needle until approximately 15 cm of the tube remained in the vein. Once the needle was removed, a small sample of blood was drawn and replaced with 5 mL of physiological grade sterile saline. The catheter was then flushed with 2% Na-EDTA to maintain patency during the experimental procedure. The tubing near the jugular vein was taped (Elastikon; Johnson &

Johnson, New Brunswick, NJ) to the calf's neck to reduce catheter movement and the remaining length of the catheter tubing was secured under flexible veterinary wrap (VetWrap; 3M, Maplewood, MN) for blood collection the following day.

3.3.5 Experimental Procedure

On d 0 of the experimental procedure, all calves were fit with a HR monitor and underwent treatment between 0700 h and 1000 h. Briefly, the disbudding procedure for MED and NoMED calves consisted of one study personnel restraining the calf's body while another personnel stood over the calf's neck to limit movement of the calf's head and perform the procedure. A hot-iron dehorner (Express Gas Dehorner; The Coburn Company, Inc., Whitewater, WI) was applied to the horn bud area for approximately 5 to 7 seconds to detach and cauterize the surrounding skin and dermis before removing the bud with the dehorner. Sham disbudded calves received no analgesic treatment and underwent a sham disbudding procedure, where the personnel conducting the study simulated the process of hot-iron disbudding using the large end of a plastic balling gun. All calves were then left alone in their hutches and HRV measurement took place for 8 h post-treatment. Blood (5 mL) was collected at -0.5, 0.5, 1, 2, 4, 8, and 24 h relative to disbudding treatment for analysis of plasma cortisol. After each blood draw, the calf received 5 mL of physiological grade sterile saline before flushing the tube with 2% Na-EDTA to maintain patency. The catheter was removed following the 24 h blood draw.

On d 1, 3, and 5 of the experimental procedure, 2 h of HRV data were collected from each calf to evaluate temporal changes to HRV following disbudding. No additional blood collection took place during this period.

3.3.6 Behavioral Analysis

Two video cameras (LBV2723B; Lorex Technology, Inc., Markham, Ontario, Canada) were used to record calf behavior both inside and outside of each calf's hutch using a digital video system (Geo Vision VMS Software; Geo Vision Inc., Taipei, Taiwan). Posture (standing and lying) was recorded continuously for the entirety of HRV collection in order to identify periods of inactivity for HRV data selection and analysis. Additionally, the amount of time spent in each posture was quantified as an indicator of activity level throughout the experimental procedure.

3.3.7 Blood Analysis

Each blood sample collected during the experimental procedure was immediately transferred to a plastic blood collection tube (Vacutainer Serum Plastic Blood Collection tubes; Becton-Dickinson, Franklin Lakes, NJ) and allowed to clot at room temperature for 2 h before undergoing 15 min of centrifugation at 1,900 x g (4℃). Plasma was then collected, aliquoted, and stored at -80℃ for subsequent cortisol analysis. Cortisol concentrations (ng/mL) were determined via radioimmunoassay per manufacturer's instructions [Cortisol RIA (CT); IBL International, Hamburg, Germany). Average intra- and inter- assay CV were 9.9% and 6.2% respectively.

3.3.8 HRV Analysis

Heart rate variability data sets were reviewed following data collection and a single 5-min data set was identified for each calf from each HRV measurement period. Heart rate variability data from d 0 (treatment) of the experimental procedure was taken during the last 2 to 4 h of HRV collection, as this was the period of time when calves were the least disturbed by experimental personnel. All data sets occurred during times of inactivity (lying) and contained less than 10%

erroneous beats. All errors were identified and edited manually using previously published guidelines (Marchant-Forde et al., 2004).

Previous guidelines on the use of HRV recommend the inclusion of multiple linear HRV measures for HRV analysis (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). Therefore, the current study utilized several linear (RR, SDNN, RMSSD, LF/HF; Table 3.1) and nonlinear (SampEn, $DFA\alpha_1$, %DET, %REC, Lmean; Table 3.1) measures which were quantified using available HRV analysis software (Kubios HRV Standard; Kubios Oy, Kuopio, Finland; RHRV package in R 3.3.3; R Foundation for Statistical Computing; Vienna, Austria). Prior to analysis, data used for measuring RMSSD, SDNN, LF/HF, SampEn and $DFA\alpha_1$ were detrended (first-order differencing) to obtain stationarity. Data used for measuring LF/HF were first re-sampled at 4 Hz to obtain equidistant sampling and then transformed using a fast Fourier transformation. Spectral limits for measuring LF/HF were set using previously published guidelines (LF: 0.0 to 0.5, HF: 0.5 to 0.83; von Borrell et al., 2007). The spectral window length was set to 50% to decrease spectral leakage in the signal.

Percent determinism, %REC, and Lmean were obtained via recurrence quantification analysis (**RQA**). Briefly, RQA allows data in multidimensional space to be visualized using a two-dimensional plot (Eckmann et al., 1987). Recurring points, patterns, and pattern trajectories within the data can then be visualized and quantified using several measures such as %DET, %REC, and Lmean. An average time delay parameter (τ) of 7 for all datasets was determined using the average mutual information method (**AMI**; "mutual" command in the 'tserieschaos' package; R Foundation for Statistical Computing; Vienna, Austria). An average embedding dimension (m) of 5 was selected in a similar manner for all data sets using the false nearest neighbors method ("FNN"

command in the ‘fractal’ package; R Foundation for Statistical Computing; Vienna, Austria; Parameters: dimension = 15, lag = determined from AMI, R_{tol} = 14, A_{tol} = 2). A radius of 20 beats was used so the majority of data values had a %REC value between 1% and 5% (Wallott, 2017).

3.3.9 Statistical Analysis

Data were analyzed using a linear mixed model (Proc GLIMMIX) with repeated measures in SAS 9.4 (SAS Institute Inc., Cary, NC). Calf nested within treatment was the experimental unit. Each HRV measure, cortisol concentration, and time spent in an active posture were used as dependent variables in individual models. Treatment (MED, NoMED, SHAM), time (HRV and activity: d 0, d 1, d 3, d 5; Cortisol: 0.5, 1, 2, 4, 8, 24 h relative to treatment) and their interaction (trt*time) were used as independent fixed factors. Weight, age, baseline measurement (for all HRV, activity, and cortisol measures), and treatment month (July, August, September, October) were also included as covariates in the model. Data sets not meeting the model assumptions (residual normality and homogeneity of variance) were transformed (Log_{10} transform: RR, SDNN, LF/HF, %REC; Square root transform: SampEn, cortisol concentration) as needed prior to analysis. A Kenward-Rogers degrees of freedom approximation was used for all analyses. Multiple comparisons were evaluated using Tukey’s HSD test. A statistically significant result was characterized as having an alpha level less than or equal to 0.05. A tendency was defined as $0.05 \leq P \leq 0.1$. All results are presented as least squares means ± SE, however, results from datasets that were transformed prior to analysis are presented in the results and figures as back-transformed least squares means ± approximated SE obtained via the delta method. Data sets from 5 calves were not included in the analysis due to extensive HRV error. Therefore, all results are based on data from 9 SHAM calves, 8 MED calves, and 8 NoMED calves.

3.4 Results

3.4.1 Activity

Calves in the SHAM treatment group spent more time in an active posture (32.7 ± 2.6%) than MED (21.2 ± 2.8%; $t_{32.91}$ = -2.92; P = 0.02; Fig. 3.1) and NoMED (22.1 ± 2.8%; $t_{32.89}$ = -2.76; P = 0.03; Fig. 3.1) calves. There were no additional effects of time, the interaction between treatment and time, treatment month, age, or weight on time spent in an active posture (P > 0.05).

3.4.2 Cortisol

Plasma cortisol concentrations for NoMED calves were greater (45.9 ± 7.6 ng/mL) at 0.5 h post-disbudding compared to MED (11.4 ± 4.8 ng/mL; $t_{92.21}$ = -3.73; P = 0.03; Fig. 3.2) and SHAM calves (10.5 ± 3.7 ng/mL; $t_{96.5}$ = 4.42; P = 0.003; Fig. 3.2). No other differences between treatments were found for any other time (P > 0.05). No additional effects of treatment month, age, or weight on plasma cortisol concentration were detected for plasma cortisol concentration (P > 0.05).

3.4.3 Linear HRV Measures

Mean R-R interval was lower in MED calves (547.0 ± 8.1 ms; $t_{20.46}$ = -3.74; P = 0.004; Fig. 3.3) and tended to be lower in NoMed calves (584.3 ± 10.3 ms; $t_{19.66}$ = -2.32; P = 0.08; Fig. 3.3) compared to SHAM calves (664.5 ± 11.1 ms). Mean R-R interval did not differ between MED and NoMed calves (P > 0.05). There was no effect of time, the interaction between treatment and time, treatment month, age, or weight on R-R (P > 0.05).

Standard deviation of the R-R intervals (Fig. 3.4) for all calves tended to be lower (25.52 ± 1.36 ms; $t_{22.35}$ = 2.49; P = 0.08) on d 3 and was lower (23.75 ± 1.32 ms; $t_{19.82}$ = 2.92; P = 0.03) on d 5 compared to day of treatment (d 0; 33.56 ± 1.09 ms). No effects of treatment, the interaction between treatment and time, treatment month, age, or weight were detected for SDNN (P > 0.05).

No effects of treatment, time, the interaction between treatment and time, treatment month, age, or weight were detected for RMSSD ($P > 0.05$; Fig. 3.5).

Calves disbudded without pain mitigation tended to have greater LF/HF on d 0 compared to SHAM calves [132.5 ± 27.8 vs. 13.7 ± 2.7 (arbitrary units; AU); $t_{48.56} = 3.35$; $P = 0.06$; Fig. 3.6]. Overall LF/HF was greater on d 0 (53.4 ± 5.9 AU) compared to d 1 (20.4 ± 2.3 AU; $t_{39.77} = 3.22$; $P = 0.01$) and d 3 (19.1 ± 2.2 ms; $t_{60.02} = 2.96$; $P = 0.02$), which was mainly due to a large numerical increase in LF/HF exhibited by NoMED and MED calves compared to SHAM calves on d 0. No effects of treatment, time, treatment month, age or weight on LF/HF were detected ($P > 0.05$).

3.4.4 Nonlinear HRV Measures

Sample entropy tended to be lower for NoMED calves compared to SHAM calves (1.23 ± 0.06 vs. 1.60 ± 0.08 ms; $t_{22} = -2.17$; $P = 0.10$; Fig. 3.7). Sample entropy was lower on d 0 (1.08 ± 0.05 ms) compared to d 1 (1.45 ± 0.06 ms; $t_{29} = -3.05$; $P = 0.02$) and d 3 (1.59 ± 0.06 ms; $t_{33.64} = 3.78$; $P = 0.004$) due to a numerical decrease in SampEn exhibited by NoMED and MED calves on d 0, whereas SampEn in SHAM calves remained relatively consistent throughout the study period (Fig. 3.7). No effects of the interaction between treatment and time, treatment month, age, or weight were detected for Sample Entropy ($P > 0.05$).

Calves disbudded with pain mitigation exhibited greater DFAα_1 than SHAM calves (1.31 ± 0.07 AU vs. 1.03 ± 0.08 AU; $t_{20.62} = 2.60$; $P = 0.04$; Fig. 3.8). No additional treatment effects were detected ($P > 0.05$). Detrended fluctuation analysis values were greater on d 0 (1.36 ± 0.05 AU) compared to d 1 (1.15 ± 0.07 AU; $t_{26.11} = 3.06$; $P = 0.02$) and d3 (1.10 ± 0.06 AU; $t_{33.59} = 3.38$; $P = 0.01$), which was largely due to increased DFAα_1 exhibited by NoMED and MED calves on d 0 (Fig. 3.8). There were no additional effects of treatment by time interaction, treatment month, age, or weight on DFAα_1 ($P > 0.05$).

Calves disbudded with pain mitigation tended to have higher %DET on d 0 compared to SHAM calves (70.1 ± 6.7% vs. 38.4 ± 7.2%; $t_{53.44} = 3.21$; $P = 0.09$; Fig. 3.9). Percent determinism was greater on d 0 compared to d1(60.0 ± 4.1 vs. 46.4 ± 4.3%; $t_{40.38} = 2.72$; $P = 0.04$) due to a numerical increase in %DET exhibited by NoMED and MED calves on d 0 (Fig. 3.9). Additionally, %DET for all calves was lower on d 1 (46.4 ± 4.3%; $t_{60.11} = -3.24$; $P = 0.01$) and tended to be lower on day 3 (52.5 ± 4.3%; $t_{15.32} = -2.86$; $P = 0.05$) compared to d 5 (67.1 ± 5.1%). No effects of treatment, treatment month, age, or weight were detected for %DET ($P > 0.05$).

No effect of treatment, time, treatment by time interaction, treatment month, age, or weight were detected for %REC or Lmean ($P > 0.05$; Fig. 3.10; Fig. 3.11).

3.5 Discussion

Previous research has shown that linear measures of HRV are altered immediately after hot-iron disbudding without pain mitigation, indicating increased pain-related stress as a result of the procedure (Stewart et al., 2008). However, little work has evaluated whether HRV alterations are long lasting and could be used an indicator of pain severity in calves. Therefore, the purpose of the current study was to evaluate HRV over a 5-day period following hot-iron disbudding in female Heifer calves, with particular emphasis placed on the ability of nonlinear HRV measures for distinguishing between treatments.

In the absence of pain mitigation, calves who undergo hot-iron disbudding exhibit greater plasma cortisol concentrations and altered behavior, indicating increased pain as a result of the procedure (Stilwell et al., 2012). Cortisol is produced and secreted by the adrenal cortex in response to activation of the hypothalamic-pituitary-adrenal axis during times of stress. In an attempt to maintain bodily homeostasis, cortisol acts to induce gluconeogenesis, protein and lipid catabolism, and suppresses some immune function (Buckingham, 2006). Accordingly, cortisol is

a commonly used indicator of physiological stress in studies focused on animal welfare. In the current study, plasma cortisol concentration was greater for NoMED calves compared to both MED and SHAM calves 0.5 h after disbudding, indicating a greater stress response during the period immediately following the procedure. However, no differences were observed between any treatment beginning at 1 h post-disbud until the end of the blood collection period. This result is similar to those found in previous disbudding studies, where an acute cortisol increase is observed in calves disbudded without pain mitigation immediately following the procedure but approaches baseline approximately 1 to 3 h after (Petrie et al., 1996; Heinrich et al., 2009; Stilwell et al., 2012). Therefore, while cortisol may be a sufficient indicator of acute disbudding pain, it is generally not useful for evaluating chronic pain.

Calves in the NoMED and MED groups (calves that were disbudded) spent less time in an active position than SHAM calves, which may indicate increased pain or stress as a result of the procedure, however, the observed decrease in time spent in an active position by MED calves was unexpected since lidocaine and meloxicam have been found to be effective for mitigating disbudding pain (Heinrich et al. 2009, 2010). Meloxicam is commonly used in combination with an injectable local anesthetic (*i.e.* lidocaine) in studies focused on disbudding pain since local anesthetics are fast-acting but begin to wear off within several hours (Fierheller et al., 2012). Meloxicam, on the other hand, is slower to respond (maximal serum concentration is approximately 12 h post-administration; Coetzee et al., 2009) but provides a longer-term solution for pain mitigation in calves (half-life is approximately 28 h post-administration; Coetzee et al., 2009). In the current study, postural data were collected during the 2-h period from which HRV data were taken. Therefore, the postural data collected on d 0 would have taken place during the period after lidocaine had worn off (approximately 4 to 8 h post-lidocaine injection) and before

meloxicam had reached maximal serum concentration (T_{max}). It's possible that the MED calves' behavior mirrored that of NoMED calves on d 0 because meloxicam was not effective for reducing pain prior to T_{max}. However, that would not explain the large numerical difference exhibited on d 1 of the experimental procedure. Another explanation may be that the meloxicam was not effective in fully reducing disbudding pain, resulting in altered MED calf behavior that closely mirrored the behavior of NoMED calves. Previous studies have also found that meloxicam treated calves continue to perform pain related behaviors (*i.e.* head shaking, ear flicking, tail flicking) during the 44 h following disbudding (Heinrich et al., 2010), which suggests that disbudding-related pain persists despite being given meloxicam, and additional pain mitigation may be needed. Therefore, a single dose of meloxicam may not be entirely sufficient for providing pain mitigation following disbudding.

The observed HRV treatment differences in the current study were mostly observed between the SHAM and MED treatment groups, which was unexpected. Specifically, calves in the MED group had lower R-R and greater DFAα_1 overall compared to SHAM calves throughout the study period, indicating increased stress as a result of the disbudding procedure. However, similar to the behavioral results previously described, the temporal R-R and DFAα_1 responses exhibited by MED and NoMED calves were similar to each other in contrast with SHAM calves, providing further evidence that the meloxicam was not sufficient for alleviating pain-related stress. The average R-R interval measure is an inverse function of average heart rate (*i.e.* a decrease in average R-R indicates an increase in HR) and is recommended for inclusion in all HRV analyses, but its prognostic power is limited compared to the remaining HRV measures (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; von Borrell et al., 2007). Nevertheless, the observed R-R decrease for MED calves may

indicate increased pain-related stress throughout the experimental period, however, the sustained decrease in R-R throughout the experimental period is unusual when compared with previous studies measuring HR following disbudding, where an increase in HR has been observed for approximately 150-200 minutes before returning to baseline (Grøndahl-Nielsen et al., 1999; Stewart et al., 2009).

Short term detrended fluctuation analysis (DFAα_1) is a measure of long-term correlation that is based on fractal theory and can be used for evaluating stress in physiological systems. Fractals, which are present in multiple natural phenomena including physiological time series data, are objects or quantities that display statistical self-similarity at different levels of magnification. However, during times of stress or illness, these statistical similarities begin to dwindle, and long-term correlations in the data start to break down (Goldberger et al., 2002a). Detrended fluctuation analysis evaluates these changes to long term correlation by measuring the relationship between HRV fluctuations and scale of measurement over several different data lengths. The integrated HRV data set is first divided into a series of boxes containing *n* heart beats. Within each box, a least square line is fit, the data is detrended, and the root mean square deviation is calculated. Once this procedure has been carried out for all boxes of a particular length, an average value is plotted onto a log-log graph. This procedure is then repeated for the remaining boxes of different lengths and a slope of best fit is applied to the log-log graph, which returns a scaling exponent (α). In general, scaling exponents close to 1 indicate long-term correlations within the data, which is often viewed as an indicator of health in complex physiological systems (Goldberger et al., 2002b). Scaling exponents closer to 1.5 indicate shorter-term correlations in the data, and therefore, may be an indicator of increased stress in physiological systems (Goldberger et al., 2002a). In the current study, MED calves had a scaling exponent closer to 1.5, indicating short-term correlations

indicative of greater stress, while SHAM calves exhibited long-term correlational structure. The intermediate response of the NoMED group, while not statistically different from either the MED or SHAM groups, was numerically similar to MED calves throughout the study.

Calves in the MED and NoMED groups displayed numerically similar temporal changes to several additional HRV measures (RMSSD, LF/HF, SampEn, %DET) compared to SHAM calves. While it seems likely that these results indicate that MED calves could still feel some pain after disbudding, one additional factor that may help explain this observation is the role of the autonomic nervous system in wound healing. Sympathetic nerve fibers innervate the dermis and play a critical role in blood circulation, lymphatic function, and regulation of sweat glands, apocrine glands, and hair follicles (Ashrafi et al., 2016). In response to a soft tissue wound, several neuropeptides are released by the sympathetic and sensory nerve fibers in the dermis that lead to inflammation, angiogenesis, and remodeling of the soft tissue (Ashrafi et al., 2016). While it's not clear whether HRV is also affected by the increase in sympathetic nerve fiber activity during wound healing, this confounding effect would prohibit HRV from being useful for evaluating pain severity if that were the case. Future studies on the topic should evaluate the effect of sympathetic activity during wound healing on HRV.

3.6 Conclusion

In response to disbudding, NoMED calves had higher plasma cortisol concentrations than MED and SHAM calves immediately following the procedure and spent less time in an active posture compared to SHAM calves. Calves that received lidocaine and meloxicam also spent less time in an active posture and exhibited lower R-R and greater $DFA\alpha_1$ compared to SHAM calves. Calves in the NoMED group displayed an intermediate HRV response when compared to MED and SHAM calves, however numeric temporal changes to RR and $DFA\alpha_1$ in NoMED calves

clearly mirrored MED calves instead of SHAM calves. These results may indicate that calves in the MED group also experienced pain-related stress as a result of the disbudding procedure. Therefore, a single dose of meloxicam may not be sufficient for mitigating disbudding pain. However, future research should rule out potential confounding factors such as the role of the ANS in wound healing that may impact the use of HRV as an indicator of disbudding pain severity in dairy calves.

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Table 3.1. Definitions of Heart Rate Variability Parameters

Parameter	Practical Definition
Linear Measures	
Time Domain	
Average RR Interval (RR), ms	Average interval between adjacent heart beats over a period of time.
Standard deviation of RR intervals (SDNN), ms	The standard deviation of all RR intervals over a period of time.
Root Mean Square of Successive Differences (RMSSD), ms	The root mean square of successive RR intervals over a period of time. Greater levels indicate increased parasympathetic input.
Frequency Domain	
Low frequency to high frequency ratio (LF/HF)	The ratio between low and high frequency spectra after fast Fourier transformation of RR interval data. Greater values indicate increased sympathetic input.
Nonlinear Measures	
Sample Entropy (SampEn)	Measures the likelihood that runs of data patterns (vector length of *m* data points) that are close to each other will remain close if the vector length is increased by one ($m + 1$; Pincus 1995). Lower values indicate increased regularity in the HRV data.
Short-term detrended fluctuation analysis ($DFA\alpha_1$)	A short-term measure of RR fluctuations at various time lengths to evaluate HR signal self-similarity. $\alpha_1 > 0.5$: Data are negatively-correlated. $\alpha_1 = 0.5$: Data are random, no long-range correlations. $0.5 < \alpha_1 < 1$: Data have long-range correlations. $1 < \alpha_1 < 2$: Data are correlated but do not have long-range correlations. Long-range correlations indicate increased self-similarity of the HRV data at different time lengths.
Recurrence rate (%REC), %	Determined using recurrence quantification analysis (RQA), the percentage of recurrent points (within some radius, *r*) in the recurrence plot. Greater values indicate increased HR regularity.
Determinism rate (%DET), %	Determined using recurrence quantification analysis (RQA), the percentage of recurrent points that form a diagonal line in the recurrence plot. Larger values indicate greater incidence of periodicity in the HRV data.
Mean line length of diagonal lines (Lmean), beats	Determined using recurrence quantification analysis (RQA), the mean length of diagonal lines in the recurrence plot. Greater values indicate periodicities with longer durations in the HRV data.

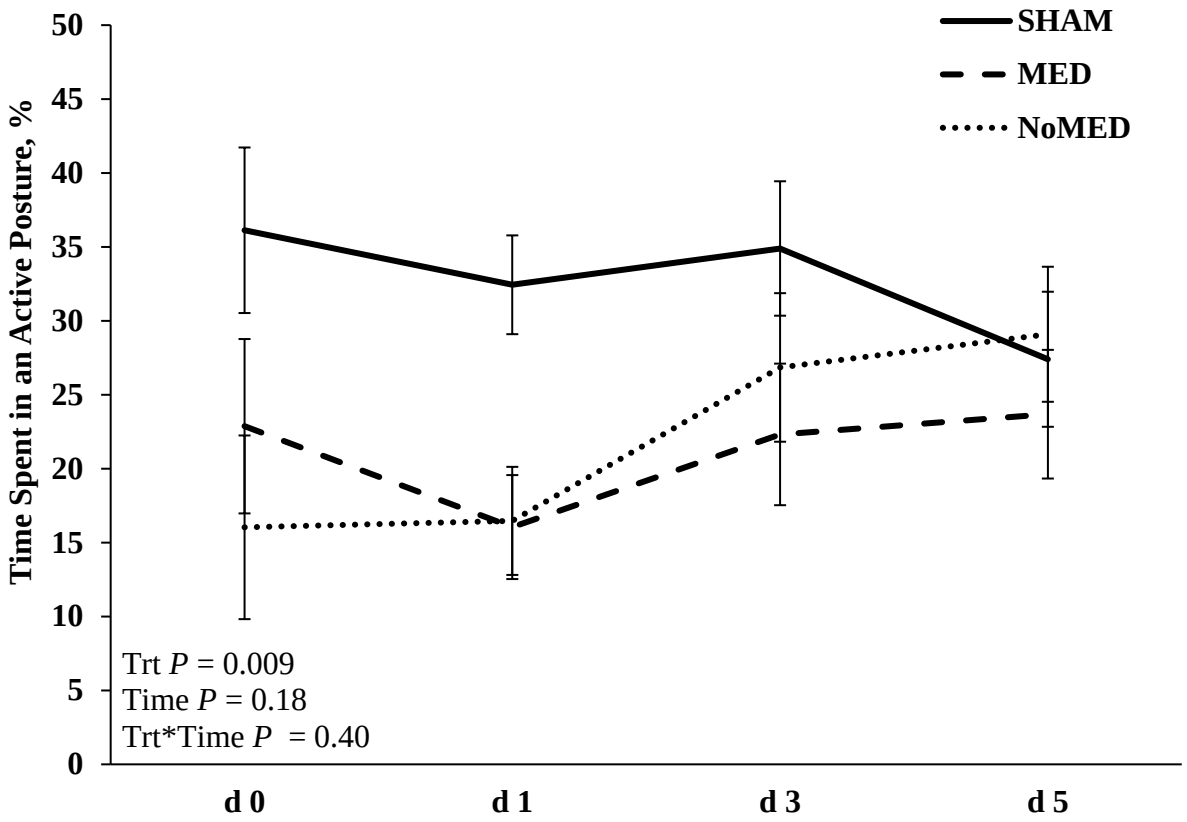

Figure 3.1. Least squares means ± SE of time spent in an active position (%) for all calves over the 5-d experimental period. Disbudding took place on d 0. Calves in the MED treatment group received lidocaine and meloxicam prior to the disbudding procedure. Calves in the NoMED treatment group did not receive any pain mitigation prior to the disbudding procedure. Disbudding was only simulated on SHAM calves.

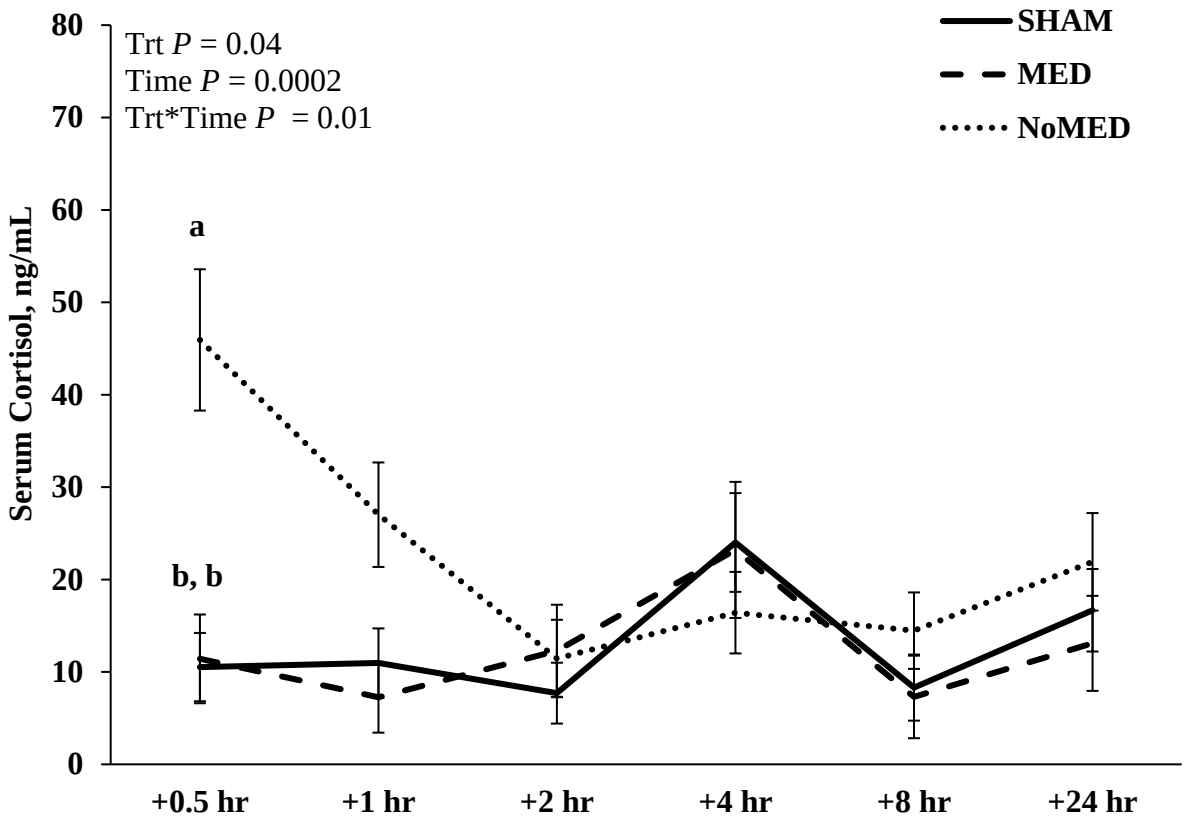

Figure 3.2. Back-transformed least squares means ± approximated SE of serum cortisol concentration for all calves over the 5-d experimental period. Disbudding took place on d 0. Calves in the MED treatment group received lidocaine and meloxicam prior to the disbudding procedure. Calves in the NoMED treatment group did not receive any pain mitigation prior to the disbudding procedure. Disbudding was only simulated on SHAM calves. Different superscripts indicate differences ($P < 0.05$) between treatments.

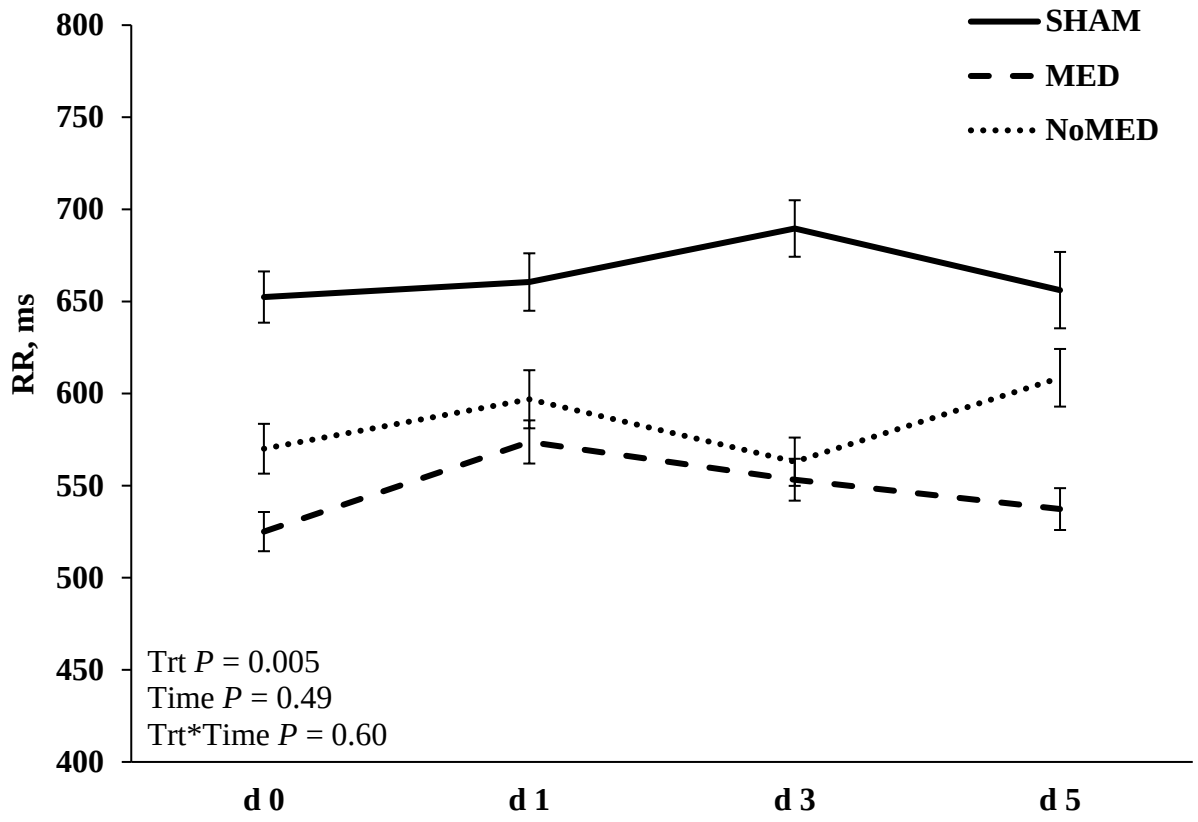

Figure 3.3. Back-transformed least squares means ± approximated SE of mean R-R interval (RR) over the 5-d experimental period. Disbudding took place on d 0. Calves in the MED treatment group received lidocaine and meloxicam prior to the disbudding procedure. Calves in the NoMED treatment group did not receive any pain mitigation prior to the disbudding procedure. Disbudding was only simulated on SHAM calves.

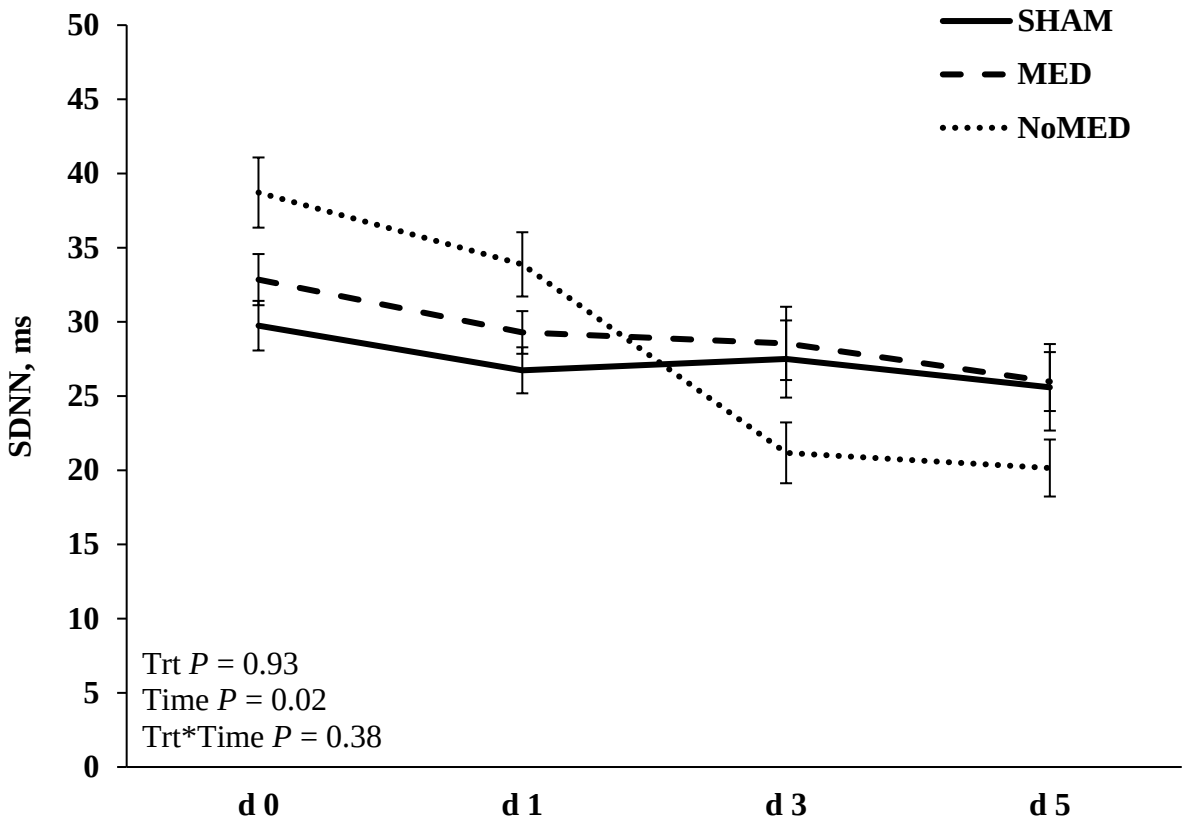

Figure 3.4. Back-transformed least squares means ± approximated SE of standard deviation of the R-R intervals (SDNN) over the 5-d experimental period. Disbudding took place on d 0. Calves in the MED treatment group received lidocaine and meloxicam prior to the disbudding procedure. Calves in the NoMED treatment group did not receive any pain mitigation prior to the disbudding procedure. Disbudding was only simulated on SHAM calves

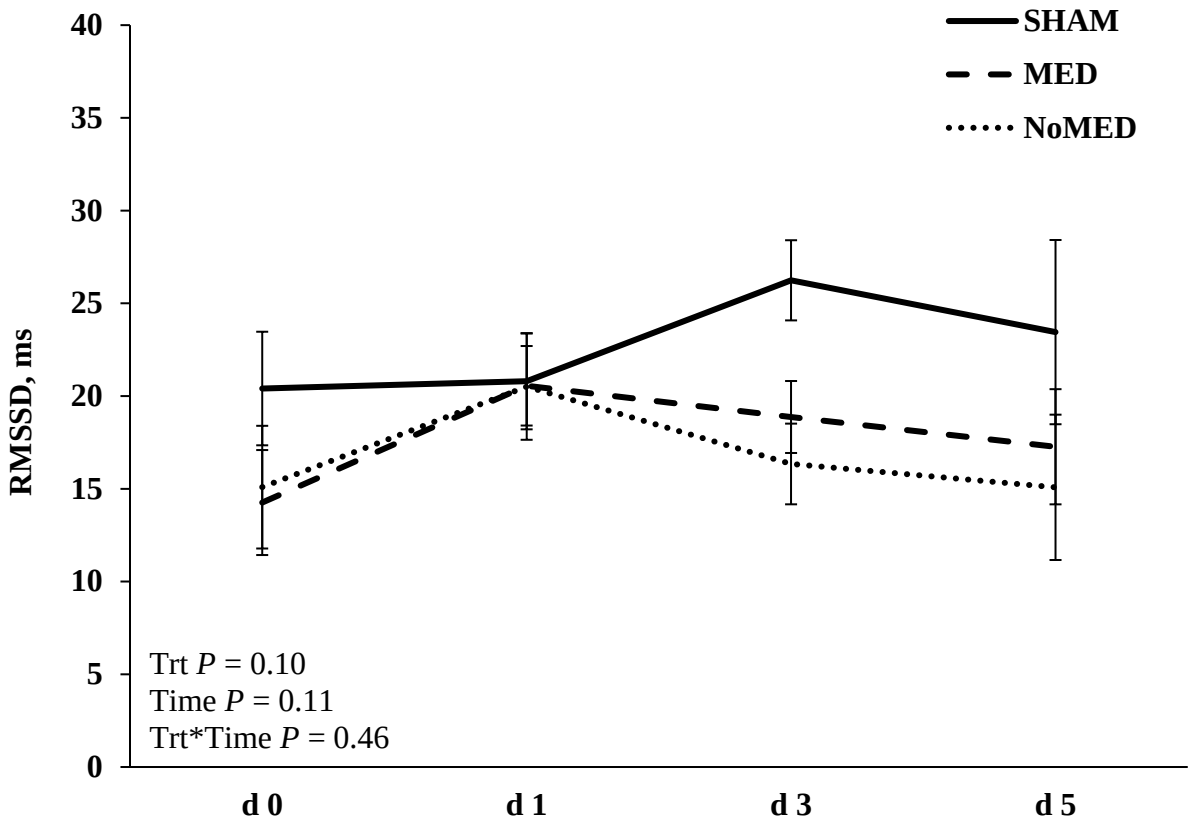

Figure 3.5. Least squares means ± SE of root mean square of successive differences (RMSSD), over the 5-d experimental period. Disbudding took place on d 0. Calves in the MED treatment group received lidocaine and meloxicam prior to the disbudding procedure. Calves in the NoMED treatment group did not receive any pain mitigation prior to the disbudding procedure. Disbudding was only simulated on SHAM calves.

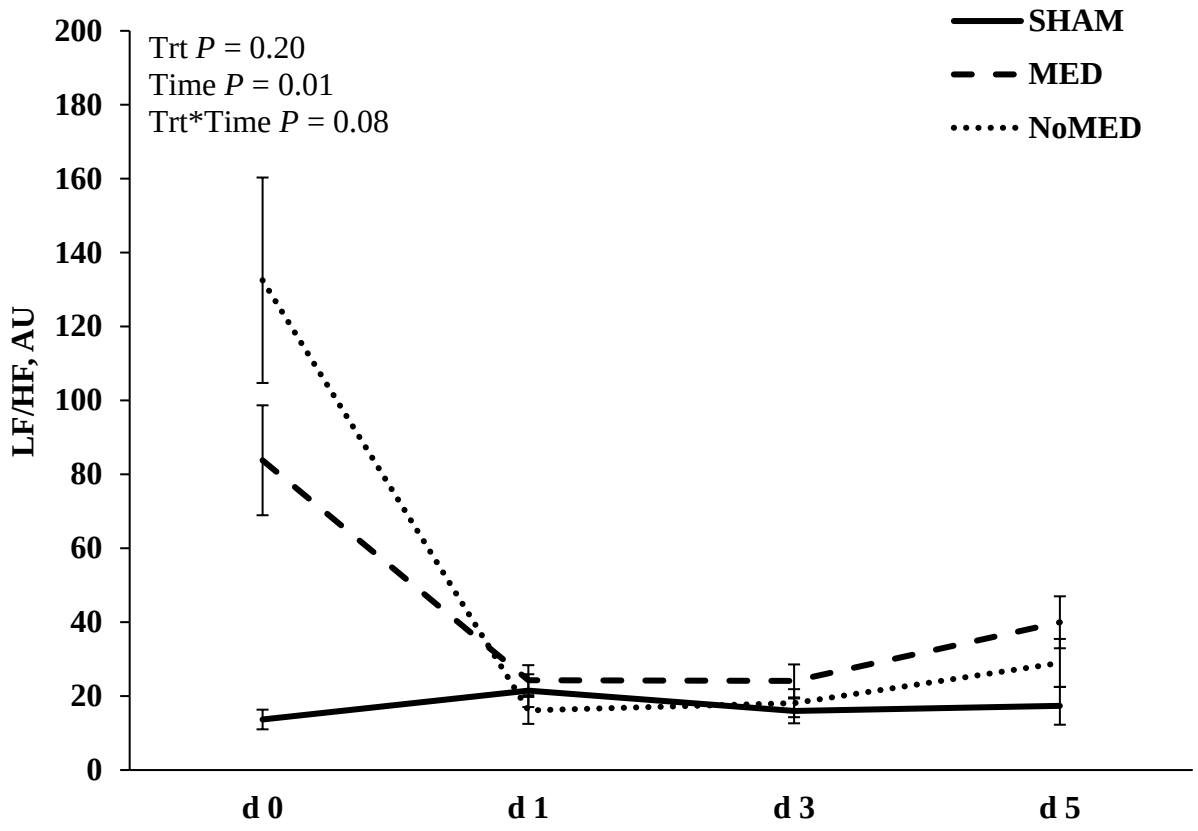

Figure 3.6. Back-transformed least squares means ± approximated SE of low frequency to high frequency ratio (LF/HF) over the 5-d experimental period. Disbudding took place on d 0. Calves in the MED treatment group received lidocaine and meloxicam prior to the disbudding procedure. Calves in the NoMED treatment group did not receive any pain mitigation prior to the disbudding procedure. Disbudding was only simulated on SHAM calves.

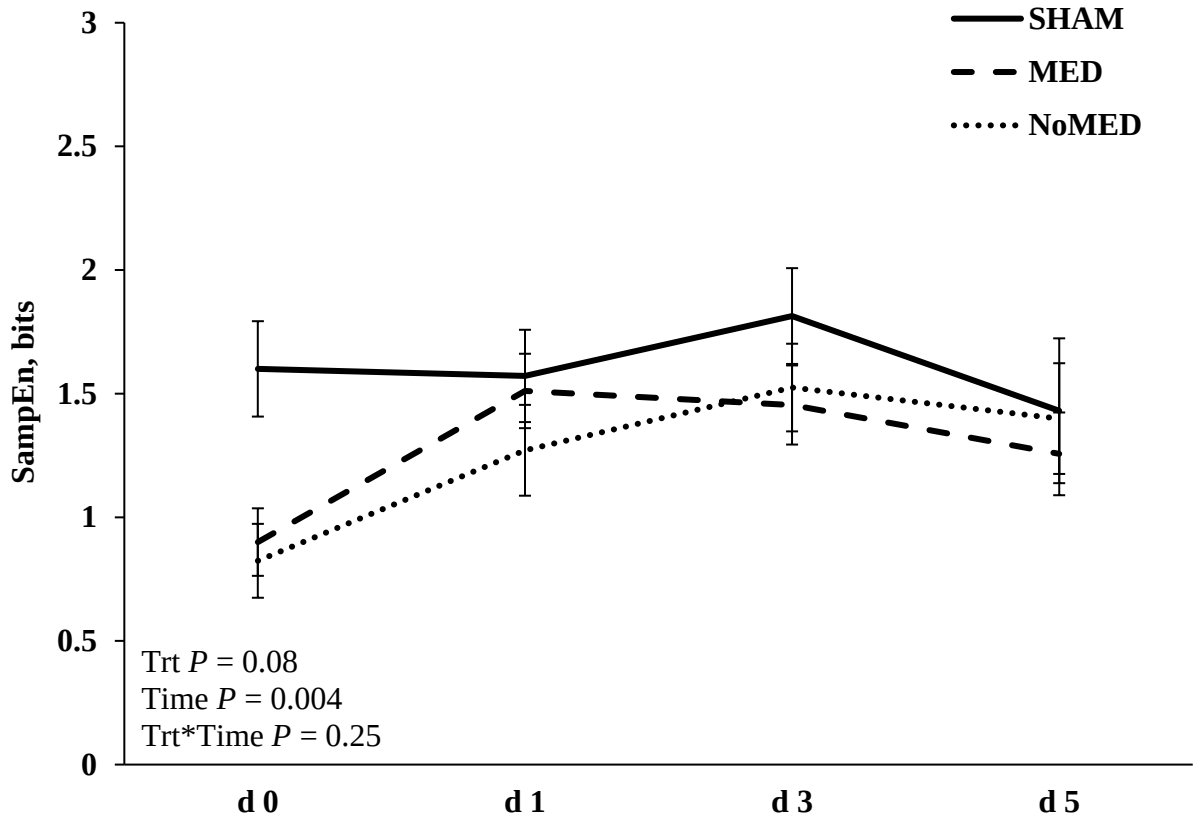

Figure 3.7. Back-transformed least squares means ± approximated SE of Sample Entropy (SampEn) over the 5-d experimental period. Disbudding took place on d 0. Calves in the MED treatment group received lidocaine and meloxicam prior to the disbudding procedure. Calves in the NoMED treatment group did not receive any pain mitigation prior to the disbudding procedure. Disbudding was only simulated on SHAM calves.

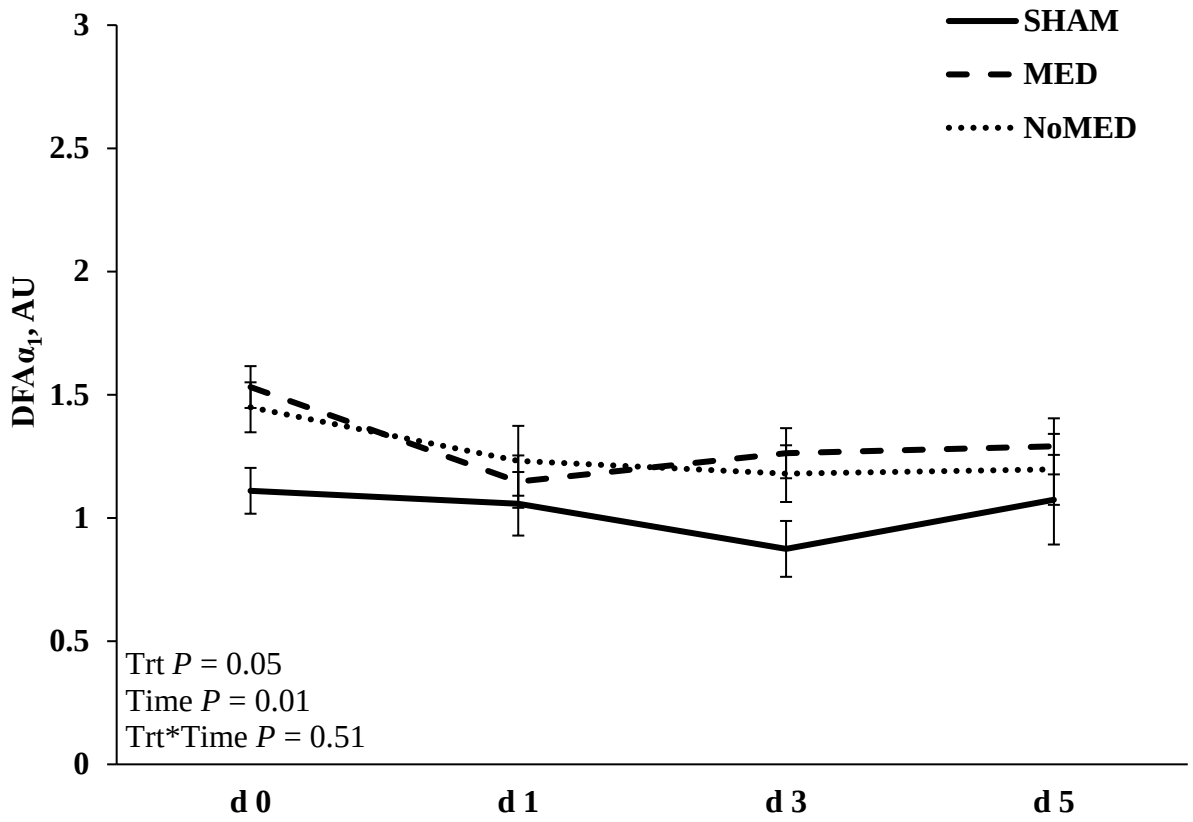

Figure 3.8. Least squares means ± SE of the short-term detrended fluctuation analysis exponent (DFAα_1) over the 5-d experimental period. Disbudding took place on d 0. Calves in the MED treatment group received lidocaine and meloxicam prior to the disbudding procedure. Calves in the NoMED treatment group did not receive any pain mitigation prior to the disbudding procedure. Disbudding was only simulated on SHAM calves.

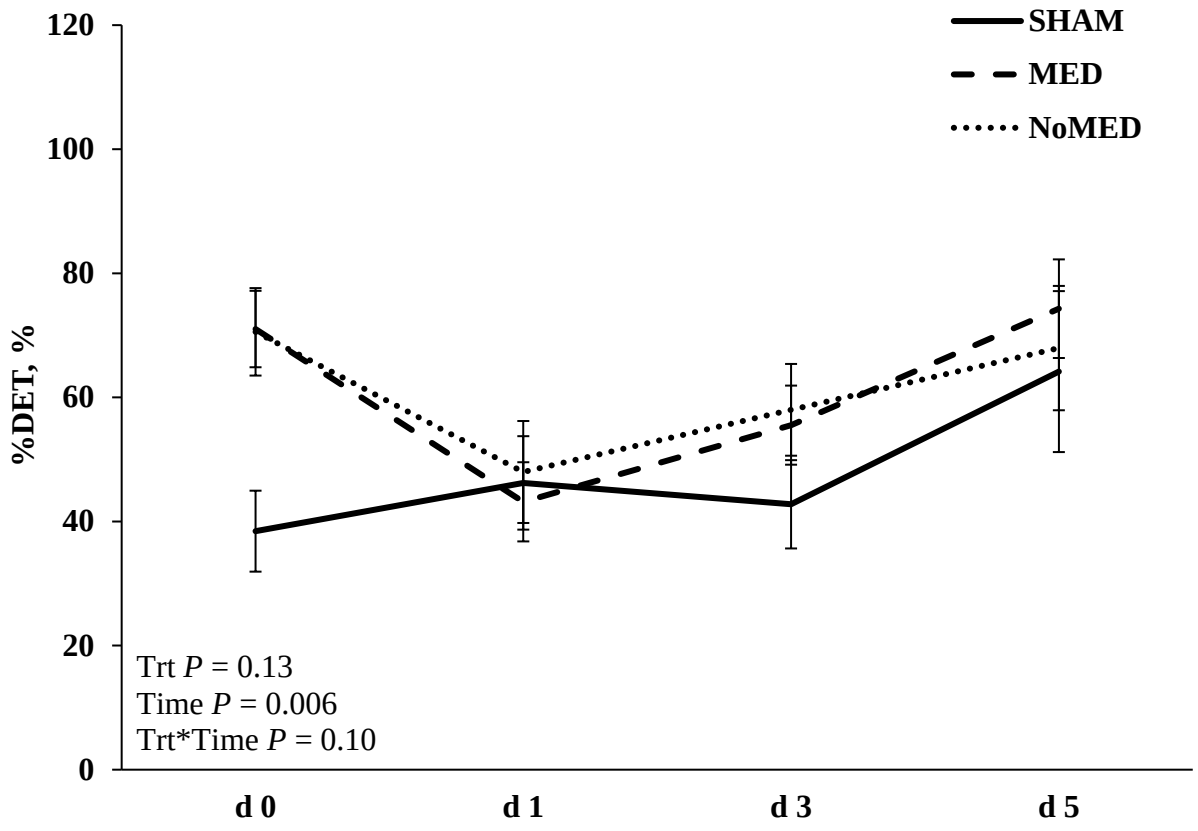

Figure 3.9. Least squares means ± SE of percent determinism (%DET) over the 5-d experimental period. Disbudding took place on d 0. Calves in the MED treatment group received lidocaine and meloxicam prior to the disbudding procedure. Calves in the NoMED treatment group did not receive any pain mitigation prior to the disbudding procedure. Disbudding was only simulated on SHAM calves.

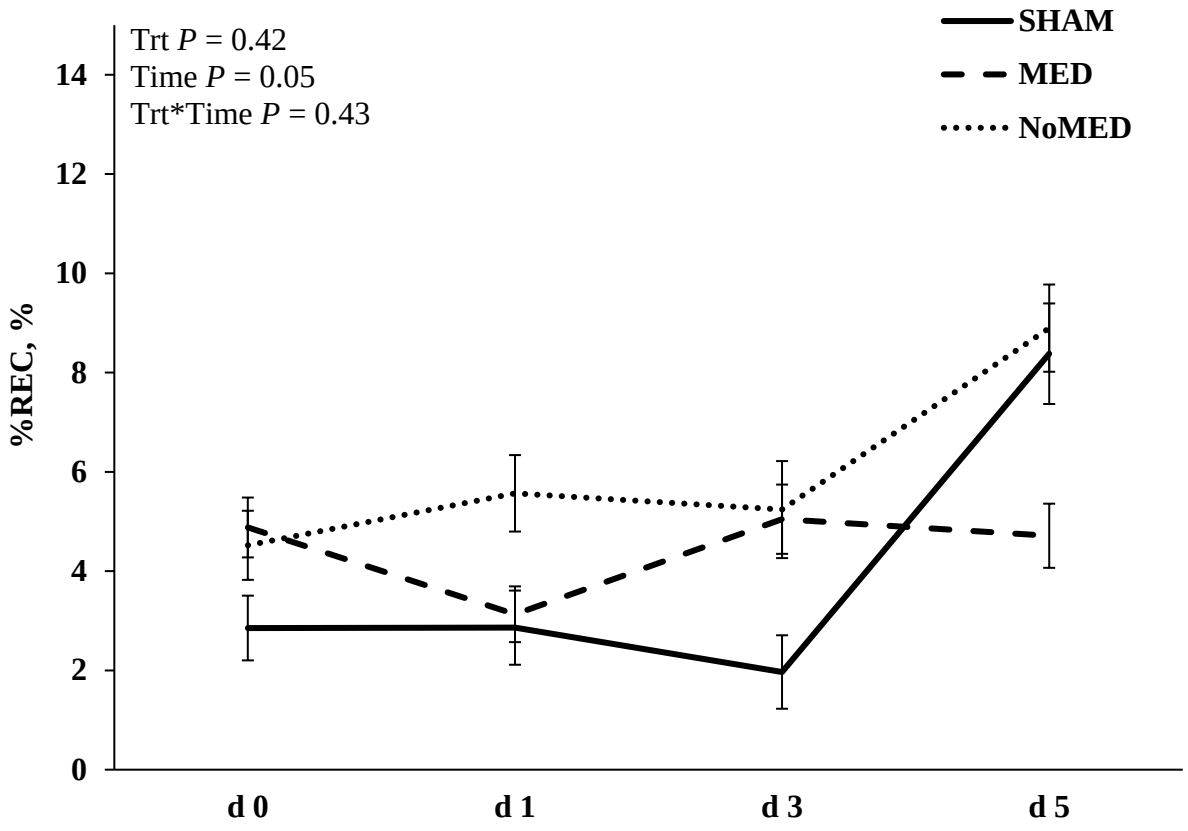

Figure 3.10. Back-transformed least squares means ± approximated SE of percent recurrence (%REC) over the 5-d experimental period. Disbudding took place on d 0. Calves in the MED treatment group received lidocaine and meloxicam prior to the disbudding procedure. Calves in the NoMED treatment group did not receive any pain mitigation prior to the disbudding procedure. Disbudding was only simulated on SHAM calves. After adjustment for multiple comparisons, no differences were observed for time ($P > 0.05$).

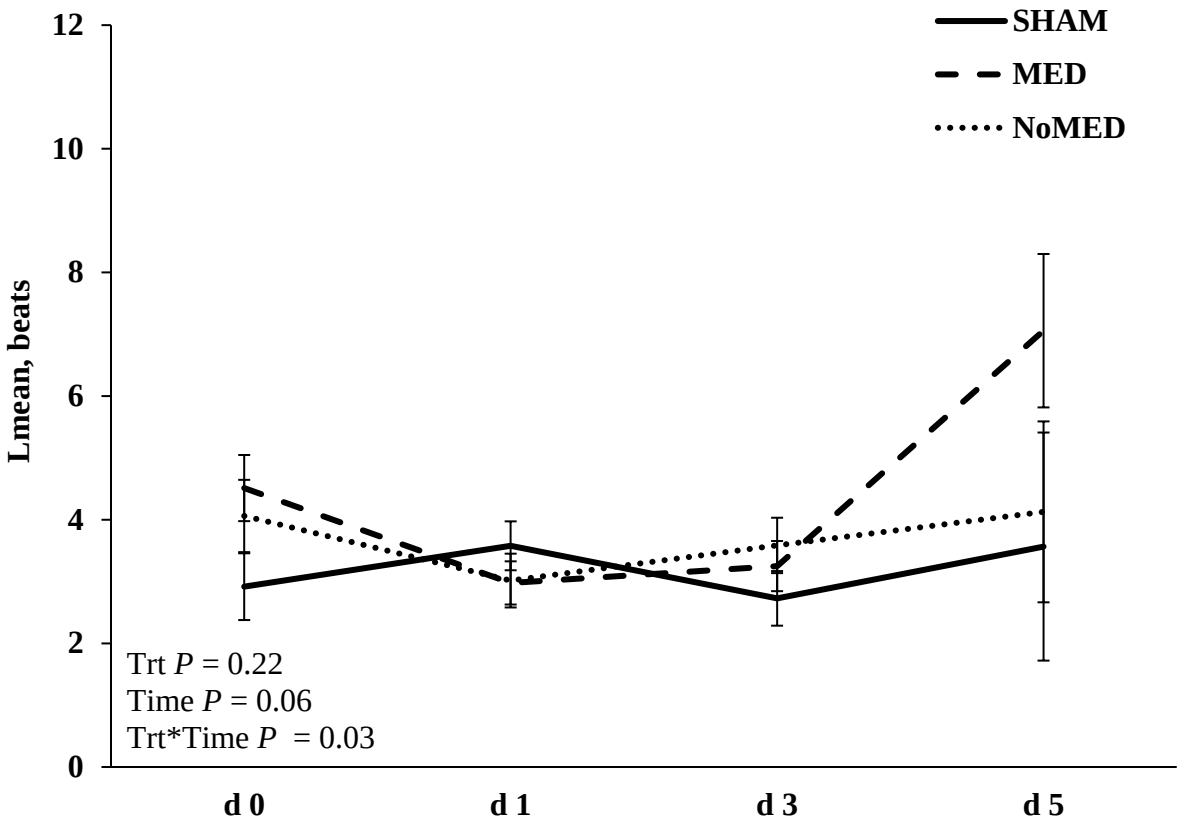

Figure 3.11. Least squares means ± SE of mean length of diagonal lines in a recurrence plot (Lmean) over the 5-d experimental period. Disbudding took place on d 0. Calves in the MED treatment group received lidocaine and meloxicam prior to the disbudding procedure. Calves in the NoMED treatment group did not receive any pain mitigation prior to the disbudding procedure. Disbudding was only simulated on SHAM calves. After adjustment for multiple comparisons, no differences were observed for the treatment by time interaction ($P > 0.05$).

CHAPTER 4. EVALUATING THE RESPONSE OF GROWING PIGS TO AN ACUTE HEAT EPISODE USING NONLINEAR MEASURES OF HEART RATE VARIABLITY

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[2]Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. The U.S. Department of Agriculture is an equal opportunity provider and employer. No conflicts of interest, financial or otherwise are declared by the author (s).

4.1 Abstract

Heart rate variability (**HRV**) is a proxy measure of autonomic function and can be used as an indicator of stress in swine. While traditional linear measures are commonly used to distinguish between stressed and unstressed treatments, inclusion of nonlinear HRV measures that evaluate data structure and organization show promise for improving HRV interpretation. Therefore, the objective of this study was to evaluate the inclusion of nonlinear HRV measures in response to an acute heat stress episode. Twenty, 12 to 14 wk-old growing pigs were individually housed in thermoneutral conditions and randomly allocated to 1 of 2 treatments: 1) Thermoneutral control (**TN**; n = 10 pigs) or 2) acute heat stress (**HS**; n = 10 pigs). In Phase 1 of the experimental procedure (**P1**), all pigs underwent a baseline HRV measurement period in thermoneutral conditions before undergoing treatment (Phase 2; **P2**), where HS pigs were exposed to heated conditions (32.6 ± 0.1°C; 26.2 ± 0.1% RH) and TN pigs remained in thermoneutral conditions (20.35 ± 0.01°C; 67.6

± 0.2% RH). After P2, all pigs were moved back to thermoneutral conditions (Phase 3; **P3**). Gastrointestinal temperature (**T_g**) data were collected for each pig every 5 min. Behavioral data were collected to evaluate the amount of time each pig spent in an active posture (sitting or standing). Linear (time and frequency domain) and nonlinear (sample entropy, detrended fluctuation analysis, percent recurrence, percent determinism, mean diagonal line length in a recurrence plot) HRV measures were quantified. Data were analyzed using Proc Glimmix in SAS 9.4. Heat stressed pigs exhibited greater T_g (P = 0.002) and spent less time in an active posture than TN pigs during P2 (P = 0.0003). Additionally, low frequency to high frequency ratio was greater in HS pigs during P3 compared to TN pigs (P = 0.02). Sample entropy was reduced in HS pigs during P2 (P = 0.007) and P3 (P = 0.03) compared to TN pigs. Heat stressed pigs exhibited greater percent determinism (**%DET**) during P3 (P = 0.03) and tended to have greater %DET (P = 0.09) during P2 compared to TN pigs. No differences between treatments were detected for the remaining HRV measures. In conclusion, linear HRV measures were largely unchanged during P2. However, changes to SampEn and %DET suggest increased stress as a result of the acute heat episode. Future work should focus on nonlinear measures such as SampEn and %DET for regular inclusion in HRV studies on swine heat stress.

Keywords: animal welfare, heart rate variability, heat stress, nonlinear analysis, stress, swine

4.2 Introduction

Heart rate variability (**HRV**), or the variation in time between adjacent heart beats (known as R-R intervals), is an established proxy for measuring autonomic nervous system (**ANS**) function and is useful for evaluating the autonomic response to various stressors in livestock species (von Borell et al., 2007). The parasympathetic and sympathetic branches of the ANS directly innervate

the heart and are largely responsible for changes to heart rate (Shaffer et al., 2014). As a result, changes to heart rate over time have been used to approximate the balance between the ANS branches with various linear measures of mean and variance, or spectral frequency analysis.

One drawback to the use of linear measures stems from their inability to evaluate the nonlinear processes involved in heart rate modulation (Young and Benton, 2015). Nonlinearity in heart rate modulation arises from the multiple physiological processes that act concurrently via the ANS or directly on the heart to alter heart rate. As a result, the branches of the ANS can interact with each other in a nonlinear manner (*i.e.* input from both branches may increase or decrease at the same time, or act in an antagonistic manner), resulting in nonlinear changes to the output variability of the heart rate signal that cannot be quantified by linear HRV measures that are typically used to estimate some aspect of balance between ANS branches (Kawada et al., 1999). To evaluate these nonlinear properties, nonlinear HRV measures attempt to quantify how a stressor affects correlational and structural properties of the heart rate signal instead of linear measures such as mean or standard deviation of the R-R intervals. Several nonlinear measures have been used in human HRV studies with varying degrees of success, and investigation of robust nonlinear measures for inclusion in HRV analysis has been recommended for measuring the physiological stress response (Sassi et al., 2015).

For the swine industry, increased environmental temperature is a major source of physiological stress that negatively affects swine welfare. Specifically, increased heat load results in decreased feed intake, altered metabolism, reduced intestinal integrity, decreased reproductive performance and an altered immune response (Ross et al., 2015). With increases in global temperatures expected to continue (Collins *et al.*, 2013), tools to measure the stress caused by heat are as pertinent as ever if further mitigation strategies are to be developed. Therefore, the objective

of this study was to evaluate whether nonlinear HRV analyses could be used to complement traditional HRV measures in response to an acute heat stress episode (defined as having a body temperature that exceeds euthermia) in growing pigs. We hypothesized that pigs exposed to heat would exhibit increased sympathetic activity [as measured by linear HRV measures: lower average R-R interval (**RR)**, lower root mean of successive squared differences (**RMSSD**), lower standard deviation of the R-R intervals (**SDNN**), greater low frequency to high frequency ratio (**LF/HF**); Table 1]. Additionally, we hypothesized that nonlinear HRV analysis of heat stressed growing pigs would result in altered heart rate (**HR**) signal regularity [lower sample entropy (**SampEn**), greater percent determinism (**%DET**), greater percent recurrence (**%REC**), greater mean length of diagonal lines in a recurrence plot (**Lmean**); Table 1] and lower HR signal self-similarity [a short-term detrended fluctuation analysis exponent that deviates from '1' (**DFAα_1**); Table 1].

4.3 Materials and Methods

All experimental procedures involving animals were approved by the Purdue University Institutional Animal Care and Use Committee (#1704001563).

4.3.1 Animals

Twenty, 12 to 14 wk-old growing pigs were housed individually on slatted floors and provided with *ad libitum* feed and water. Feed was formulated to meet or exceed the nutritional requirements set by the National Research Council (NRC, 2012) for growing pigs. The room used for general housing was maintained at a thermoneutral temperature (20.35 ± 0.01°C; 67.6 ± 0.2% RH; Federation of Animal Science Societies, 2010) for the entirety of the experiment. Artificial lighting was provided from 0700 h to 1600 h each d. Windows in the room allowed for

supplemental lighting when the artificial lighting was off. Pigs had auditory and olfactory exposure to their conspecifics, however, pen partitions did not allow for tactile or visual contact.

4.3.2 Experimental Procedure

All pigs were randomly allocated to 1 of 2 treatments: thermoneutral control (**TN**; n = 10; gilt = 6, barrow = 4) or heat stress (**HS**; n = 10; gilt = 4, barrow = 6). Four d prior to the start of the experiment, all pigs began an acclimation period to the heart rate equipment and personnel conducting the experiment. Specifically, experimental personnel entered the individual pens for a 20 min period while attempting to interact and fit each pig with a Polar heart rate monitor (H10; Polar Electro Oy, Kempele, Finland). This procedure was repeated 2 additional times or until each pig would freely interact with the observer and allow fitting of the heart rate monitor. No pig required more than 4 acclimation periods before undergoing the experimental procedure.

Approximately 16 h prior to undergoing the experimental procedure, each pig was rope snared and orally administered a calibrated temperature sensor (CorTemp Ingestible Core Body Temperature Sensor HTI50002; accuracy ± 0.1°C; HQ Inc., Palmetto, FL) to measure gastrointestinal temperature (**T_g**) as a non-invasive approximation of core body temperature. Gastrointestinal temperature was recorded telemetrically every 5 min during the experimental procedure (Wireless Core Body Temperature Monitoring Data Recorder; HQ Inc., Palmetto, FL) by placing the recorder approximately 15 cm above the middle of the pig's back.

The experimental procedure was completed over two repetitions (10 pigs/repetition) consisting of 5 HS and 5 TN pigs per repetition. Repetition 1 [6 barrows (n = 3 TN, 3 HS) and 4 gilts (n = 2 TN, 2 HS)] occurred over 3 days, with 2, 4, and 4 pigs undergoing the experimental procedure on days 1, 2, and 3, respectively. Repetition 2 [6 gilts (n = 3 TN, 3 HS) and 4 barrows (n = 2 TN, 2 HS)] occurred over 2 days, with 4 and 6 pigs undergoing the experimental procedure

on days 1 and 2, respectively. All pigs were fitted with a heart rate monitor that was secured with flexible bandage wrap (VetWrap; 3M, Maplewood, MN), and allowed to stay in their individual home pens for a period of 60 min (Phase 1; **P1**). Phase 2 (**P2**) occurred immediately after P1 and consisted of moving one HS pig to a separate, but identical, room (32.6 ± 0.1°C; 26.2 ± 0.1% RH; Federation of Animal Science Societies, 2010) across a single hallway to undergo an acute heat episode. The pen used in the heated room had the same drinker, feeder, pen partitions, and lighting as the home pens in the thermoneutral room. After the HS pig was moved to the heated room, a single TN pig was moved away from its home pen, into the hallway, and returned to the empty home pen of the paired HS pig for a period of 60 min. This was done to account for any potential effects movement, blood collection (see below), or pen novelty may have had on the experimental results. Phase 2 lasted for 60 min and began when T_g reached 40.6℃ for the HS pigs. If T_g of the HS pig reached 41.5℃ before 60 min elapsed, the pig was promptly removed from the HS room and placed back into TN conditions to recover. Following Phase 2, TN and HS pigs were moved back into their home pens for an additional 60 min (Phase 3; **P3**). Again, TN pigs were moved into the hallway before returning to their home pens to account for handling and movement. Heart rate was monitored continuously throughout the experimental procedure.

4.3.3 Blood Collection and Analyses

To further evaluate the effect of acute heat on the physiological stress response, blood was collected following P1 and P2 for analysis of plasma cortisol. Specifically, each pig was rope snared and approximately 3 mL of blood was collected (Vacutainer Plastic K_3EDTA Blood Collection Tube; Becton-Dickinson, Franklin Lakes, NJ) via jugular venipuncture using a 20-gauge, 2.54 cm needle (Vacutainer; Becton-Dickinson; Franklin Lakes, NJ). Blood for plasma

cortisol analysis was refrigerated for approximately 2 h after each blood collection before centrifugation at 1,900 x g for 15 min (4℃). Plasma was then collected, aliquoted, and stored at -80℃. Cortisol concentrations (ng/mL) were determined via radioimmunoassay per manufacturer's instructions [Cortisol RIA (CT); IBL International, Hamburg, Germany). Inter- and intra-assay CV were 7.1% and 3.8%, respectively.

4.3.4 Behavior

One camera (KPC-N502NUB; KT&C USA, Fairfield, NJ) was mounted above each pen to record pig behavior using a digital video system (Geo Vision VMS Software; Geo Vision Inc., Taipei, Taiwan). The posture (active: standing, sitting; inactive: lying sternal, lying lateral) of each pig was then collected continuously from the recorded video as an indicator of activity and to identify periods of inactivity (lying in either a lateral or sternal position) for heart rate variability data selection.

4.3.5 Heart Rate Variability Analysis

Heart rate interval (R-R interval) data were collected during the experimental procedure with a heart rate monitor (Polar V10; Polar Electro Oy, Kempele, Finland) and transmitted telemetrically to a recorder (Polar V800 Sports Watch; Polar Electro Oy, Kempele, Finland) that allows for continuous R-R interval data collection at a sampling rate of 1000 Hz. Following data collection, each dataset was reviewed, and errors were edited manually using previously published guidelines for artifact correction (Marchant-Forde et al., 2004). No dataset selected for analysis had more than 5% corrected errors. Additionally, data with more than three consecutive erroneous heart beat intervals were not used. A single dataset of 512 R-R intervals for each phase was selected for each pig. Datasets selected for analysis occurred during the final available periods of inactivity in each phase and were at least 10 min after contact with experimental personnel.

Several HRV parameters were measured, as is recommended for HRV analysis (Table 4.1; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). These included measures in the time domain (RR, RMSSD, SDNN; Table 4.1) and frequency domain (LF/HF; Table 4.1). Additionally, nonlinear measures based on HR regularity (SampEn; Table 4.1), self-similarity ($DFA\alpha_1$; Table 4.1) and RQA (**%REC, %DET,** and **Lmean**; Table 4.1) were included to evaluate whether nonlinear HRV measures provided additional information resulting from acute heat exposure

All measures except those obtained through RQA were evaluated using a software package available for HRV analysis (Kubios HRV Standard; Kubios Oy, Kuopio, Finland). Data analyzed in Kubios were detrended (first-order differencing) prior to analysis to obtain stationarity for all measures except RR. Each data set used for frequency domain analysis was re-sampled at 4 Hz and underwent fast Fourier transformation. Spectral limits were set according to previously published guidelines on frequency domain analysis of heart rate variability in growing pigs (Poletto et al., 2011): LF (0.00 to 0.09), HF (0.09 to 2.0). The window size was set so each dataset was divided into two with an overlapping function of 50% to reduce spectral leakage in the signal. For analysis of SampEn, the embedding dimension was set to 2 beats with a threshold of 0.15 x SD, as is commonly recommended for HRV analysis (Pincus, 1995, Zhao et al., 2015). Finally, $DFA\alpha_1$ was evaluated using a range of 4 to 16 beats.

Recurrence Quantification Analysis was carried out using a statistical package for obtaining HRV output in R (RHRV package in R 3.3.3; R Foundation for Statistical Computing; Vienna, Austria). These data were not detrended prior to analysis. A time delay (τ) of 9 and embedding dimension (m) of 7 were used. Selection of τ was determined by taking the mean τ value of all datasets obtained by the average mutual information method ("mutual" command in

the ‘tserieschaos’ package). Embedding dimension was determined in a similar fashion using the false nearest neighbor method (“FNN” command in the ‘fractal’ package; Parameters: dimension = 15, lag = determined from AMI, R_{tol} = 14, A_{tol} = 2). Using these parameters, a radius of 15 beats was employed to standardize %REC so the majority of data sets fell between 1% and 5% recurrence, an appropriate range for RQA analysis (Wallott, 2017)

4.3.6 Statistical Analysis

All data were analyzed using a linear mixed model (Proc GLIMMIX) with repeated measures (subject = pig nested within treatment and sex) in SAS 9.4 (SAS Institute Inc., Cary, NC). Treatment (HS, TN), phase (P1, P2, or P3), sex (male, female), weight, repetition (1 or 2), the interaction between treatment and phase, and the interaction between treatment and sex were included as fixed factor independent variables. Time spent in an active posture (standing, sitting; %), body temperature (℃), cortisol concentrations (ng/mL) and each HRV measure (RR, RMSSD, SDNN, LF, HF, LF/HF, SampEn, %REC, %DET, Lmean) were included as dependent variables in individual models. Degrees of freedom were determined using the Kenward-Rogers degrees of freedom approximation. Prior to analysis, variables were transformed, as needed, to meet the residual normality and homogeneity of variance assumptions associated with the model. Specifically, SDNN, %REC, and Lmean were log transformed. The low frequency to high frequency ratio was square root transformed. All variables are presented as least squares means ± SE, however, transformed variables are presented as back-transformed least squares means ± approximate SE obtained via the delta method. Multiple comparisons were evaluated using a Bonferroni correction. A statistically significant difference between comparisons was defined as $P \leq 0.05$. A trend in the data was defined as $0.05 \leq P \leq 0.1$. Data from 1 TN pig was removed from the data set due to an elevated body temperature above 40.5 ℃ during all phases. No suitable HRV

data could be identified during phase 2 for 2 TN pigs. Therefore, Phase 2 HRV estimations are based on data from 7 TN pigs and 10 HS pigs. Estimates for P1 and P3 are based on data from 9 TN pigs and 10 HS pigs.

4.4 Results

4.4.1 Body Temperature

Heat stressed pigs exhibited greater T_g during P2 compared to TN pigs (40.9 ± 0.1 vs. 40.0 ± 0.2℃; $t_{43.65}$= -4.28; P = 0.002; Fig. 4.1). Gastrointestinal temperatures for HS pigs were greater during P2 (40.9 ± 0.1℃) compared to P1 (39.7 ± 0.1℃; $t_{31.9}$= -7.91; P < 0.0001; Fig. 4.1) and P3 (40.1 ± 0.1℃; $t_{31.9}$= 5.03; P = 0.0002; Fig. 4.1), and tended to be greater during P3 compared to P1 ($t_{31.9}$= -2.88; P = 0.1; Fig. 4.1). No changes to T_g were observed in TN pigs over the course of the experiment (P > 0.05; Fig. 4.1).

4.4.2 Activity

No changes to time spent in an active posture were observed for HS pigs over the course of the study (P > 0.05; Fig. 4.2), however, TN pigs spent more time in an active posture during P2 (75.6 ± 4.6 %; Fig. 4.2) compared to P1 (27.9 ± 4.0 %; $t_{25.71}$ = -8.38; P < 0.0001; Fig. 4.2) and P3 (29.6 ± 5.5 %; $t_{24.66}$ = 6.86; P < 0.0001; Fig. 4.2). Consequently, TN pigs spent more time ($t_{15.65}$ = 5.10; P = 0.0003; Fig. 4.2) in an active posture during P2 compared to HS Pigs.

4.4.3 Cortisol

No effects of treatment, phase, sex, weight, or repetition were found for cortisol concentrations (P > 0.05). There tended ($F_{1, 11.95}$ = 3.89; P = 0.07; Table 4.2) to be an interaction between treatment and phase, where cortisol concentration for HS pigs was numerically greater than TN pigs following P2.

4.4.4 Linear HRV Measures

Average R-R interval did not differ between HS and TN pigs during any phase ($P > 0.05$; Table 4.3), however, TN pigs exhibited lower RR during P2 compared to P1 ($t_{22.55} = 3.39$; $P = 0.02$; Table 4.3) and P3 ($t_{24.62} = -3.13$; $P = 0.04$; Table 4.3). Overall, gilts exhibited greater RR than barrows (449.7 ± 7.6 vs. 418.6 ± 7.9 ms; $F_{1,14.84} = 7.14$; $P = 0.02$). Weight was weakly, but positively, associated with RR ($F_{1,14.39} = 6.36$; $P = 0.02$), where pigs with higher BW exhibited greater RR ($R^2 = 0.10$).

Standard deviation of the R-R intervals (SDNN) tended to be greater for TN pigs during P1 compared to P2 ($t_{32.67} = 2.86$ $P = 0.07$; Table 4.3). No other effects of treatment, phase, sex, repetition, or interaction on SDNN were found ($P > 0.05$).

Root mean square of successive differences (RMSSD) was associated with weight ($F_{2,24.61} = 1.15$; $P = 0.03$), where larger weights were weakly correlated with greater values of RMSSD ($R^2 = 0.01$). No other effects of treatment, phase, sex, or any interaction were detected for RMSSD ($P > 0.05$).

The ratio between low and high frequency power (LF/HF) was greater for HS pigs during P3 compared to TN pigs ($t_{17.62} = -3.35$; $P = 0.02$; Table 4.3). Thermoneutral pigs tended to have lower LF/HF during P2 ($t_{26.98} = 2.99$; $P = 0.06$; Table 4.3) and P3 ($t_{25.06} = 2.79$; $P = 0.09$; Table 4.3) compared to P1. Heat Stressed Pigs had greater LF/HF during P3 compared to P2 ($t_{20.45} = -3.07$; $P = 0.05$; Table 4.3).

4.4.5 Nonlinear Measures of HRV

Heat stressed pigs exhibited lower SampEn than TN pigs during P2 ($t_{34.67} = 3.87$; $P = 0.01$; Table 4.4) and P3 ($t_{34.24} = 3.25$; $P = 0.03$; Table 4.4). Sample entropy was lower in HS pigs during

P2 compared to P1 ($t_{18.65}$ = 3.34; P = 0.03; Table 4.4). Thermoneutral pigs had greater SampEn during P3 compared to P1 ($t_{17.98}$ = -4.75; P = 0.001; Table 4.4).

Gilts tended to have lower DFAα_1 compared to barrows (1.41 ± 0.04 vs. 1.52 ± 0.04; $F_{1,14.94}$ = 3.21; P = 0.09). No other effects of treatment, phase, repetition, or any interaction on DFAα_1 were detected (P > 0.05; Table 4.4).

Overall, HS pigs tended to have greater Lmean than TN pigs ($F_{1,15.65}$ = 4.05; P = 0.06; Table 4.4). No effects of phase, sex, weight, repetition, or any interaction were detected for Lmean (P > 0.05; Table 4.4).

No effects of treatment, phase, sex, weight, repetition, or any interaction on %REC were detected (P > 0.05; Table 4.4).

Overall, HS pigs had greater %DET than TN pigs (69.6 ± 3.3 vs. 54.4 ± 3.7%; $F_{1,15.68}$ = 8.35; P = 0.01), where %DET for HS pigs was greater during P3 ($t_{36.65}$ = -3.14; P = 0.03; Table 4.4) and tended to be greater during P2 ($t_{40.75}$= -2.73; P = 0.09; Table 4.4) compared to TN pigs. There was also a tendency for an interaction between treatment and sex, where TN gilts had lower %DET than TN barrows and all HS pigs (data not shown; $F_{1,15.55}$ = 3.46; P = 0.08).

4.5 Discussion

Increased ambient temperature can be detrimental to swine welfare and poses a significant challenge for the swine industry, particularly during the summer months. When pigs are exposed to temperatures above their thermoneutral zone, body temperatures increase, and blood vessels are dilated to shunt blood away from the body's core as a method to dissipate body heat (Collin et al., 2001). Consequently, HR is typically thought to increase while blood vessels constrict in an attempt to preserve homeostatic maintenance of the tissues; however, the specific effect of heat on HR is likely variable depending on exposure length (Sapkota et al., 2016). Therefore, heart rate

variability was evaluated as a potential non-invasive tool for measuring changes to autonomic and cardiac function in response to an acute heat episode, with a particular emphasis placed upon the use of nonlinear HRV measures for distinguishing between stressed and non-stressed treatments.

In the current study, HS pigs exhibited lower SampEn in response to heat exposure (P2 and P3) and greater %DET during P3 compared to TN pigs. Sample entropy is a measure of regularity, where runs of data patterns (vector length of *m* data points) that are close to each other will remain close if the vector length is increased by one (*m* + 1; Pincus, 1995). In general, SampEn of HRV is reduced in response to stress and disease (Lake et al., 2002; Batchinsky et al., 2007). Accordingly, it could be concluded that the lower SampEn values exhibited by HS pigs are an indication of greater stress as a result of the heat episode. Percent determinism (%DET), on the other hand, is a measure obtained via recurrence quantification analysis (RQA) that quantifies the percentage of recurrence points that form diagonal lines in an RQA plot. Recurrence quantification analysis is a method used to visualize multi-dimensional dynamic systems in a two-dimensional plot and obtain information about the system dynamics and trajectories (see Eckmann et al., 1987). Diagonal lines on the plot indicate recurring trajectories (periodicity) in the data, so larger values of %DET indicate a greater incidence of periodicity. Therefore, greater %DET in HS pigs following P3 suggest more periodicity in the data compared to TN pigs and may also suggest greater stress (Frondelius et al., 2015). While the only difference in the interaction term between HS and TN pigs was observed during P3, it's notable that the numerical level of %DET was maintained in the HS pigs throughout the study, while %DET decreased over time for TN pigs. Ultimately, this led to an overall difference between HS and TN pigs, indicating that HS pigs were likely more stressed throught the study. Taken together, changes to SampEN and %DET in the current study do provide some evidence for decreased nonlinear heart activity in HS pigs,

suggesting a possible increase in physiological stress and a reduction in overall physiological complexity.

The concept of physiological complexity describes the interaction of multiple physiological processes on different scales that are needed for the body to adapt and respond to stressors (Goldberger et al., 2002). While its details are somewhat overwhelming, an overarching theme of physiological complexity is that the output of healthy physiological systems is characterized by: 1) low output predictability, 2) sensitivity to initial conditions, and 3) nonlinear interactions between the components of the system. However, these characteristics begin to deteriorate in response to stress, disease, and aging (Goldberger et al., 2002). Since the heart receives input from multiple physiological processes and heart rate displays nonlinear characteristics, nonlinear measures of HRV may be more sensitive indicators of stress than linear HRV measures in response to certain stressors. In the current study, however, differences between treatments resulting from acute heat stress were not evident for the remaining nonlinear measures during P2, a result that mirrors previously reported preliminary results (Byrd et al., 2017).

Despite evidence that HS pigs were heat stressed during P2 (greater body temperature, decreased behavioral and nonlinear HRV activity compared to TN pigs), cortisol concentrations and traditional linear measures of HRV were mostly unreliable for detecting differences between treatments. We expected plasma cortisol concentrations to increase following P2, however, cortisol concentrations did not differ between treatments during P2, which indicates little effect of the acute heat episode on the hypothalamic-pituitary-adrenal (HPA) axis response. Cortisol secretion by the adrenal glands results from activation of the HPA-axis in response to a stressor (such as increased environmental temperature) and acts to induce gluconeogenesis, protein and lipid catabolism, and suppresses the inflammatory immune response to maintain bodily

homeostasis (Buckingham, 2006). Accordingly, cortisol is a commonly used measure of stress and has been shown to increase in response to high environmental temperatures (Becker et al., 1997); but this result is not consistent (Hicks et al., 1998). In the current study, HS and TN pigs exhibited a relatively large divergence in mean plasma cortisol concentration following the acute heat episode, so it seems plausible that the sample size used for cortisol analysis was insufficient given the amount of variability present in the data.

A decrease in RR (and, therefore, an increase in HR) during the heat stress phase was expected as a result of increased sympathetic activity. However, RR did not change for HS pigs in response to the heat stress phase during P2 and was not different from TN pigs throughout the study. While unexpected, changes to RR are not always evident in response to acute heat exposure since RR is the least sensitive measure for assessing autonomic function (Sapkota et al., 2016; von Borell et al., 2007). However, RR decreased in the TN group during P2 indicating greater mean heart rates resulting from movement to an unfamiliar pen in a thermoneutral environment. This result coincided with a large increase in the amount of time spent in an active posture (standing, sitting). Therefore, it is possible the decrease in RR, although sampled from a period of inactivity, was a result of increased activity throughout P2.

The remaining linear measures (SDNN and RMSSD) did not change for either treatment throughout the experimental phase. Standard deviation of the R-R intervals is commonly used as an overall indicator of autonomic function, while RMSSD is thought to be more closely tied to parasympathetic activity (von Borell et al., 2007). Greater values of both measures are generally indicative of lower levels of stress, however, the lack of observed change for both measures during P2 would indicate that pigs in both treatments were not stressed throughout the study despite the

T_g, behavioral, and nonlinear HRV evidence that the acute heat stress period was stressful for HS pigs.

The LF/HF ratio is one of the most commonly used HRV measures, but also one of the more challenging measures to accurately interpret (Billman, 2013). Through blockade studies, previous work has shown that administration of anti-cholinergic agents, such as atropine, reduce the LF power and almost completely remove the HF component of the HRV spectra (Kuwahara et al., 1994). In contrast, beta-adrenergic receptor blockers, such as propranolol, enhance the HF power and remove a large portion of the LF spectrum (Poletto et al., 2011). Therefore, the LF/HF ratio is commonly used as a measure of balance between the ANS branches, where HF spectra is interpreted as parasympathetic activity and the LF spectra represents both parasympathetic and sympathetic activity. As a result, greater LF/HF values are thought to be indicative of greater sympathetic activity (Kuwahara et al., 1994; Poletto et al., 2011). In the current study, the LF/HF ratio was greater for HS pigs in P3 compared to TN pigs, however no differences between the two treatments were detected during P2. Since LF/HF in HS pigs did not increase during P2, the increased LF/HF ratio during P3 may indicate a delayed increase in sympathetic activity or decreased parasympathetic response to the additive effect of increased body temperature and stress associated with blood collection at the end of P2.

One caveat to the use of LF/HF is that the HRV spectrum can be affected by respiration. Studies in humans have shown that slowed respiration rates can dramatically increase low frequency spectral power, which may lead to an erroneous conclusion regarding increased sympathetic activity (Brown et al., 1993; Koh et al., 1995). Given the likelihood of diverging respiration rates between HS and TN pigs during the acute heat period, it's possible that LF/HF ratio for each treatment are biased and not comparable. Therefore, since the present study was

unable to incorporate controlled breathing and did not monitor respiration rate, the LF/HF results reported here remain difficult to interpret in the absence of changes to additional linear HRV measures.

There is some indication that certain HRV measures are altered in a sex-specific manner after exposure to a stressor (Zupan et al., 2016). In the present study, gilts exhibited greater RR than barrows throughout the study. However, post-hoc analysis did not reveal any interaction between sex, phase, or treatment in terms of RR. Since sex-specific changes to HRV in the absence of an interaction with phase or treatment were not a focus of this study, further evaluation is needed to clarify how both linear and nonlinear aspects of HRV are altered in a sex-specific manner.

4.6 Conclusion

In response to an acute heat stress period, HS pigs experienced greater body temperatures and were less active than TN pigs when placed in a novel pen for heat stress treatment. However, plasma cortisol concentrations and linear measures of HRV (RR, SDNN, RMSSD, and LF/HF) were largely unaffected by acute heat exposure. Heat stressed pigs exhibited lower SampEn during the acute heat episode and greater %DET in the period immediately following the acute heat episode compared to TN pigs. No other effects of the heat stress period on nonlinear measures were found. Inclusion of nonlinear HRV measures (such as SampEn and %DET) may be beneficial for inclusion in studies on animal welfare, however, further studies are needed.

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Table 4.1. Definitions of Heart Rate Variability Parameters.

Parameter	Practical Definition
Linear Measures	
Time Domain	
Average RR Interval (RR), ms	Average interval between adjacent heart beats over a period of time.
Standard deviation of RR intervals (SDNN), ms	The standard deviation of all RR intervals over a period of time.
Root Mean Square of Successive Differences (RMSSD), ms	The root mean square of successive RR intervals over a period of time. Greater levels indicate increased parasympathetic input.
Frequency Domain	
Low frequency to high frequency ratio (LF/HF)	The ratio between low and high frequency spectra after fast Fourier transformation of RR interval data. Greater values indicate increased sympathetic input.
Nonlinear Measures	
Sample Entropy (SampEn)	Measures the likelihood that runs of data patterns (vector length of *m* data points) that are close to each other will remain close if the vector length is increased by one ($m + 1$; Pincus 1995). Lower values indicate increased regularity in the HRV data.
Short-term detrended fluctuation analysis (DFAα_1)	A short-term measure of RR fluctuations at various time lengths to evaluate HR signal self-similarity. $\alpha_1 > 0.5$: Data are negatively-correlated. $\alpha_1 = 0.5$: Data are random, no long-range correlations. $0.5 < \alpha_1 < 1$: Data have long-range correlations. $1 < \alpha_1 < 2$: Data are correlated but do not have long-range correlations. Long-range correlations indicate increased self-similarity of the HRV data at different time lengths.
Recurrence rate (%REC), %	Determined using recurrence quantification analysis (RQA), the percentage of recurrent points (within some radius, *r*) in the recurrence plot. Greater values indicate increased HR regularity.
Determinism rate (%DET), %	Determined using recurrence quantification analysis (RQA), the percentage of recurrent points that form a diagonal line in the recurrence plot. Larger values indicate greater incidence of periodicity in the HRV data.
Mean line length of diagonal lines (Lmean), beats	Determined using recurrence quantification analysis (RQA), the mean length of diagonal lines in the recurrence plot. Greater values indicate periodicities with longer durations in the HRV data.

Table 4.2. Least squares means ± SE for plasma cortisol concentrations (ng/mL). Plasma was collected immediately following Phase 1 (pre-treatment baseline period) and Phase 2, where heat stress (HS) pigs were exposed to an acute heat episode while thermoneutral control (TN) pigs remained in thermoneutral conditions.

			P-Value
Phase	TN	HS	Trt*Phase
1	53.9 ± 9.4	48.7 ± 9.1	> 0.05
2	37.8 ± 9.4	67.7 ± 8.7	

Table 4.3. Least squares means ± SE for time and frequency domain heart rate variability measures. HRV collection occurred over three phases, where heat stress (HS) pigs were exposed to an acute heat episode during phase 2 while thermoneutral control (TN) pigs remained in thermoneutral conditions. Phases 1 and 3 occurred under thermoneutral conditions and served as baseline and post-treatment measurement periods for all pigs.

				P-Value
Parameter	Phase	TN	HS	Trt*Phase
RR, ms	1	448.8 ± 9.1[x]	429.8 ± 8.9	
	2	409.0 ± 11.1[y]	434.43 ± 9.0	0.02
	3	446.34 ± 9.5[x]	436.6 ± 9.2	
SDNN[1], ms	1	24.7 ± 1.2	21.0 ± 1.0	
	2	17.4 ± 1.0	26.0 ± 1.2	> 0.05
	3	19.3 ± 1.0	20.1 ± 1.0	
RMSSD, ms	1	9.1 ± 1.1	7.9 ± 1.1	
	2	7.9 ± 0.9	8.3 ± 0.7	> 0.05
	3	9.9 ± 1.5	7.4 ± 1.4	
LF/HF[1]	1	6.2 ± 1.2	5.3 ± 1.1[x,y]	
	2	2.6 ± 0.7	3.9 ± 0.6[x]	0.01
	3	2.7 ± 0.8[a]	7.4 ± 1.2[b,y]	

[a,b] Differences between TN and HS treatments within phase ($P \leq 0.05$).
[x,y] Differences between phases (1, 2, 3) within treatment ($P \leq 0.05$).
[1] Variable was transformed for analysis. Back-transformed least squares means ± approximated SE are presented.

Table 4.4. Least squares means ± SE for nonlinear heart rate variability measures. HRV collection occurred over three phases, where heat stress (HS) pigs were exposed to an acute heat episode during phase 2 while thermoneutral control (TN) pigs remained in thermoneutral conditions. Phases 1 and 3 occurred under thermoneutral conditions and served as baseline and post-treatment measurement periods for all pigs.

				P-Value
Parameter	Phase	TN	HS	Trt*Phase
SampEn	1	1.18 ± 0.09^{x}	1.29 ± 0.09^{x}	
	2	$1.46 \pm 0.12^{a,x,y}$	$0.90 \pm 0.09^{b,y}$	< 0.0001
	3	1.52 ± 0.09^{y}	$1.11 \pm 0.09^{x,y}$	
DFAα_1	1	1.54 ± 0.06	1.38 ± 0.06	
	2	1.41 ± 0.07	1.54 ± 0.05	>0.05
	3	1.40 ± 0.06	1.51 ± 0.05	
%REC[1], %	1	2.44 ± 0.33	4.75 ± 0.48	
	2	3.71 ± 0.47	3.10 ± 0.32	> 0.05
	3	3.25 ± 0.35	4.36 ± 0.44	
%DET, %	1	61.9 ± 5.0	64.3 ± 4.5	
	2	53.6 ± 5.7	73.5 ± 4.5	0.05
	3	47.6 ± 4.7^{a}	68.1 ± 4.5^{b}	
Lmean[1], beats	1	3.35 ± 0.12	3.07 ± 0.10	
	2	2.81 ± 0.12	3.83 ± 0.13	> 0.05
	3	2.76 ± 0.10	3.70 ± 0.12	

[a,b] Differences between TN and HS treatments within phase ($P \leq 0.05$).
[x,y] Differences between phases (1, 2, 3) within treatment ($P \leq 0.05$).
[1] Variable was transformed for analysis. Back-transformed least squares means ± approximated SE are presented.

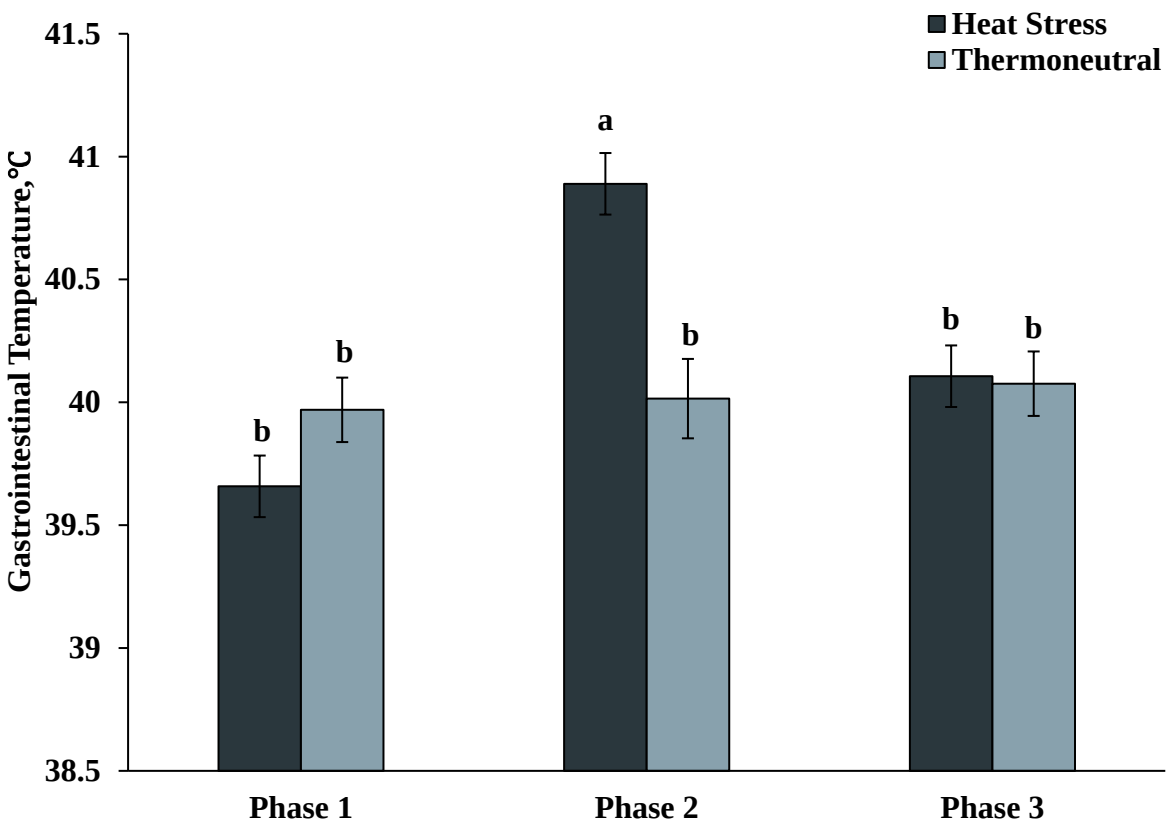

Figure 4.1. Least squares means ± SE of gastrointestinal temperature (T_g) for all pigs. Data collection occurred over three phases, where heat stress pigs were exposed to an acute heat episode during phase 2 while thermoneutral pigs remained in thermoneutral conditions. Phases 1 and 3 occurred under thermoneutral conditions and served as baseline and post-treatment measurement periods for all pigs. Different superscripts (a, b) indicate $P \leq 0.05$.

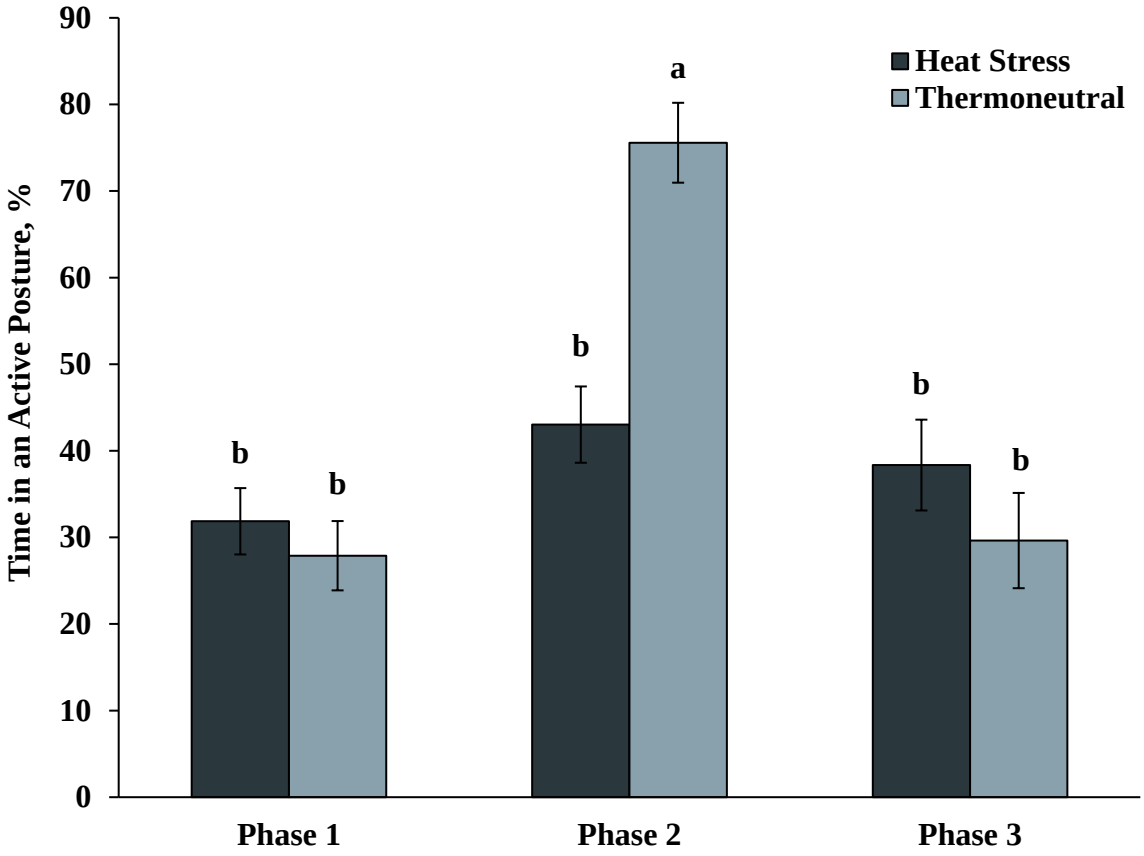

Figure 4.2. Least squares means ± SE of time spent in an active posture (standing or sitting; %) for all pigs. Data collection occurred over three phases, where heat stress pigs were exposed to an acute heat episode during phase 2 while thermoneutral pigs remained in thermoneutral conditions. Phases 1 and 3 occurred under thermoneutral conditions and served as baseline and post-treatment measurement periods for all pigs. Different superscripts (a, b) indicate $P \leq 0.05$.

CHAPTER 5. EVALUATION OF BASELINE HEART RATE VARIABILITY AS AN INDICATOR OF THE BEHAVIORAL AND PHYSIOLOGICAL RESPONSE TO A LIPOPOLYSACCHARIDE CHALLENGE IN GROWING PIGS

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5.1 Abstract

The objective of this study was to evaluate baseline heart rate variability (**HRV**) as a potential indicator of the subsequent pro-inflammatory cytokine response to a lipopolysaccharide (**LPS**) challenge in growing pigs. One day prior to the LPS challenge, baseline HRV was recorded from 20 experimental pigs. The following day, all experimental pigs were administered 2μg/kg BW LPS (*Escherichia coli,* 0111:B4). Rectal temperature and sickness behavior (evaluated by a human approach test; **approach time**) data were collected at -0.5, 1, 2, 3, 4, 8, and 24 h relative to LPS administration to evaluate the relationship between baseline HRV and the subsequent febrile and behavioral response. Additionally, blood was collected immediately before LPS administration and 1, 2, 4, 8, and 24 h post-LPS administration to evaluate the relationship between baseline HRV and the cortisol and pro-inflammatory cytokine response (TNF-α, IL-1α, IL-1β, IL-1ra, IL-6, and IL-8). Maximal change (**MAX**; maximum value – baseline) and area under the curve

(**AUC)** values were calculated for body temperature, human-approach behavior (AUC only), cortisol, and all cytokines after LPS administration. All relationships between baseline HRV values and indicators of the pro-inflammatory immune response to LPS (body temperature, approach time, serum cortisol concentration, serum cytokine concentration) were evaluated using multiple regression analysis in SAS 9.4. Approach time prior to LPS administration was inversely related to baseline standard deviation of the R-R intervals (t = -2.25; $P = 0.04$), and directly related to mean R-R interval length (t = 2.69; $P = 0.02$) and the mean length of diagonal lines in a recurrence plot (**Lmean**; t = 2.33; $P = 0.03$). This result may have implications for the use of HRV as a potential measure of temperament in future swine welfare studies. Area under the curve values for approach time following LPS administration were inversely related to high frequency spectral power (**HF**; $t_1 = -2.55$; $P = 0.02$) and directly related to body weight ($t_1 = 2.95$; $P = 0.009$), where pigs with low baseline HF values and higher body weights exhibited lower approach time AUC values following LPS administration. Body temperature MAX was directly related to Lmean (t = 4.21; $P = 0.0005$), indicating that pigs with greater Lmean values had a greater change in body temperature from baseline following LPS administration. In conclusion, while baseline HRV measures were not directly representative of the cortisol or cytokine response following an LPS challenge, HF and Lmean may be useful indicators for evaluating certain aspects (sickness behavior and fever) of the innate immune response to an LPS challenge.

Keywords: cytokine, heart rate variability, lipopolysaccharide, sickness, stress, swine

5.2 Introduction

The autonomic nervous system (**ANS**) exerts a suppressive effect on the pro-inflammatory cytokine response to an immune challenge. While the exact mechanism for how this occurs is not

known, several studies have implicated various aspects of the ANS that may play an active role in innate cytokine suppression. These include modulation of the sympathetic ganglion by the efferent arm of the vagus nerve via the cholinergic anti-inflammatory pathway (Huston and Tracey, 2011), or as of late, via cooperation between the afferent arm of the vagus nerve and splanchnic sympathetic nerves (Martelli et al., 2016; Komegae et al., 2018). Regardless of mechanism, however, it is clear that the ANS is an important factor in suppressing the innate cytokine response to avoid overexpression, tissue damage, and injury (Haensel et al., 2008)

Previous research in humans has found that various measures of heart rate variability (**HRV**; a non-invasive method used to evaluate autonomic function) are inversely correlated with the production of innate pro-inflammatory cytokines (Sloan et al., 2007; Cooper et al., 2015). Additionally, individuals with lower baseline levels of certain HRV measures exhibit a more pronounced cytokine response and, in some cases, impaired post-stress cytokine recovery (Jan et al., 2010; Weber et al., 2010). Therefore, baseline HRV may be a useful indicator of future risk for tissue damage and injury due to cytokine overexpression in swine.

The objective of this study was to evaluate baseline HRV as a potential indicator of the subsequent pro-inflammatory cytokine response to a lipopolysaccharide (**LPS**) challenge in growing pigs. We hypothesized that pigs with greater baseline parasympathetic activity would exhibit a more tempered cytokine and febrile response than pigs with low baseline parasympathetic activity. Accordingly, we predicted that pigs with greater mean R-R interval (**RR**; Table 5.1), greater standard deviation of the R-R intervals (**SDNN**; Table 5.1), greater root mean of successive squared differences (**RMSSD**; Table 5.1), and greater high frequency spectral power (**HF**; Table 5.1) would exhibit lower area under the curve (**AUC**) and maximal response (**MAX**; maximum

concentration – baseline concentration) values for body temperature, serum cortisol, and serum circulating cytokines (IL-1β, IL-1α, IL-1ra, TNF-α, IL-6, and IL-8) following LPS administration.

Nonlinear HRV measures were also included for evaluating their ability to provide predictive information regarding the subsequent innate cytokine response to LPS administration (Sassi et al., 2015). Specifically, nonlinear measures indicative of HR signal regularity, complexity, and periodicity [sample entropy (**SampEn**; Table 5.1), detrended fluctuation analysis (**DFAα_1**; Table 5.1), mean line length of diagonal lines in a recurrence plot (**Lmean**; Table 5.1), determinism **(%DET**; Table 5.1), recurrence rate (**%REC**; Table 5.1)] were quantified. We predicted that pigs with lower sample entropy, lower %DET, lower %REC, lower Lmean, and a DFAα_1 exponent of approximately 1 would exhibit lower AUC and MAX values for body temperature, serum cortisol, and serum circulating cytokines (IL-1β, IL-1α, IL-1ra, TNF-α, IL-6, and IL-8) following LPS administration.

5.3 Materials and Methods

All experimental procedures were approved by Purdue University's Institutional Animal Care and Use Committee (protocol #1708001614).

5.3.1 Animals and Housing

Thirty experimental pigs [8-wk-old; mean body weight (**BW**): 26.6 ± 0.6 kg] were housed individually in single nursery pens (1.22 m x 1.37 m) with plastic slatted flooring and were provided *ad libitum* feed and water. Diets were formulated to meet or exceed NRC requirements set for the appropriate stage of production (NRC, 2012). All pigs had tactile, visual and auditory contact with pigs in adjacent pens. Temperature within the experimental facility was controlled (25.1 ± 0.1℃; 69.1 ± 0.3% relative humidity) and artificial light was provided between

approximately 0730 h and 1500 h, however, several windows within the facility provided supplementary natural lighting. The experimental procedure occurred over 2 repetitions, where sample size per repetition was balanced for treatment (see below) and sex.

5.3.2 Acclimation Protocol

All experimental pigs underwent a 4-d acclimation protocol, where they were exposed to study personnel and heart rate equipment (Polar H10 HR monitor; Polar Electro Oy; Kempele, Finland) in their home pen for 1 session (20 min) each day. The initial 10 min of each session consisted of study personnel entering each pen and standing on the opposite side of the pig. When the pig approached and made contact (*e.g.* nose to leg) with the observer, the pig was given 1 marshmallow before the observer walked to the opposite side of the pen and repeated the protocol for up to 10 min. At 10 min, the observer attempted to fit the pig with the HR monitor. Once the monitor was fit, the observer continued to interact with the pig until 20 min had elapsed. For each acclimation session, study personnel wore brown rubber boots, white disposable coveralls with a hood (DuPont Tyvek 400; DuPont, Wilmington, DE), black nitrile gloves, and a dust mask (N35; 3M, Maplewood, MN) to conceal their face.

5.3.3 Baseline HRV Measurement

One day after the final acclimation session, each pig underwent HRV data collection for 1 h in their home pen. Briefly, ECG gel was applied to the electrode area of the HR monitor before placing the HR monitor around the pigs' thorax immediately behind the front legs. Heart rate was checked briefly by palpation prior to placing the heart rate monitor and securing the HR monitor with flexible veterinary bandage (Vet Wrap; 3M, Maplewood, MN). Data were transmitted telemetrically to a data recorder (Polar V800 Sports Watch; Polar Electro Oy; Kempele, Finland) and stored until subsequent HRV analysis. Experimental personnel left the experimental facility

during HRV data collection. After 1 h, HR monitors were removed, and each pig was weighed to accurately formulate lipopolysaccharide (LPS) doses based on BW.

5.3.4 Experimental Procedure

Ten pigs (5 gilts, 5 barrows) were randomly selected as controls (**CON**) to remain in their home pens throughout the LPS sickness challenge, while the remaining pigs (**TRANSPORT**; n = 20; 10 gilts, 10 barrows) were transported for 1 h, mixed for 30 min, and individually penned in another facility prior to the LPS sickness challenge. This was done to investigate whether exposure to a common stressor alters the relationship between baseline HRV and the subsequent immune response to a sickness challenge. As such, transportation treatment (CON or TRANSPORT) was included as a covariate in the model (see *Statistical Analysis* below). A steel livestock trailer (4.9 m L x 2 m W x 2 m H) used for transportation was bedded with straw to assist with thermoregulation. Transportation occurred over a 33.5-mile route and consisted of approximately 25% unpaved and 75% paved roads. Additionally, a 15-min stop occurred at a busy rest stop to simulate normal transportation conditions (*e.g.* fueling). Once the trailer arrived at the destination facility, TRANSPORT pigs were unloaded and penned together in a single pen (1.52 m x 1.83 m) for 30 min on grooved concrete flooring to simulate typical mixing procedures experienced on-farm. Each TRANSPORT pig was then moved to an individual pen (1.52 m x 1.83 m) on grooved concrete with *ad libitum* feed and water for the remainder of the study.

The LPS challenge for TRANSPORT pigs began 1 h after all pigs were moved to individual pens. Pigs in the CON group were not moved prior to LPS administration. Briefly, each pig was restrained on their backs in a v-trough and 2 μg/kg BW LPS (*Escherichia coli*, 0111:B4, Sigma-Aldrich Corporation; St. Louis, MO) was administered intravenously via jugular venipuncture

using a sterile 20-gauge, 3.8 cm needle. Pigs were monitored throughout the experimental procedure and taken off study 24 h after LPS-administration.

5.3.5 Human Approach Test and Rectal Temperature Collection

To evaluate the relationship between baseline HRV and sickness behavior, a human approach test occurred at - 0.5, 1, 2, 3, 4, 8, and 24 h relative to LPS administration. Briefly, a single study personnel, wearing the same attire worn during the acclimation phase, entered each pen on the opposite side of the pig. The amount of time it took for the pig to approach and make contact (*e.g.* nose to leg) with the observer was measured (**approach time**). If a pig failed to make contact within 120 s, the test ended. Immediately after the test, the observer approached the pig and collected rectal temperature at similar time intervals (-0.5, 1, 2, 3, 4, 8, and 24 h relative to LPS administration).

5.3.6 Blood Collection and Analysis

Blood collection occurred following the human approach test and body temperature collection period at 0, 1, 2, 4, 8, and 24 h relative to LPS administration. Study personnel who also participated in the human approach test left the experimental room to remove their white coveralls, boots, and masks prior to the blood collection period. For each collection, pigs were restrained on their backs in a v-trough and 5 mL of blood were collected in a 10 mL plastic serum collection tube (Vacutainer Plastic Serum Collection Tube; Becton-Dickinson, Franklin Lakes, NJ) via jugular venipuncture with a 20-gauge 3.8 cm needle (Vacutainer; Becton-Dickinson; Franklin Lakes, NJ). Each blood collection sample was allowed to clot for 2 h at room temperature before undergoing centrifugation (4℃; 1,900 x g for 15 min). The resulting serum was then collected, aliquoted, and stored at -80℃. Serum concentrations of TNF-α, IL-1α, IL-1β, IL-1ra, IL-6, and IL-8 were determined via immunology multiplex assay (Milliplex MAP Porcine Cytokine and

Chemokine Magnetic Bead Panel; EMD Millipore, Billerica, MA) by a third-party (Cytokine Reference Laboratory; University of Minnesota, St. Paul, MN) using Luminex platform technology. Intra-assay CV for TNF-α, IL-1α, IL-1β, IL-1ra, IL-6, and IL-8 concentrations were 11.8, 17.6, 8.0, 4.8, 6.9, and 5.4 %, respectively. Inter-Assay CV for TNF-α, IL-1α, IL-1β, IL-1ra, IL-6, and IL-8 concentrations were 7.1, 7.4, 6.3, 5.2, 6.2, and 8.4%, respectively. Serum cortisol concentrations were determined using a commercially available RIA kit (ImmuChem Cortisol Coated Tube Kit; MP Biomedicals, Inc., Santa Ana, CA). Intra- and inter-assay CV for serum cortisol concentrations were 9.4% and 7.0%, respectively.

5.3.7 Behavioral Analysis

Piglet postural behavior (standing, sitting, lying) was recorded continuously using a digital video recorder system (GeoVision VMS Software; Geo Vision Inc., Taipei, Taiwan) and 8 mounted cameras (1 camera for 2 pens per repetition; KPC-N502NUB; KT&C USA, Fairfield, NJ). Subsequent behavioral analysis identified 5-min periods of inactivity (lying) for use in HRV analysis.

5.3.8 HRV Analysis

One 5-min segment of R-R interval data during a period of inactivity was identified for each pig. The first suitable 5-min segment to occur at least 10 min after interaction with study personnel was used. All data sets contained less than 5% erroneous R-R intervals and were corrected prior to HRV analysis using previously published artefact correction guidelines (Marchant-Forde et al., 2004).

All linear measures (RR, SDNN, RMSSD, and HF) and 2 of 5 nonlinear measures (SampEn and $DFA\alpha_1$) were quantified using available HRV analysis software (Kubios HRV Standard; Kubios Oy, Kuopio, Finland). Prior to analysis of SDNN, RMSSD, HF, SampEn, and $DFA\alpha_1$ data

were detrended using first-order differencing to obtain data stationarity. Data were subsequently re-sampled at 4 Hz for determination of HF spectral power (0.09-2.0 Hz; Poletto et al., 2011) using fast Fourier transformation (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). A spectral window length of 50% was used to reduce spectral leakage in the signal. Sample entropy was evaluated using an embedding dimension of 2 heart beats and threshold value of 0.15 x SD (Yentes et al., 2013). A range of 4-16 heart beats was used to conduct DFAα_1.

The remaining nonlinear measures (%REC, %DET, and Lmean) were determined by recurrence quantification analysis (**RQA**) using an available software package (RHRV package in R 3.3.3; R Foundation for Statistical Computing; Vienna, Austria). The average mutual information method ("mutual" command in the 'tserieschaos' package) was used for each data set to determine a mean time delay value of 12. A mean embedding dimension of 6 was determined in a similar fashion using the false nearest neighbors method ("FNN" command in the 'fractal' package; Parameters: dimension = 15, lag = determined from AMI for each data set, R_{tol} = 15, A_{tol} = 2). Finally, a radius of 9 beats was used so the majority of data sets had a %REC value between 1% and 5% (Wallott, 2017).

5.3.9 Statistical Analysis

Temporal changes to body temperature, approach time, serum cortisol concentration, and cytokine concentrations following LPS administration were evaluated using a linear mixed model with repeated measures (Proc GLIMMIX; SAS Institute Inc., Cary, NC). Body temperature, approach time, serum cortisol concentration, and serum concentrations of each cytokine (TNF-α, IL-1α, IL-1β, IL-1ra, IL-6, and IL-8) were used as dependent variables in individual models with time (0, 1, 2, 4, 8, and 24 h relative to LPS administration) serving as the independent variable. A

Kenward-Rogers degrees of freedom approximation was applied to all analyses and multiple comparisons were conducted using Tukey's honest significance test. Data were transformed as needed to meet model assumptions (residual normality and homogeneity of variance) and are presented as least squares means ± SE. Transformed data (Log_{10}: TNF-α, IL-1α, IL-1β, IL-6, and IL-8; Square Root: IL-1ra) are presented as back-transformed least squares means ± approximated SE that were determined using the delta method.

Relationships between baseline HRV and body temperature, approach time, serum cortisol concentration, and serum cytokine concentrations were analyzed using multiple regression analysis in SAS 9.4 (Proc REG; SAS Institute Inc., Cary, NC). Several of the HRV parameters investigated were highly correlated with each other (Table 5.2). Therefore, RMSSD, SampEn, and %DET were removed from the analysis to reduce multicollinearity within the model (*e.g.* variance inflation factor values for terms in model were 5 or less). Serum cortisol concentration and human approach time values obtained immediately prior to LPS administration were included as dependent variables in individual regression models to evaluate their relationship with baseline HRV following transport and mixing. Maximal change from baseline (**MAX**; maximum value - baseline value) and area under the curve (**AUC**) values were calculated for body temperature, approach time (AUC only), serum cortisol concentration, and serum cytokine (TNF-α, IL-1α, IL-1β, IL-1ra, IL-6, and IL-8) concentrations. All MAX and AUC values were included as dependent variables in individual regression models to evaluate their relationship with baseline HRV measures following LPS administration. Heart rate variability measures (RR, SDNN, HF, $DFA\alpha_1$, %REC, and Lmean) were included as independent variables in all models. Data were transformed as needed in order to maintain residual normality and homogeneity of variance. Specifically, baseline approach time, approach time AUC, body temperature AUC, TNF-α AUC,

and IL-1α MAX were log-transformed for analysis. Additionally, Cortisol AUC, TNF-α MAX, IL-1α AUC, IL-1β MAX, IL-1ra AUC, IL-6 MAX/AUC, and IL-8 MAX/AUC were square root transformed prior to analysis. A variable selection procedure was employed using the BIC criterion as an indicator of model-fit. Any reduced model with $P \leq 0.05$ was then re-analyzed with potential confounding variables [repetition, weight, sex, treatment (CON or TRASNPORT)] and re-fit using the model selection procedure previously described. A final model (HRV measures + covariates) with $P \leq 0.05$ was considered significant. Regression data in figures are presented as they were used in the model. Therefore, no back-transformation took place. Data from 5 TRANSPORT (1 barrow, 4 gilts) and 5 CON (2 barrows, 3 gilts) pigs were removed due to extensive HRV error, therefore data were analyzed with 15 TRANSPORT (9 barrows, 6 gilts) and 5 CON (3 barrows, 2 gilts) pigs.

5.4 Results

5.4.1 Temporal Changes to Body Temperature, Approach Time, Serum Cortisol Concentration, and Serum Cytokine Concentrations Following LPS Administration

Body temperature (Fig. 5.1), approach time (Fig. 5.2) serum cortisol concentration (Fig. 5.3), and all analyzed serum cytokine (TNF-α, IL-1α, IL-1β, IL-1ra, IL-6, and IL-8; Fig. 5.4-5.9) concentrations were related to time ($P < 0.05$) and were characterized by an elevation and return to baseline in the 24-h period following LPS administration.

5.4.2 Multiple Regression Analysis Prior to LPS Administration

Approach behavior prior to LPS administration was inversely related to SDNN ($t_1 = -2.25$; $P = 0.04$; Table 5.3; Fig. 5.10), and directly related to RR ($t_1 = 2.69$; $P = 0.02$; Table 5.3; Fig. 5.11) and Lmean ($t_1 = 2.33$; $P = 0.03$; Table 5.3; Fig. 5.12). No additional effects of any remaining HRV variables, repetition, sex, weight, or treatment were detected ($P > 0.05$).

Serum cortisol concentration prior to LPS administration was not affected by any HRV measure, repetition, sex, weight, or treatment ($P > 0.05$).

5.4.3 Multiple Regression Analysis Following LPS Administration

Approach behavior AUC values were directly related to RR ($t_1 = 2.70$; $P = 0.02$; Table 5.3) and DFAα_1 ($t_1 = 2.33$; $P = 0.03$; Table 5.3), and inversely related to HF ($t_1 = -2.98$; $P = 0.009$; Table 5.3) prior to adjustment for covariates (repetition, sex, weight, treatment). However, following covariate adjustment and subsequent variable selection, only HF ($t_1 = -2.55$; $P = 0.02$; Table 5.3; Fig. 5.13) and BW ($t_1 = 2.95$; $P = 0.0089$; Table 5.3; Fig. 5.14) remained in the model.

Body temperature MAX was directly related to Lmean ($t = 4.21$; $P = 0.0005$; Table 5.3; Fig. 5.15). No additional effects of any remaining HRV variables, repetition, sex, weight, or treatment on body temperature MAX were detected ($P > 0.05$).

Prior to covariate adjustment, body temperature AUC was directly related to Lmean ($t_1 = 2.57$; $P = 0.02$; Table 5.3). However, Lmean was no longer related to body temperature AUC following covariate adjustment ($t_1 = 1.67$; $P = 0.11$). Subsequent variable selection analysis removed Lmean in place of treatment ($t_1 = -3.87$; $P = 0.001$; Table 5.3; Fig 5.16) and sex ($t_1 = -2.06$; $P = 0.05$; Table 5.3), where TRANSPORT pigs had lower body temperature AUC values compared to CON pigs and barrows exhibited greater body temperature AUC values compared to gilts (data not shown).

Prior to adjustment for repetition, sex, weight, and treatment, TNF-α AUC values were inversely related to SDNN ($t_1 = -2.16$; $P = 0.04$; Table 5.3). However, following covariate adjustment, SDNN was no longer related to TNF-α AUC ($t_1 = -1.39$; $P = 0.19$). Subsequent variable selection analysis based on the BIC criterion removed all variables except sex ($t_1 = 0.06$; $P = 0.04$; Table 5.3), where gilts had greater TNF-α AUC values than barrows (data not shown).

No additional relationships were detected between any remaining dependent variables and HRV measures (data not shown; $P > 0.05$).

5.5 Discussion

While the exact mechanism is not known, it is clear that the ANS plays a role in suppressing the production of pro-inflammatory cytokines to reduce overexpression, tissue damage and injury (Haensel et al., 2008). Previous research in humans has found some evidence that baseline heart rate variability can be used as an indicator of the ANS's subsequent effect on the innate immune response during stress and illness (Jan et al., 2010; Weber et al., 2010; Ohira et al., 2013). However, there was little evidence in the current study that baseline HRV was indicative of the subsequent growing pig pro-inflammatory cytokine response following an LPS challenge.

Of the cytokines investigated, only area under the curve values for TNF-α were found to be related to HRV prior to covariate adjustment. Standard deviation of the r-r intervals (SDNN) was directly related to TNF-α AUC, however, after controlling for covariates, SDNN was not a significant factor and was removed from the model by the variable selection process in place of sex. Previous work in humans has shown that baseline HRV spectral measures are positively (but somewhat weakly) correlated with the TNF-α response following LPS administration and subjects with low baseline RMSSD display a delayed serum TNF-α recovery response to a stressful task (Jan et al., 2010; Weber et al., 2010). While these studies show a relationship between baseline HRV and the TNF-α response, neither study evaluated whether TNF-α expression could be more readily explained by sex differences, which have been shown previously in pigs exposed to an LPS challenge (Williams et al., 2009).

The strongest finding in the current study was that approximately 47% of the variability in body temperature MAX could be explained by baseline Lmean, where pigs that had a greater

change in body temperature following LPS administration had greater baseline Lmean values. The mean length of diagonal lines in a recurrence plot (Lmean) is obtained via RQA, which allows a data set to be evaluated in multi-dimensional state space and visualized on a 2-dimensional recurrence plot (Eckmann et al., 1987). The RQA plot is then used to quantify recurring data points and periodicities within the data set. The mean length of diagonal lines in a recurrence plot provides a mean length of recurring periodicities that occur throughout the data set (Wallott, 2017). In general, longer Lmean values indicate more regularity within the data set and are consistent with greater stress in livestock species (Mohr et al., 2002). Therefore, while baseline HRV was not able to adequately characterize the cytokine response to an LPS challenge in the current study, particular aspects of the febrile response were readily characterized by Lmean.

In contrast with traditional linear HRV measures, physiological interpretations of changes to nonlinear HRV measures, like Lmean, are not well understood. However, evidence of nonlinearity in physiological systems is an integral component of physiological complexity, which is described as the complex interaction between physiological systems and processes that allow an organism to adapt to stressors (Goldberger et al, 2002). Accordingly, these nonlinear properties begin to break down in response to stress and illness (Goldberger et al., 2002). Therefore, it might be assumed that pigs with greater baseline Lmean values display reduced physiological complexity and could be more prone to the deleterious effects of regulatory physiological responses, such as fever, in response to illness.

Following covariate adjustment, area under the curve values for body temperature following LPS administration were related to treatment, where TRANSPORT pigs exhibited lower body temperature AUC values compared to CON pigs. One potential explanation could be that exposure to a prior stressor may alter the morphology and activation of cells required for a

subsequent pro-inflammatory immune response to a sickness challenge. A previous study in rats evaluating the role of different stressors (repeated restraint or variable stress) on the prefrontal cortical inflammatory response to LPS reported divergent effects depending on stressor type, where repeated restraint promoted a microglial response while variable stress was immunosuppressive (Smith et al., 2016). Therefore, it's possible that the activity of the cells involved in producing cytokines responsible for fever production were altered following transport and mixing, leading to their suppression and a less-severe febrile response. Unfortunately, however, the CON treatment sample size was too small to make any strong conclusions regarding the effect of a prior stressor on the subsequent immune response to an LPS challenge.

Interestingly, approach time prior to LPS administration (-0.5 h) was related to baseline values of RR, SDNN, and Lmean. Specifically, RR and Lmean were greater, and SDNN was lower in pigs that took longer to approach a human observer compared to pigs that approached more quickly. The direct relationship between RR and approach time was unexpected, since RR is typically inversely related to stress. However, the relationships between individual HRV measures in the model and time to approach were relatively weak. Therefore, while RR was selected to remain in the model it may be less important than SDNN and Lmean for distinguishing between stressed and non-stressed animals. Nevertheless, the combined variability explained by the three HRV measures in the model may provide further evidence that HRV can be used as an indicator of animal temperament, which has been investigated previously in livestock species (Sutherland et al., 2012; Frondelius et al., 2015).

After controlling for possible confounding factors, area under the curve values for approach time following LPS administration were inversely related to HF and directly related to BW. High frequency spectral power is indicative of vagally mediated changes to respiration and is typically

used as an indicator of parasympathetic activity, where greater values indicate greater parasympathetic tone (von Borell et al., 2007). Therefore, pigs with greater baseline BW and lower baseline parasympathetic tone took longer to approach the experimental personnel after LPS administration. Whether this reluctance to approach was a result of the innate immune response (as opposed to stress caused by the presence of experimental personnel) is not completely clear, however, temporal changes to approach times closely followed those of the febrile, cytokine, and cortisol responses to LPS. Given the temporal structure and additional evidence that decreased parasympathetic activity leads to a more severe inflammatory response (Bernik et al., 2002; The et al., 2011), baseline parasympathetic activity (as measured by HF) may be a useful indicator of the subsequent adaptive behavioral response to a sickness challenge in the absence of additional immune indicators. Further work is needed to explain the relationship between baseline BW and the behavioral sickness response since BW was not related to any other measure in the study and was not monitored following LPS administration.

5.6 Conclusion

Following adjustment for potential confounding factors, baseline HRV values were not related to MAX or AUC values for cortisol or any of the cytokines measured during an LPS challenge. However, approach time prior to LPS administration was inversely related to SDNN, and directly related to RR and Lmean. Approach time AUC values after LPS administration were inversely related to baseline HF and directly related to BW. Additionally, body temperature MAX was directly related to baseline Lmean. As a result, while not directly representative of the cortisol or cytokine response following an LPS challenge, HF and Lmean may be useful measures for evaluating certain aspects (sickness behavior and fever) of the innate immune response to an LPS challenge.

5.7 Literature Cited

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Table 5.1. Definitions of Heart Rate Variability Parameters.

Parameter	Practical Definition
Linear Measures	
Time Domain	
Average RR Interval (RR), ms	Average interval between adjacent heart beats over a period of time.
Standard deviation of RR intervals (SDNN), ms	The standard deviation of all RR intervals over a period of time.
Root Mean Square of Successive Differences (RMSSD), ms	The root mean square of successive RR intervals over a period of time. Greater levels indicate increased parasympathetic input.
Frequency Domain	
Low frequency to high frequency ratio (LF/HF)	The ratio between low and high frequency spectra after fast Fourier transformation of RR interval data. Greater values indicate increased sympathetic input.
Nonlinear Measures	
Sample Entropy (SampEn)	Measures the likelihood that runs of data patterns (vector length of *m* data points) that are close to each other will remain close if the vector length is increased by one ($m + 1$; Pincus 1995). Lower values indicate increased regularity in the HRV data.
Short-term detrended fluctuation analysis ($DFA\alpha_1$)	A short-term measure of RR fluctuations at various time lengths to evaluate HR signal self-similarity. $\alpha_1 > 0.5$: Data are negatively-correlated. $\alpha_1 = 0.5$: Data are random, no long-range correlations. $0.5 < \alpha_1 < 1$: Data have long-range correlations. $1 < \alpha_1 < 2$: Data are correlated but do not have long-range correlations. Long-range correlations indicate increased self-similarity of the HRV data at different time lengths.
Recurrence rate (%REC), %	Determined using recurrence quantification analysis (RQA), the percentage of recurrent points (within some radius, *r*) in the recurrence plot. Greater values indicate increased HR regularity.
Determinism rate (%DET), %	Determined using recurrence quantification analysis (RQA), the percentage of recurrent points that form a diagonal line in the recurrence plot. Larger values indicate greater incidence of periodicity in the HRV data.
Mean line length of diagonal lines (Lmean), beats	Determined using recurrence quantification analysis (RQA), the mean length of diagonal lines in the recurrence plot. Greater values indicate periodicities with longer durations in the HRV data.

Table 5.2. Pearson correlations for heart rate variability measures. Refer to Table 5.1 for HRV measure definitions.

	SDNN	**RR**	**RMSSD**	**HF**	**SampEn**	**DFAα_1**	**%DET**	**%REC**
RR	0.30812							
RMSSD	0.49760[a]	0.62140[a]						
HF	0.54218[a]	0.47330[a]	0.81516[c]					
SampEn	-0.42282	0.43403	0.45651[a]	0.26974				
DFAα_1	0.29980	-0.38050	-0.54923[a]	-0.27657	-0.85030[c]			
%DET	-0.06991	-0.52333[a]	-0.70348[b]	-0.49311[a]	-0.79157[c]	0.79163[c]		
%REC	-0.56748[a]	-0.32378	-0.51222[a]	-0.51517[a]	-0.17434	0.11951	0.48312[a]	
Lmean	-0.03880	-0.54599[a]	-0.60706[a]	-0.41666	-0.81317[c]	0.71081[b]	0.89366[c]	0.55315[a]

[a] $P \leq 0.05$
[b] $P \leq 0.001$
[c] $P \leq 0.0001$

Table 5.3. Multiple regression model fitting before and after covariate adjustment. Refer to Table 5.1 for HRV measure definitions.

	Initial Model[a]				Final Model[b]			
Parameter	**Variables in Model**	**Adj R^2**	**F**	***P***	**Variables in Model**	**Adj R^2**	**F**	***P***
Baseline Approach Behavior[e]	RR, SDNN, Lmean	0.28	3.44	0.04	RR, SDNN, Lmean	0.28	3.44	0.04
Approach Behavior AUC[cf]	RR, HF, DFAα_1	0.42	5.52	0.009	HF, Weight	0.47	7.69	0.004
Body Temperature MAX[d]	Lmean	0.47	17.73	0.0005	Lmean	0.47	17.73	0.0005
Body Temperature AUC[c]	Lmean	0.23	6.62	0.02	Sex, Treatment	0.48	9.63	0.002
TNF-α AUC[c]	SDNN	0.16	4.69	0.04	Sex	0.17	4.99	0.04

[a] Initial model evaluating only HRV measures following variable selection procedure.
[b] Final model after covariate adjustment and variable selection procedure.
[c] AUC = Area under the curve
[d] MAX = Maximal change (maximum value – baseline value)
[e] Time to approach an observer following transport but prior to LPS administration.
[f] Area under the curve for time to approach an observer following LPS administration.

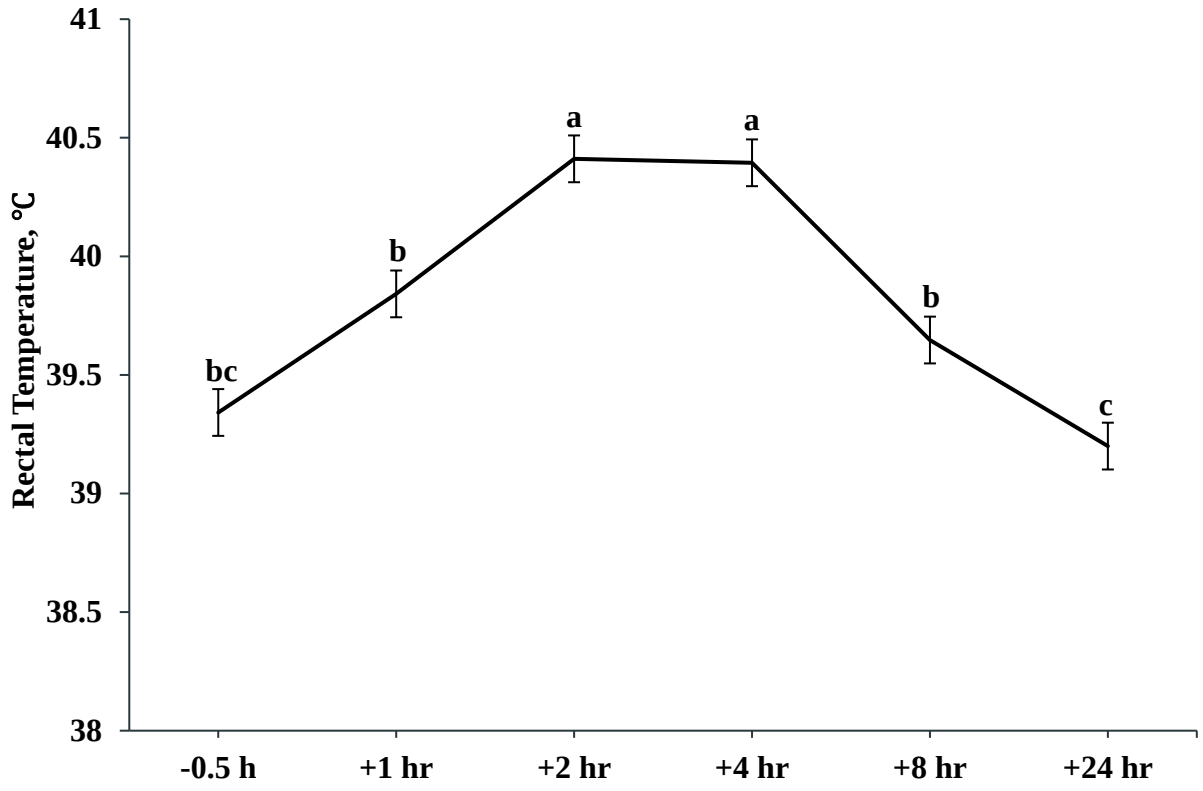

Figure 5.1. Least squares means ± SE of rectal temperatures in response to a lipopolysaccharide challenge in growing pigs. Lipopolysaccharide administration took place at time 0 (not shown in figure). Different superscripts (a, b, c) indicate differences ($P \leq 0.05$) between timepoints.

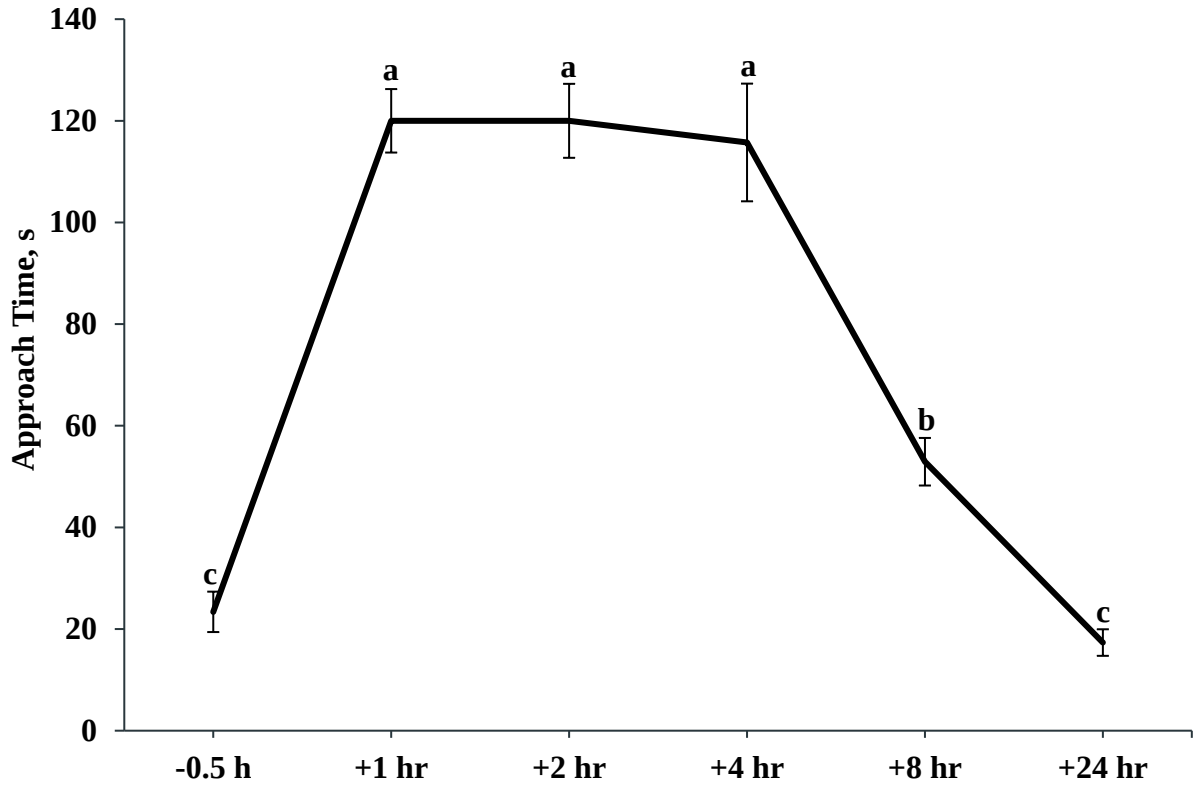

Figure 5.2. Least squares means ± SE of approach time during a human-approach test following lipopolysaccharide administration. Lipopolysaccharide administration took place at time 0 (not shown in figure). Different superscripts (a, b, c) indicate differences ($P \leq 0.05$) between timepoints.

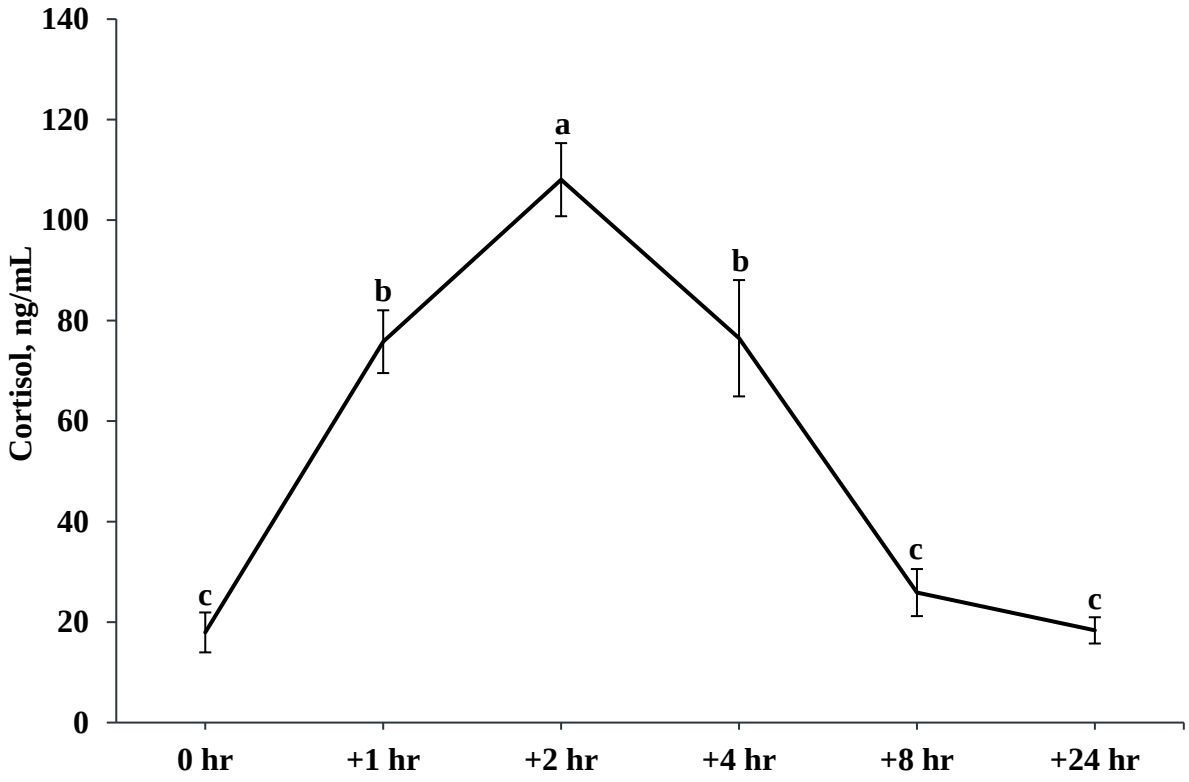

Figure 5.3. Back-transformed least squares means ± approximated SE of serum cortisol concentrations in response to a lipopolysaccharide challenge in growing pigs. Lipopolysaccharide administration took place at time 0 (not shown in figure). Different superscripts (a, b, c) indicate differences ($P \leq 0.05$) between timepoints.

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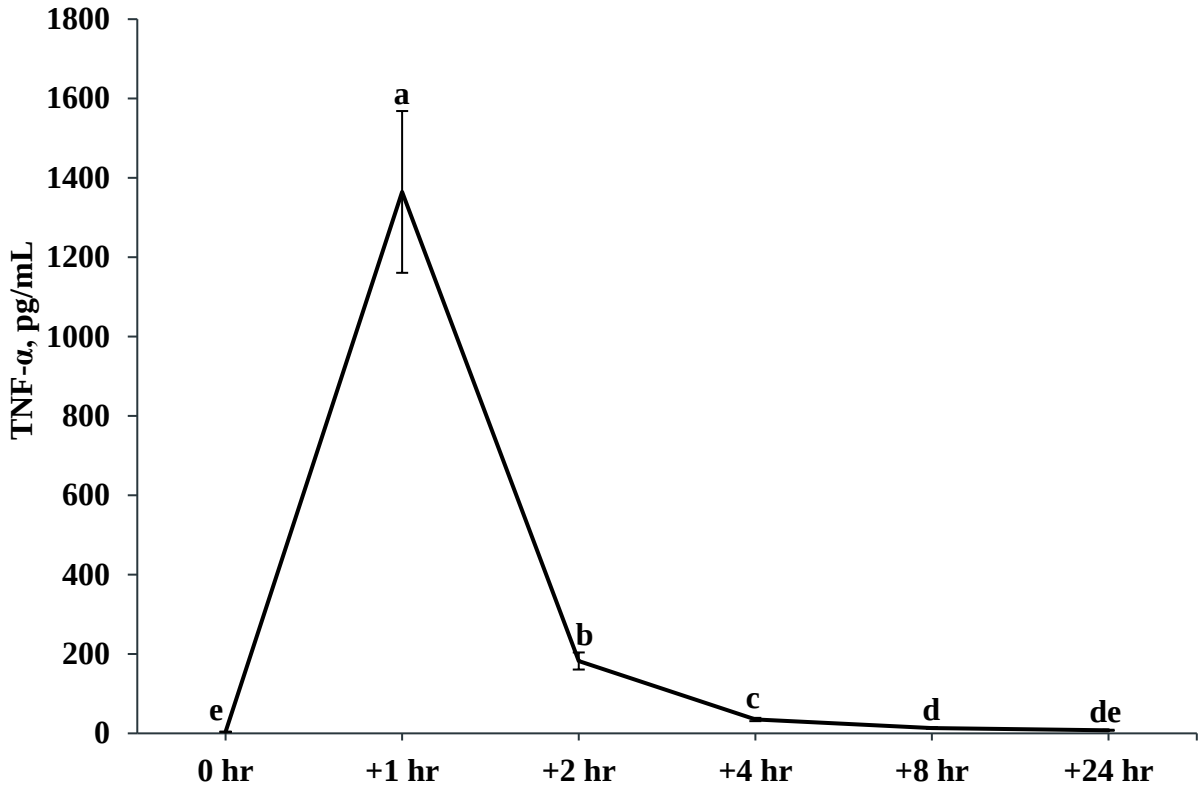

Figure 5.4. Back-transformed least squares means ± approximated SE of serum TNF-α concentrations in response to a lipopolysaccharide challenge in growing pigs. Lipopolysaccharide administration took place at time 0 (not shown in figure). Different superscripts (a, b, c, d, e) indicate differences ($P \leq 0.05$) between timepoints.

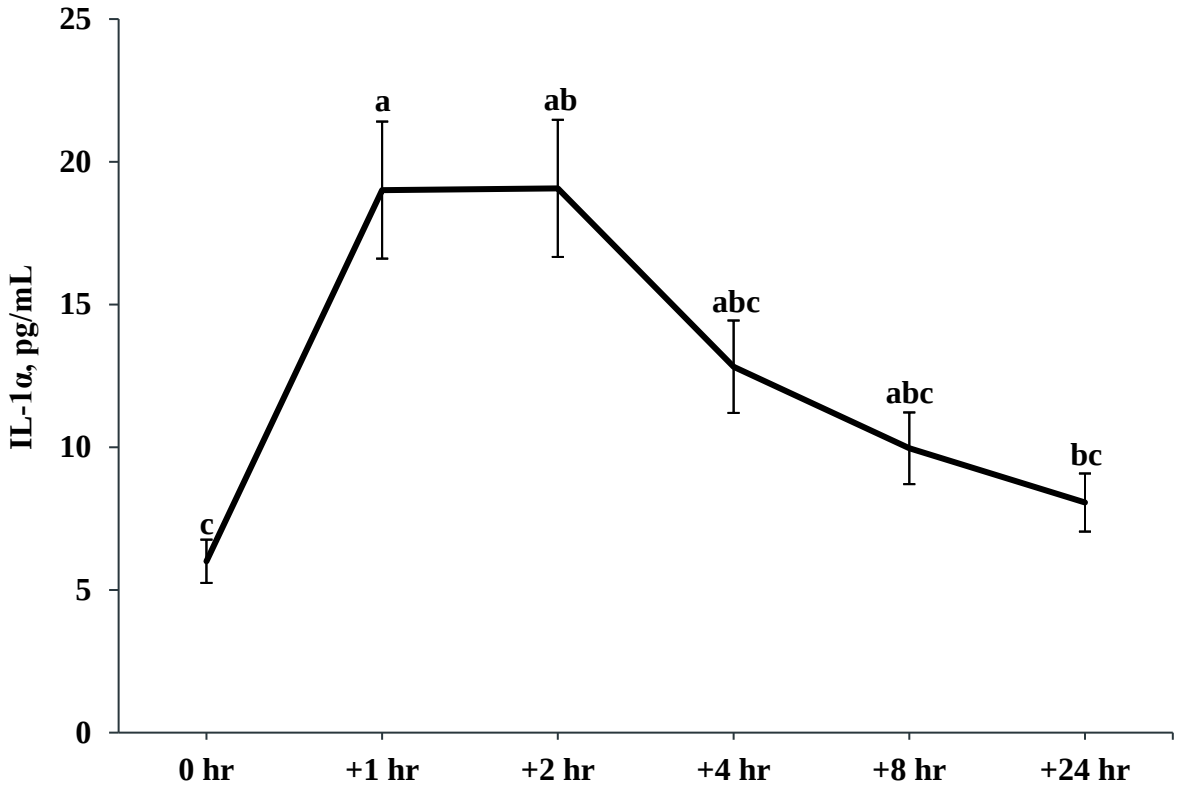

Figure 5.5. Back-transformed least squares means ± approximated SE of serum IL-1α concentrations in response to a lipopolysaccharide challenge in growing pigs. Lipopolysaccharide administration took place at time 0 (not shown in figure). Different superscripts (a, b, c) indicate differences ($P \leq 0.05$) between timepoints.

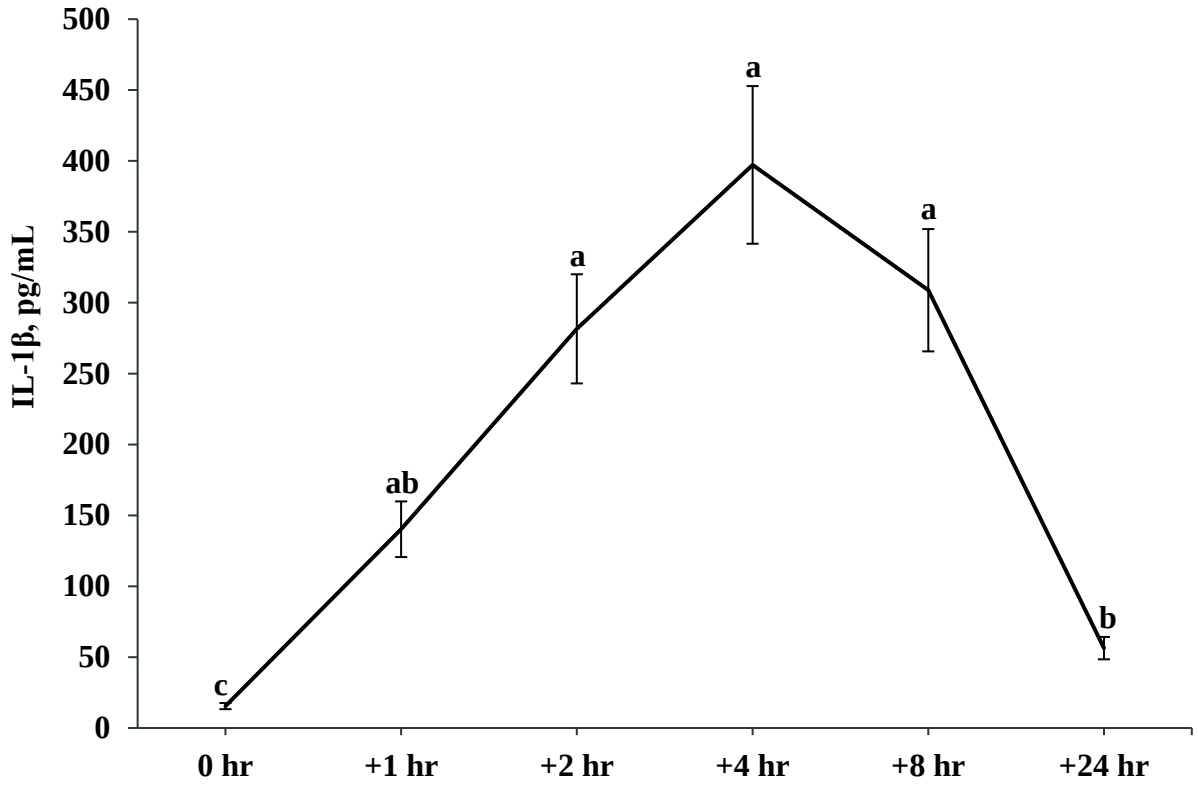

Figure 5.6. Back-transformed least squares means ± approximated SE of serum IL-1β concentrations in response to a lipopolysaccharide challenge in growing pigs. Lipopolysaccharide administration took place at time 0 (not shown in figure). Different superscripts (a, b, c) indicate differences ($P \leq 0.05$) between timepoints.

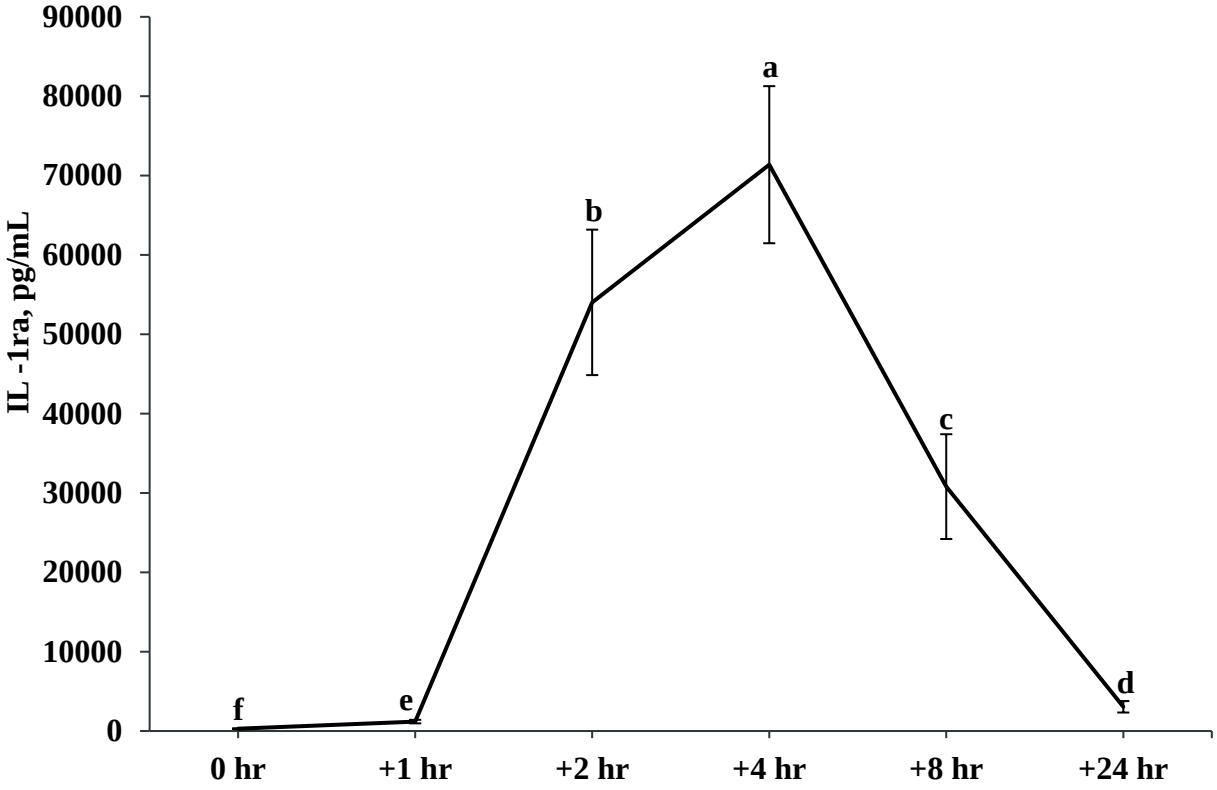

Figure 5.7. Back-transformed least squares means ± approximated SE of serum IL-1ra concentrations in response to a lipopolysaccharide challenge in growing pigs. Lipopolysaccharide administration took place at time 0 (not shown in figure). Different superscripts (a, b, c, d, e, f) indicate differences ($P \leq 0.05$) between timepoints.

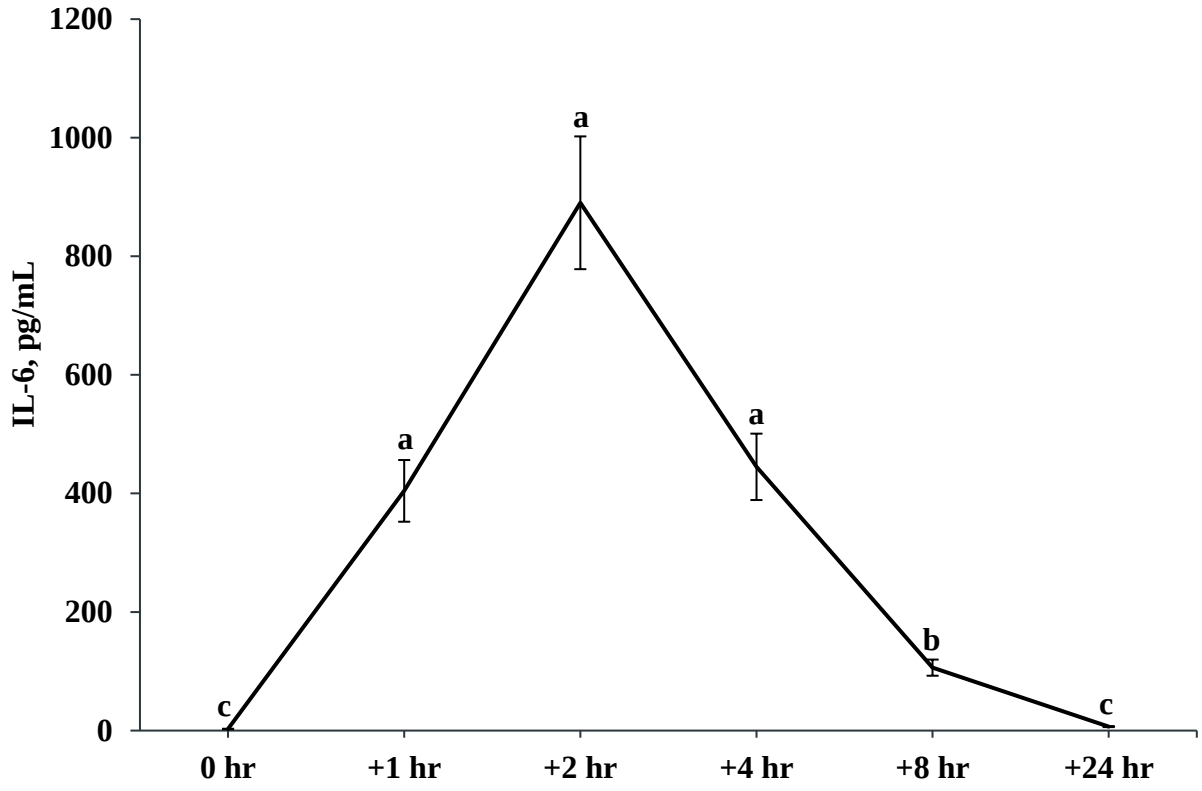

Figure 5.8. Back-transformed least squares means ± approximated SE of serum IL-6 concentrations in response to a lipopolysaccharide challenge in growing pigs. Lipopolysaccharide administration took place at time 0 (not shown in figure). Different superscripts (a, b, c) indicate differences ($P \leq 0.05$) between timepoints.

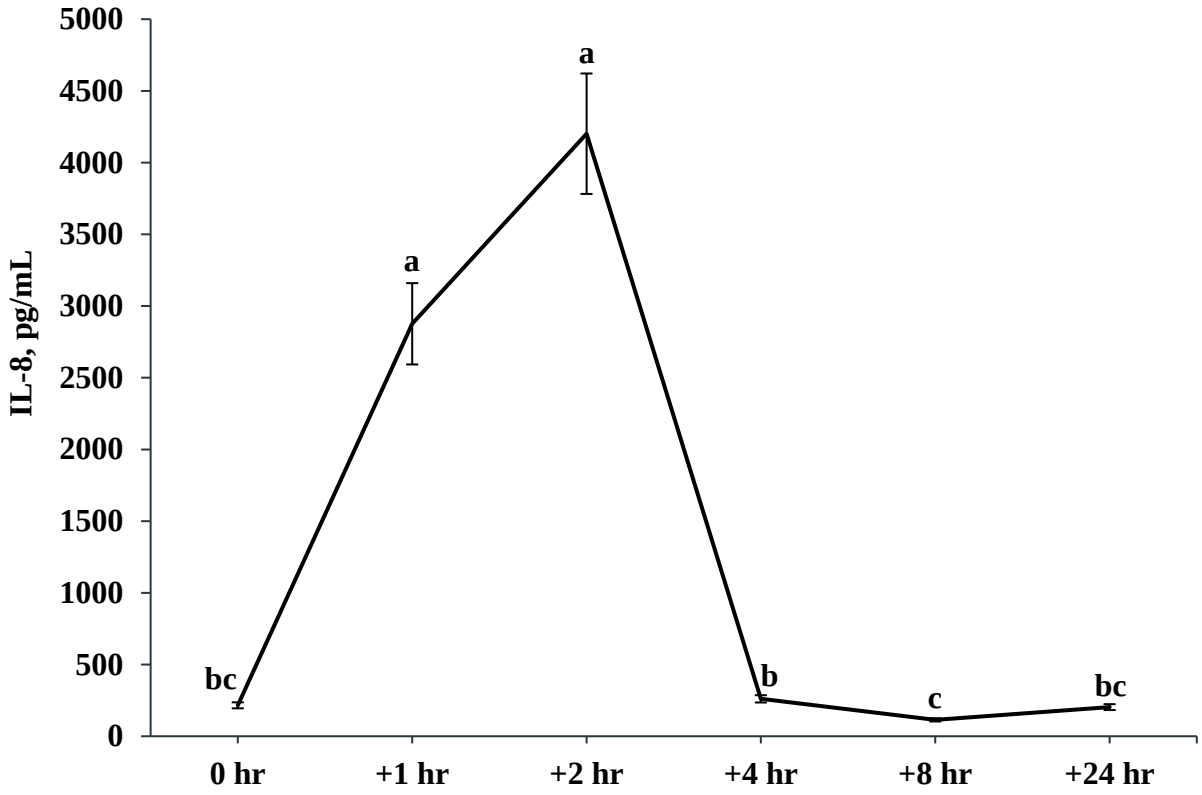

Figure 5.9. Back-transformed least squares means ± approximated SE of serum IL-8 concentrations in response to a lipopolysaccharide challenge in growing pigs. Lipopolysaccharide administration took place at time 0 (not shown in figure). Different superscripts (a, b, c) indicate differences ($P \leq 0.05$) between timepoints.

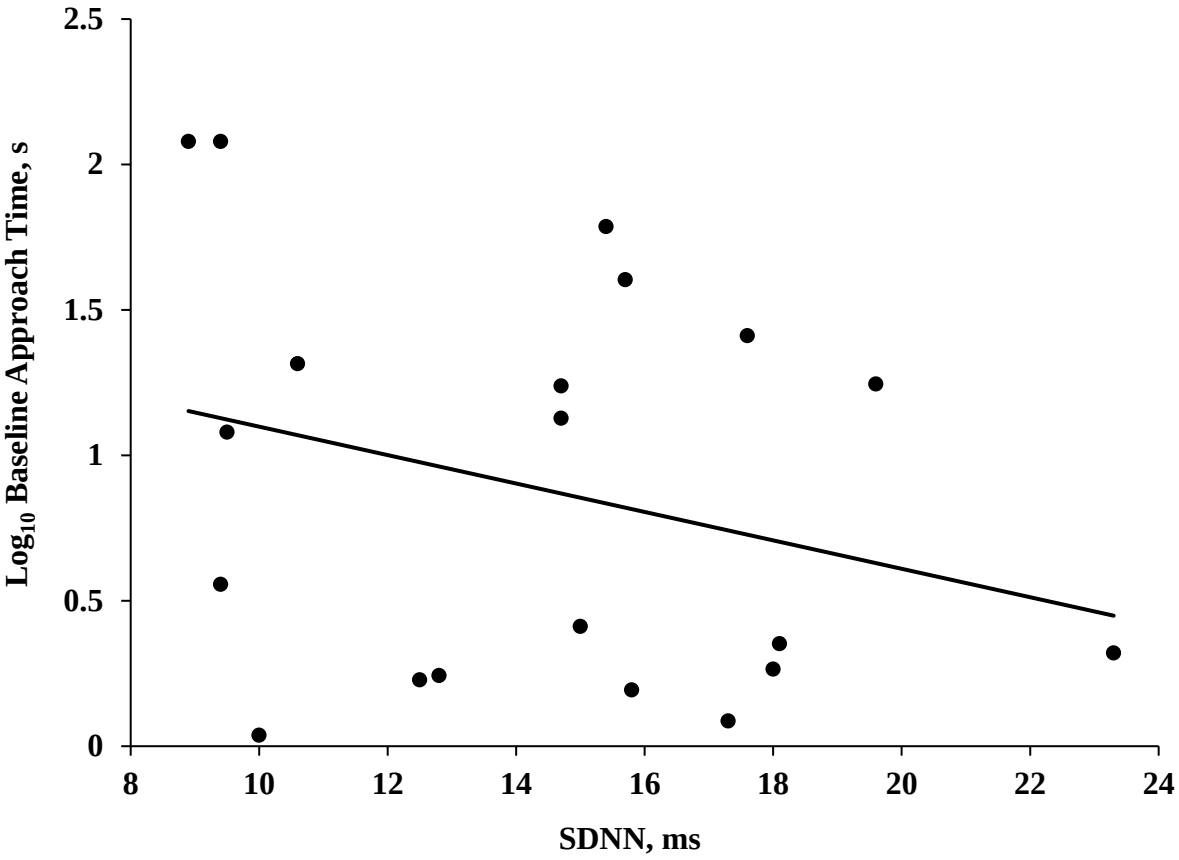

Figure 5.10. Linear relationship between log_{10}-transformed time to approach during a human-approach test and standard deviation of the R-R intervals (SDNN) prior to lipopolysaccharide challenge.

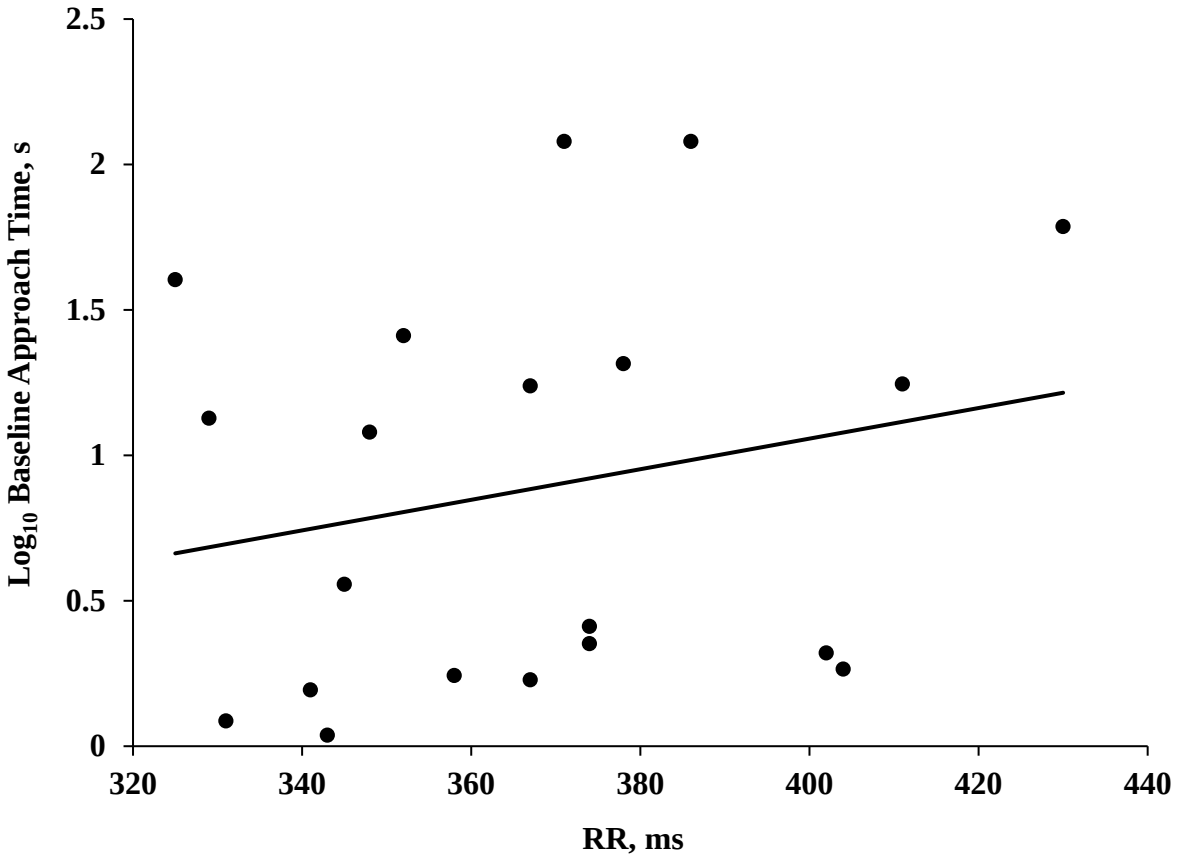

Figure 5.11. Linear relationship between log_{10}-transformed time to approach during a human-approach test and mean R-R interval (RR) prior to lipopolysaccharide challenge.

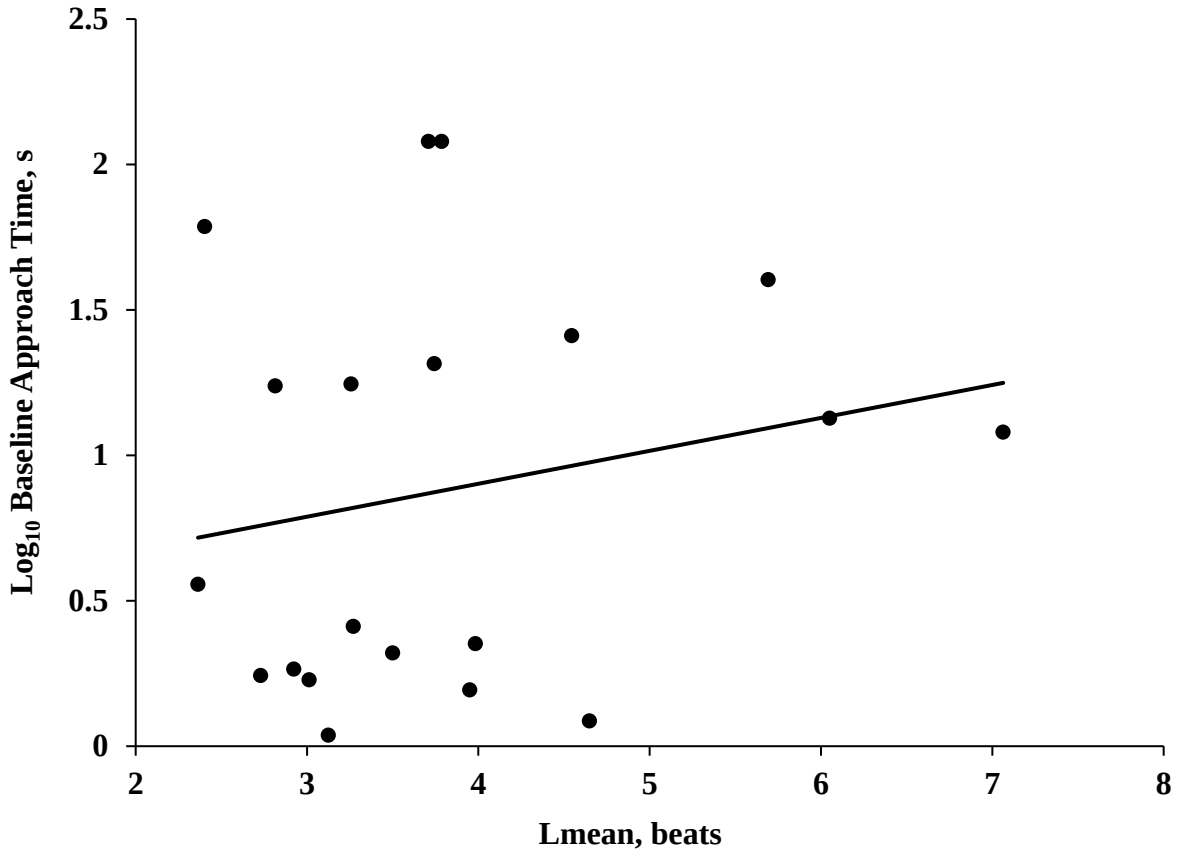

Figure 5.12. Linear relationship between log_{10}-transformed time to approach during a human-approach test and mean length of diagonal lines in a recurrence plot (Lmean) prior to lipopolysaccharide challenge.

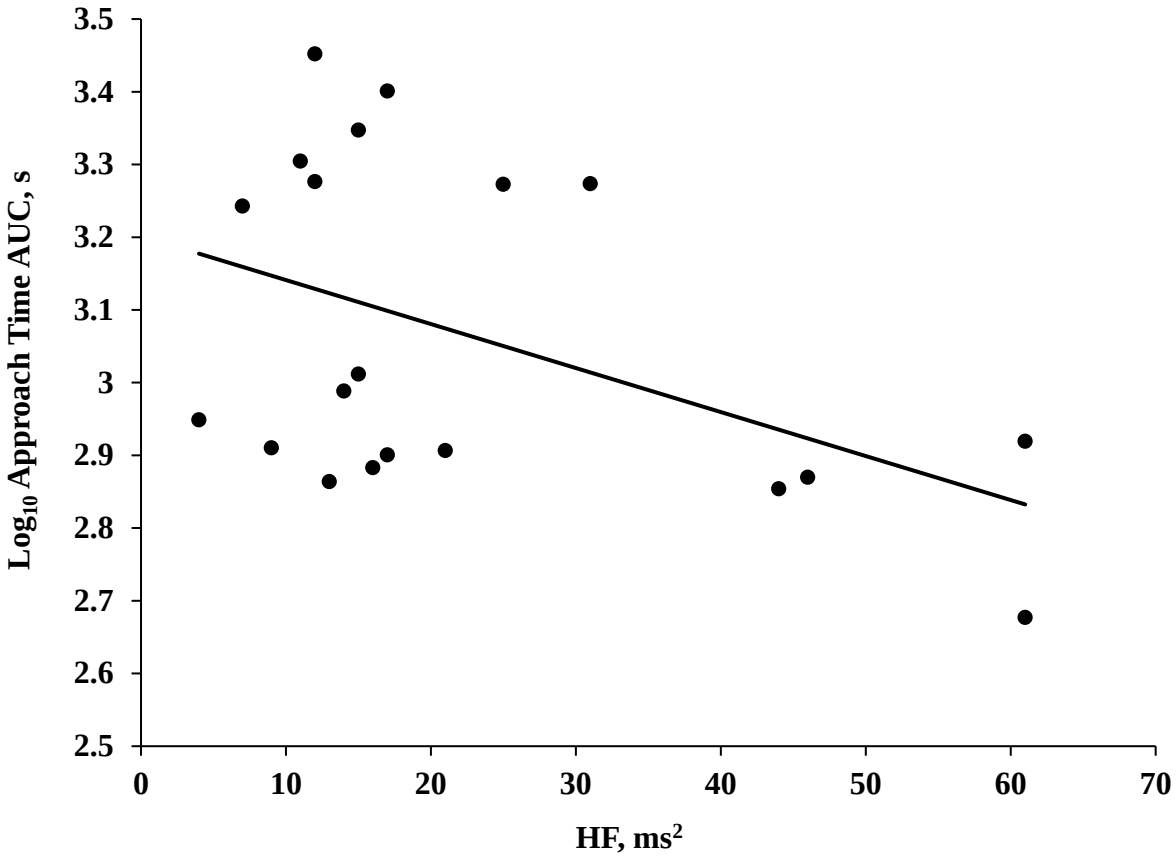

Figure 5.13. Linear relationship between log_{10}-transformed time to approach area under the curve (AUC) during a human-approach test and high frequency spectral power (HF) following lipopolysaccharide administration.

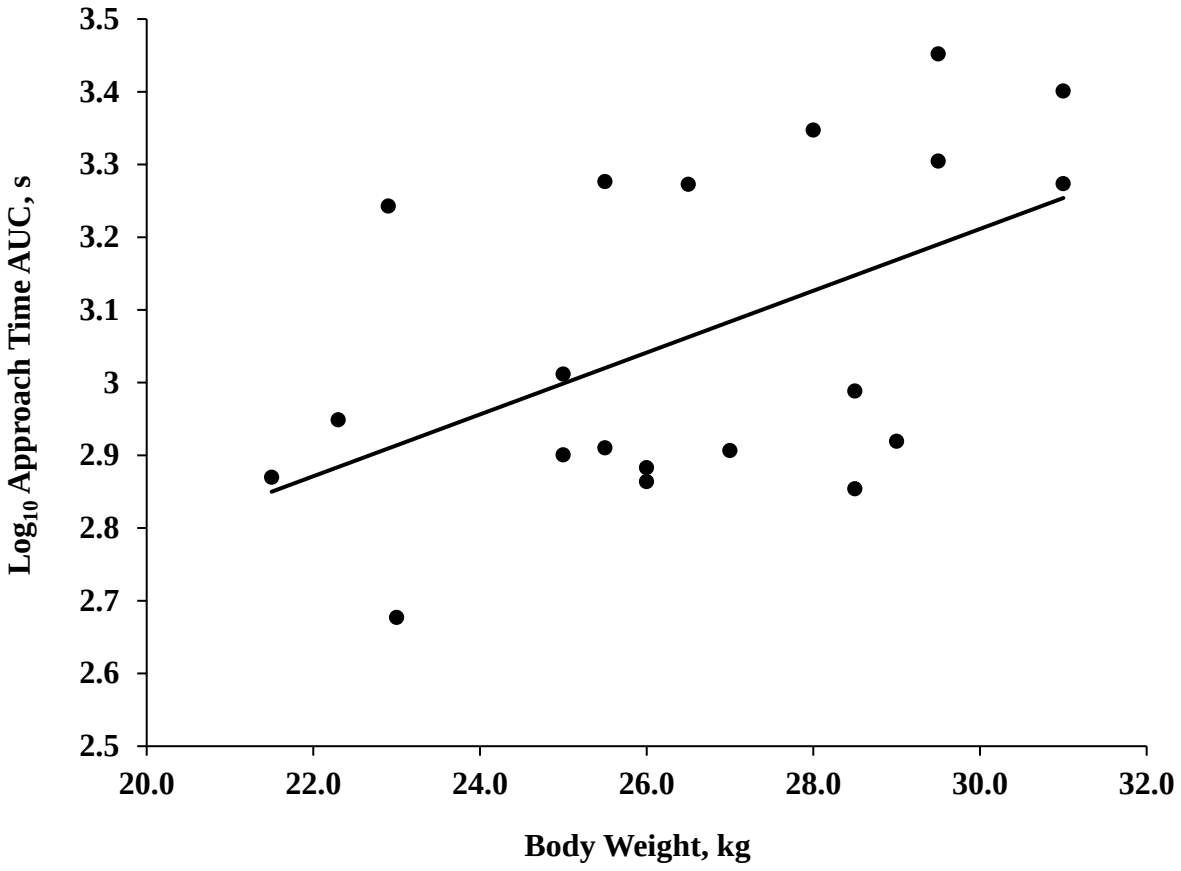

Figure 5.14. Linear relationship between log_{10}-transformed time to approach area under the curve (AUC) during a human-approach test and body weight following lipopolysaccharide administration.

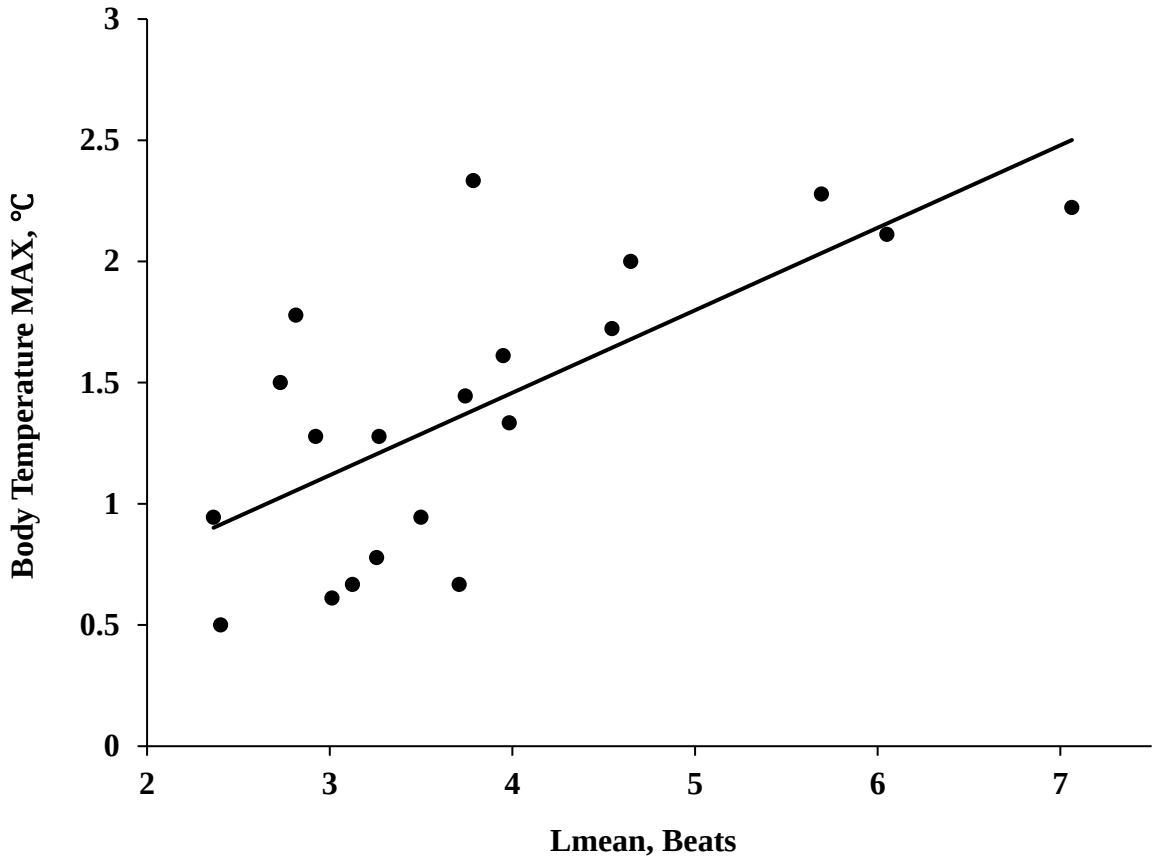

Figure 5.15. Linear relationship between maximal change in body temperature (MAX) and mean length of diagonal lines in a recurrence plot (Lmean) following lipopolysaccharide administration.

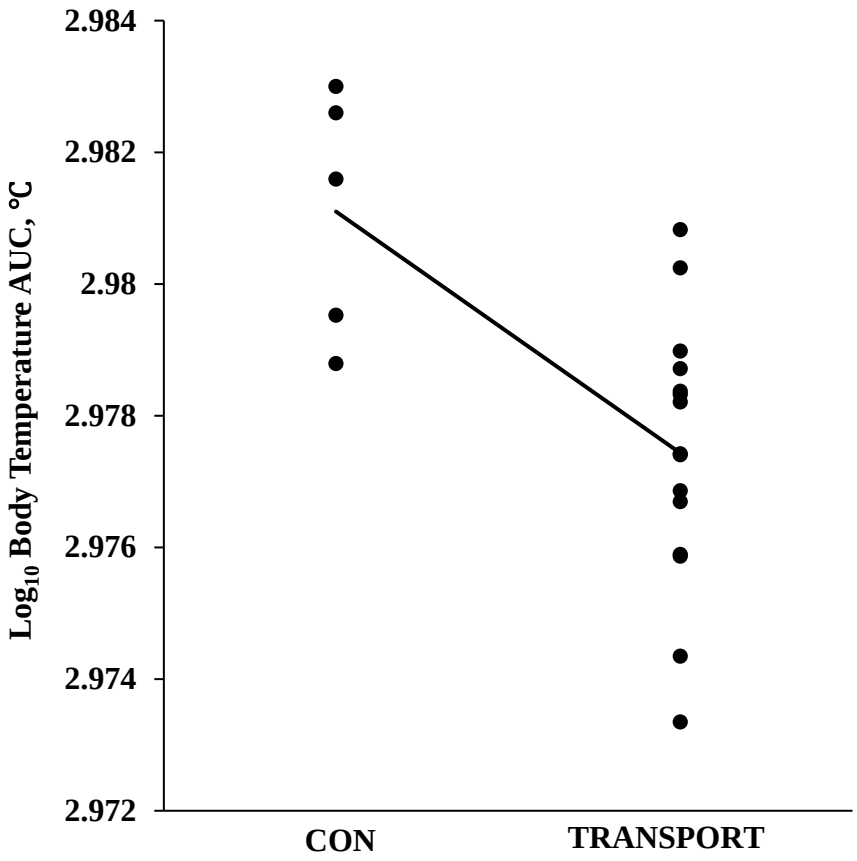

Figure 5.16. Linear relationship between log_{10}-transformed body temperature area under the curve (AUC) and transportation treatment following lipopolysaccharide (LPS) administration. Pigs in the transportation treatment (TRANSPORT) were transported to a new facility and mixed prior to the LPS challenge. Pigs in the control treatment (CON) remained in their home pens for the entirety of the experimental procedure.

CHAPTER 6. CONCLUSION

The presence of nonlinear variability in physiological processes, such a heart rate, is an integral component for maintaining healthy physiological complexity. However, very little work has sought to measure changes to nonlinear heart rate variability in relation to on-farm stressors experienced by livestock species. The work in this dissertation attempted to evaluate these nonlinear aspects by incorporating nonlinear HRV measures into studies focused on pain in dairy calves and piglets, heat stress in growing pigs, and the growing pig sickness response to a lipopolysaccharide challenge.

In response to pain, surgically castrated piglets exhibited greater regularity (SampEn) and more recurring periodicities (%DET) in their HRV data than sham castrated piglets, which indicates increased pain-related stress as a result of the procedure (Chapter 2). Additionally, calves disbudded with pain mitigation (lidocaine and meloxicam) exhibited reduced multi-scale correlation within their HRV data ($DFA\alpha_1$; Chapter 3). Variability with long-range nonlinear correlations is typically observed in the output of healthy physiological systems. However, these correlations begin to break down in response to stress and disease. Therefore, this result can be interpreted as a reduction in healthy physiological complexity as a result of the disbudding procedure. Surprisingly, this effect was observed in cattle that were given pain mitigation, whereas those disbudded without pain relief had an intermediate response that closely followed that of calves given pain mitigation. This suggests that the widely used method of pain mitigation used in the study did not provide sufficient relief of pain-related stress following disbudding.

In Chapter 4, growing pigs exposed to an acute heat episode exhibited greater regularity (SampEn) in their HRV data during the heat episode, whereas linear HRV measures that provide some evidence of greater sympathetic activity (LF/HF) remained unchanged until the recovery

period. Therefore, it was not clear whether LF/HF was altered in response to heat or another confounding factor. Accordingly, nonlinear HRV measures, such as SampEn, may be capable of providing more reliable and timely information regarding the physiological response to environmental stressors, such as heat.

Chapter 5 evaluated the use of baseline HRV as an indicator of the subsequent behavioral and physiological response to an LPS challenge in growing pigs. While there were no relationships found between baseline nonlinear HRV measures and the subsequent cortisol or pro-inflammatory cytokine response, pigs with longer periodicities in their HRV data (Lmean) exhibited a greater change in body temperature than pigs with shorter periodicities. Therefore, nonlinear HRV measures (such as Lmean) recorded prior to illness or disease may be sufficient for characterizing certain aspects of the future physiological sickness response. This finding could have positive implications for identification and management of at-risk animals before the onset of sickness or disease.

Taken together, the results reported in these chapters show that nonlinear HRV measures complement traditional linear HRV measures, and in some cases, are more sensitive indicators of the physiological response to a variety of stressors. Therefore, they should be included in studies where HRV is used to measure livestock stress. Future work should attempt to clarify the physiological significance of nonlinear HRV measures for aid in interpretation of results.