Measuring piglet castration pain using linear and non-linear measures of heart rate variability

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Abstract

The purpose of this study was to evaluate whether linear and non-linear measures of heart rate variability (HRV) could be used as indicators of piglet castration pain. Thirty piglets were allocated to 1 of 4 treatments: i) sham castrated HRV (SHRV; n = 8); ii) surgical castrated HRV (CHRV; n = 7); iii) sham castrated blood collection (SBC; n = 7); or iv) surgical castrated blood collection (CBC; n = 8). Piglets in the SHRV and CHRV treatment groups underwent a 1-h HRV and postural behaviour evaluation on day −1, day 0 (castration treatment), day 1 and day 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. Castrated piglets (CHRV) exhibited greater low to high frequency ratio, lower sample entropy and greater percent determinism compared to SHRV piglets, indicating greater pain-related stress due to the surgical castration procedure. Serum cortisol was greater in CBC pigs at 1 h post-castration compared to SBC piglets. No effect of treatment was found for amount of time spent lying post-castration. In conclusion, surgically castrated pigs exhibited greater pain-related stress than their sham castrated counterparts. Additionally, non-linear HRV measures seem to complement traditional linear HRV measures and may be valuable for assessing pain-related stress in future studies investigating swine welfare.

Keywords: *animal welfare, castration, heart rate variability, pain, piglet, stress*

Introduction

In the United States, male piglets not intended for the breeding herd are surgically castrated to reduce aggressive behaviour and improve meat quality (American Veterinary Medical Association [AVMA] 2013). Previous studies evaluating the behavioural and physiological response to castration have shown that surgically castrated piglets exhibit greater plasma cortisol concentrations (Prunier *et al* 2005; Sutherland *et al* 2012), acutely increased adrenaline and noradrenaline concentrations (Prunier *et al* 2002), may slow growth at three days of age (Kielly *et al* 1999; no effects of castration in Hay *et al* 2003 and Carroll *et al* 2006), altered behaviour (Weary *et al* 1998; Taylor *et al* 2001; Davis *et al* 2017) and, if castration is conducted at an older age, a moderated 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 minimise any lasting negative effects, it is evident that the procedure causes considerable pain and stress to the piglet.

In the past, non-invasive behavioural 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). Several interconnected factors, such as procedure duration and handling of untrained animals, may reduce the effectiveness of common physiological measures (eg cortisol or catecholamines) for evaluating stress (Marchant-Forde *et al* 2009). 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 two main components, the parasympathetic (PNS) and sympathetic (SNS) branches, which regulate essential involuntary physiological processes (eg 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).

Table 1   Definitions of heart rate variability parameters (adapted from Byrd et al 2019a).

Parameter	Practical definition
Linear measures	
Mean RR Interval (RR; ms)	Mean 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
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
Non-linear 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 and greater stress (Parois *et al* 2018; Byrd *et al* 2019b)
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 Values closer to zero indicate increased self-similarity of the HRV data at different time lengths and, therefore, lower stress (Goldberger *et al* 2002)
Recurrence (%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 and greater stress (Mohr *et al* 2002)
Determinism (%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 and greater stress (Mohr *et al* 2002)
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 and greater stress (Dua *et al* 2012)

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 it), the interaction between the ANS branches is not strictly linear (Uijtdehaage & Thayer 2000; Billman 2013). Physiological influences (eg respiration and blood pressure) exert a non-linear 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 & Thayer 2000). Therefore, the introduction of non-linear HRV measures for evaluating non-linear 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 non-linear HRV measures for measuring castration pain-related stress in piglets over a three-day experimental period. We hypothesised that castrated piglets would exhibit altered autonomic activity and increased stress compared to sham castrated piglets. Specifically, we predicted that castrated piglets would exhibit greater plasma cortisol, lower average R-R interval (RR; Table 1), lower standard deviation of the R-R intervals (SDNN; Table 1), lower root mean of successive squared differences (RMSSD; Table 1) and greater LF/HF ratios than sham castrated piglets. Additionally, we predicted that non-linear HRV analysis would result in castrated piglets having lower sample entropy (SampEn; Table 1), lower HR signal self-affinity (as measured by detrended fluctuation analysis; DFAα_1; Table 1), greater percent determinism (%DET; Table 1) and recurrence (%REC; Table 1), and greater average diagonal line length in a recurrence quantification plot (Lmean; Table 1) compared to sham castrated piglets.

Materials and methods

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

Study animals and housing

Thirty-two individually selected focal piglets, along with their littermates, from thirty-two litters were selected for enrolment in the study and housed with their sow in similar-sized farrowing stalls (2.29 × 0.61 m; length × width). All

piglets were kept under environmentally controlled conditions (24.6 [± 0.1]°C; 52.8 [± 0.1]% relative humidity) with supplemental heat provided by heat lamps in each farrowing stall. Artificial lighting was provided from approximately 0700 until 1500h 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.

Treatments and experimental design

All focal piglets were randomly allocated to 1 of 4 treatments: i) sham castrated HRV (SHRV; n = 8); ii) castrated HRV (CHRV; n = 8); iii) sham castrated blood collection (SBC; n = 8); or iv) 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 bodyweight in a separate, previously unutilised 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 two repetitions during July and December 2017 at Purdue University's Animal Sciences Research and Education Center. Twelve piglets (n = 3 SHRV; n = 3 CHRV; n = 3 SBC and n = 3 CBC) underwent treatment during the first repetition. Twenty piglets (n = 5 SHRV; n = 5 CHRV; n = 5 SBC, and n = 5 CBC) underwent treatment during the second repetition.

Acclimation to personnel and heart rate monitors

All piglets were tail-docked and had needle teeth clipped at two days of age. To reduce the likelihood that these procedures would influence subsequent experimental results, the experimental procedure was not initiated until seven days of age, where all piglets enrolled in the study began a two-day 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 m² wooden crate for approximately 5 min. A heart rate monitor (Polar H10, Polar Electro Oy, Kempele, Finland) was then fitted immediately behind the piglet's forelegs with flexible veterinary wrap (VetWrap, 3M, Maplewood, MN, USA). To reduce targeting of the HR monitor on experimental piglets by non-focal littermates, long black socks were altered to make body socks that fitted over the body of all piglets in litters containing an SHRV or CHRV piglet (Figure 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 following day. Piglets in the SBC and CBC groups were also handled for approximately 30 s each day and weighed, however no further acclimation was conducted.

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

Baseline HRV measurement

Following the acclimation procedure, at nine days of age, experimental piglets in the SHRV and CHRV treatment groups underwent a baseline HRV measurement (day –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 fitted with body socks. Piglets in the SHRV and CHRV treatment groups were also fitted 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.

Experimental procedure

All piglets underwent treatment the following day (day 0). First, all piglets in litters containing an SHRV or CHRV piglet were removed from their pens, fitted with body socks, and placed in a 0.61 m² 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 fitted 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 m² 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 two study personnel, whereby one individual 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, vertical scrotal incision, slightly left of middle. 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 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 days 1 and 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-shaped trough covered with absorbent paper. One individual held the piglet while the second 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, USA) using a 2.54-cm, 22-gauge Vacutainer hypodermic needle at each time-point. Piglets were returned to their home farrowing stall immediately following blood collection.

Behavioural analysis

Two cameras (KPC-N502NUB, KT&C USA, Fairfield, NJ, USA) 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, Tapei, Taiwan). Subsequent behavioural analysis of the stored video data was conducted by a single study personnel using behavioural coding software (The Observer, Noldus Information Technology, Wageningen, The Netherlands). Postural behaviour (lying, standing) of piglets in the SHRV and CHRV treatment groups was recorded continuously for 1 h during each HRV collection period so HRV data sets could be selected from periods of inactivity. These data were also quantified and used to evaluate whether postural differences (% time spent lying) were observed between SHRV and CHRV treatment groups. Sitting behaviours were not included in the analysis, given the scarcity of occurrence.

Heart rate variability analysis

Heart rate variability data collected by the HR monitor were transmitted telemetrically (maximum distance approximately 1 m) to a data recorder (Polar V800 Sports Watch, Polar Electro Oy, Kempele, Finland) and subsequently downloaded for screening. Erroneous beats, often occurring as a result of poor contact between skin and the HR monitor,

were edited 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 (day –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 an HRV spectrum (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996). High (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 standardised using an embedding dimension of two heart beats with a threshold of 0.15× SD. Short-term detrended fluctuation analysis (DFAα_1) was conducted using a window range of 4 to 16 heart beats.

The remaining non-linear 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 neighbour 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).

Blood analysis

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

Figure 2
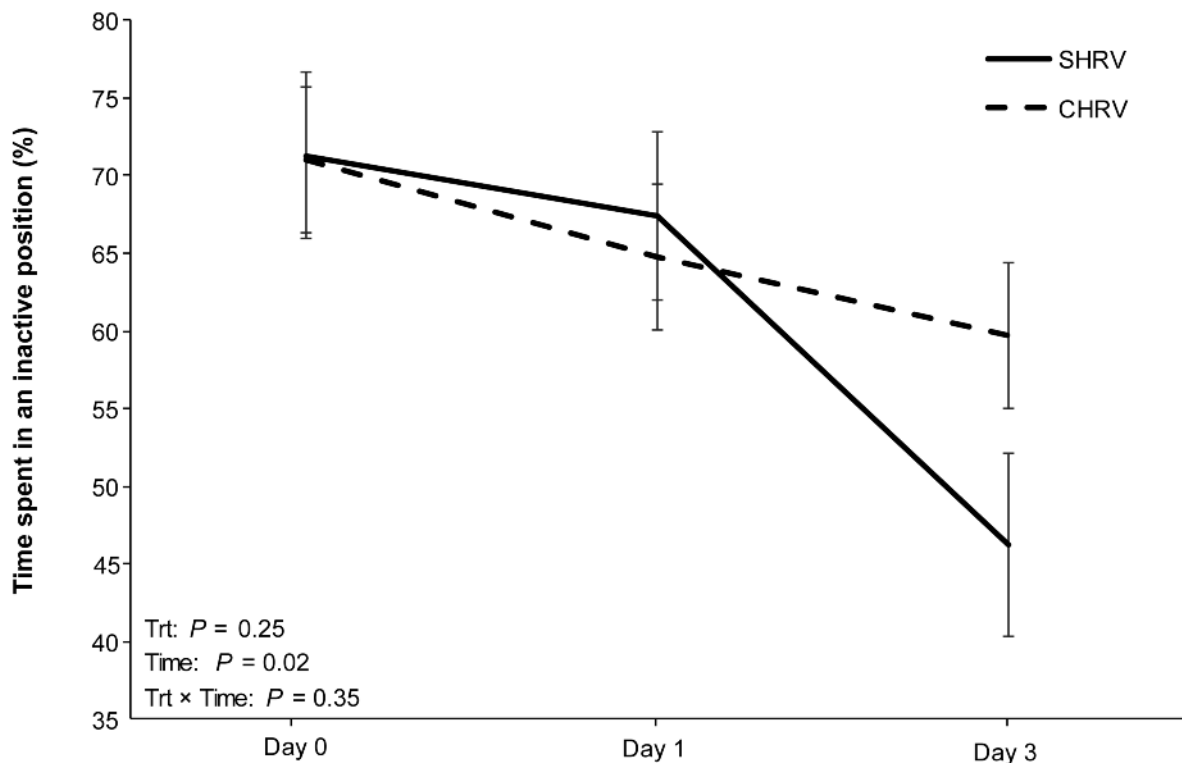
Least squares means (± SEM) of time spent lying for the three-day experimental period organised by treatment. Piglets in the CHRV treatment group were castrated on day 0. Piglets in the SHRV treatment group underwent simulated castration on day 0 but were not castrated until after the experiment ended.

Statistical analysis

Data were analysed in SAS 9.4 (SAS Institute Inc, Cary, NC, USA) using a 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: day 0, day 1 and day 3 relative to treatment; cortisol analysis: 1, 2, 3, 24, 48 and 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. An appropriate covariance structure for each model was selected using the lowest Bayesian information criterion (BIC) value. 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 (± SEM). Data that were transformed prior to analysis are presented as back-transformed least squares

means (± approximated SEM) determined using the delta method. A significant result was defined as $P \leq 0.05$. Data from one CHRV and one SBC piglet were removed from statistical analyses due to highly erroneous HRV data and a case of illness, respectively. Therefore, statistical analyses of HRV data were carried out with eight SHRV and seven CHRV piglets. Statistical analyses of serum cortisol concentrations were conducted with seven SBC and eight CBC piglets.

Results

Postural behaviour

Piglets spent more time lying on day 0 (71.3 [± 3.6]%; $t_{32.87} = 3.26$; $P = 0.01$) compared to day 3 (54.0 [± 3.9]%; Figure 2). This result was likely due to a large numerical decline in time spent lying exhibited by SHRV piglets on day 3 (Figure 2). There was no effect of treatment, the interaction between treatment and time, or weight on time spent in an inactive position ($P > 0.05$).

Cortisol

After adjustment for multiple comparisons, castrated piglets in the blood collection group exhibited greater serum cortisol concentrations than SBC piglets at 1 h (52.1 [± 8.9] vs 23.1 [± 6.5] ng ml^{-1}; $t_{52.92} = 2.73$; $P = 0.05$; Figure 3). No effects of weight or repetition on serum cortisol concentration were detected ($P > 0.05$).

Figure 3
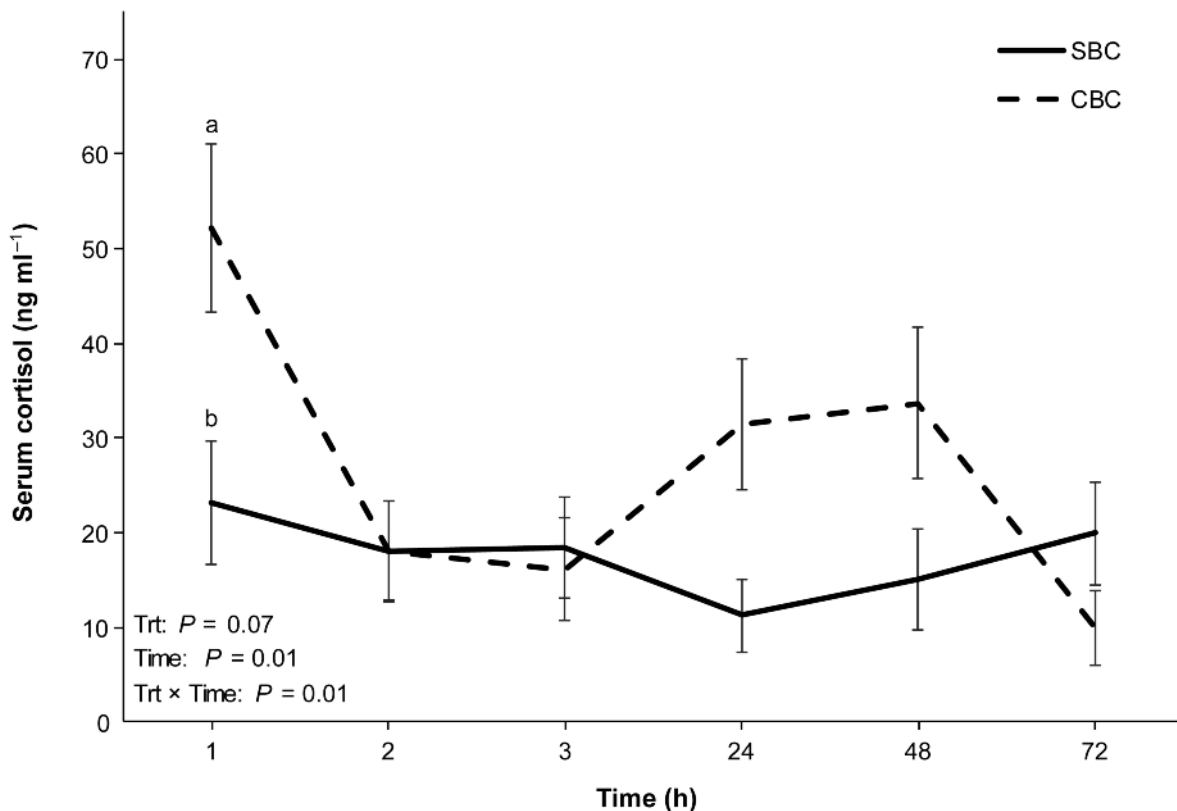
Back-transformed least squares means (± approximated SEM) of piglet serum cortisol concentrations following castration. Piglets in the CBC treatment group were castrated on day 0. Piglets in the SBC treatment group underwent simulated castration on day 0 but were not castrated until after the experiment ended. Different superscripts (a, b) indicate $P \leq 0.05$. No other differences between treatments were found.

Linear HRV measures

No effect of treatment, time, the interaction between treatment and time, weight, or repetition were detected for RR (Figure 4[a]; $P > 0.05$), SDNN (Figure 4[b]; $P > 0.05$), or RMSSD (Figure 4[c]; $P > 0.05$).

Castrated piglets exhibited greater LF/HF compared to SHRV piglets (26.9 [± 2.9] vs 9.0 [± 1.0] arbitrary units; $F_{1,27.06} = 9.30$; $P = 0.005$; Figure 4[d]). No additional effects of time, the interaction between treatment and time, weight, or repetition were detected for LF/HF ($P > 0.05$).

Non-linear HRV measures

Castrated HRV piglets exhibited lower SampEn compared to SHRV piglets (1.47 [± 0.12] vs 1.96 [± 0.12] bits; $F_{1,28.59} = 8.43$; $P = 0.007$; Figure 5[a]). 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$_1$ (Figure 5[b]; $P > 0.05$).

Castrated piglets exhibited greater %DET compared to SHRV piglets (55.0 [± 4.2] vs 40.0 [± 4.4]%; $F_{1,9.142} = 6.29$; $P = 0.03$; Figure 5[c]). 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 (Figure 5[d]) or Lmean (Figure 5[e]; $P > 0.05$).

Discussion

The purpose of the current study was to evaluate HRV as a potential non-invasive indicator of pain-related stress over a three-day period following surgical castration in piglets. Surgical castration is typically conducted without pain mitigation and results in altered piglet behaviour and physiology. For example, there is some evidence that castrated piglets lie 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 behaviour 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 behaviour is not a consistently reliable measure of pain and a more detailed behavioural observation is likely needed.

Figure 4
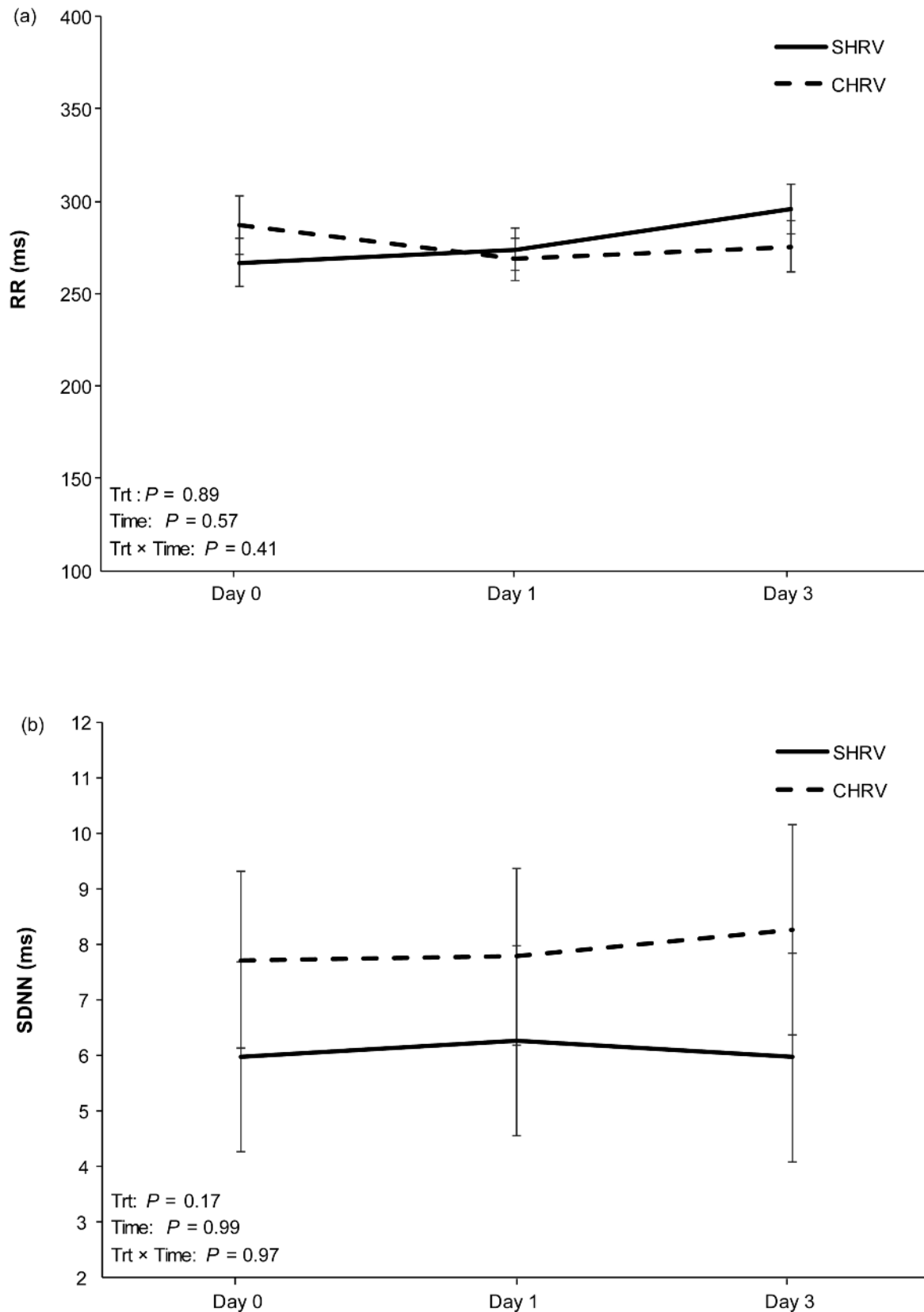
Temporal changes to linear HRV parameters over the three-day experimental period organised by treatment for (a) back-transformed least squares means (± approximated SEM) of average R-R interval (RR) and (b) back-transformed least squares means (± approximated SEM) of standard deviation of the R-R intervals (SDNN). Piglets in the CHRV treatment group were castrated on day 0. Piglets in the SHRV treatment group underwent simulated castration but were not castrated until after the experiment ended.

Figure 4 (cont)
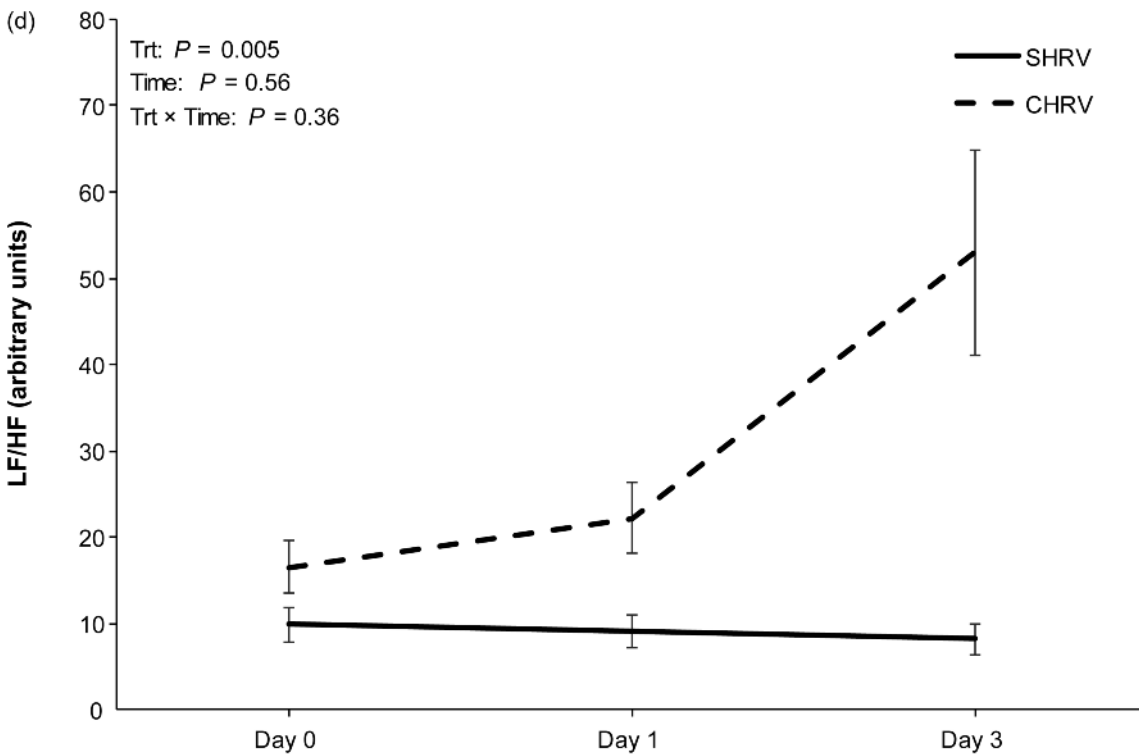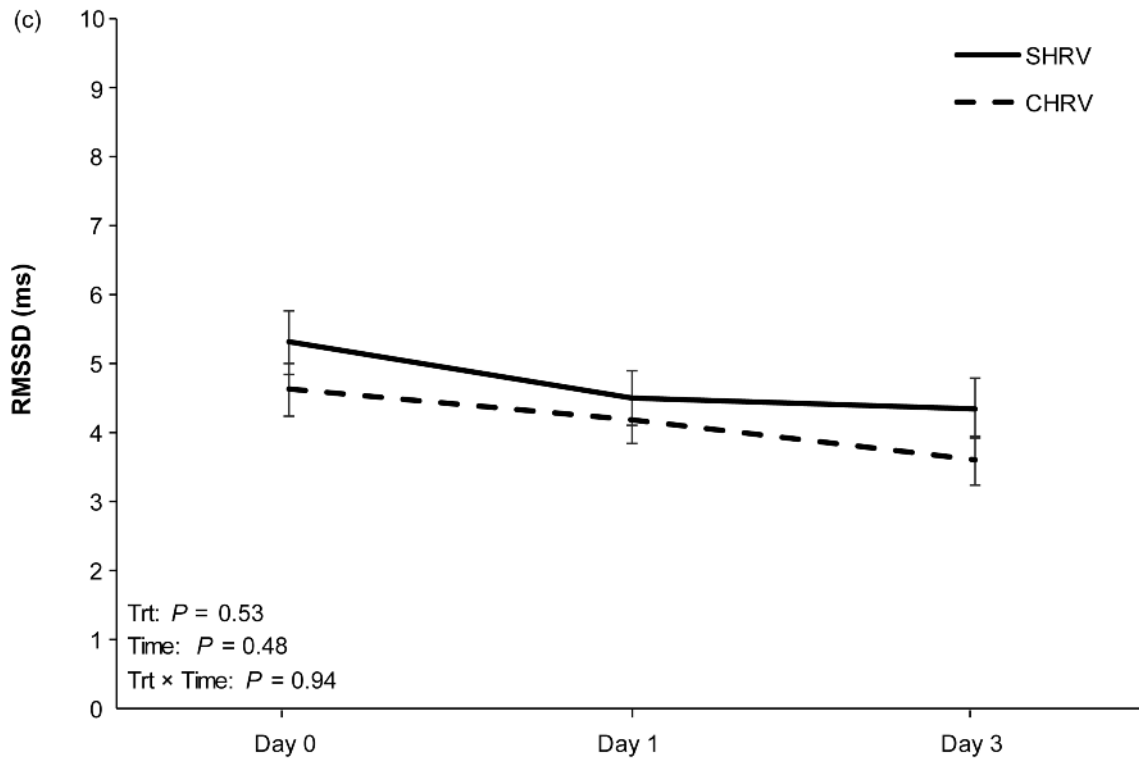
(c)

(d)

Temporal changes to linear HRV parameters over the three-day experimental period organised by treatment for (c) back-transformed least squares means (± approximated SEM) of root mean of successive squared differences (RMSSD) and (d) back-transformed least squares means (± approximated SEM) of low frequency to high frequency ratio (LF/HF). Piglets in the CHRV treatment group were castrated on day 0. Piglets in the SHRV treatment group underwent simulated castration but were not castrated until after the experiment ended.
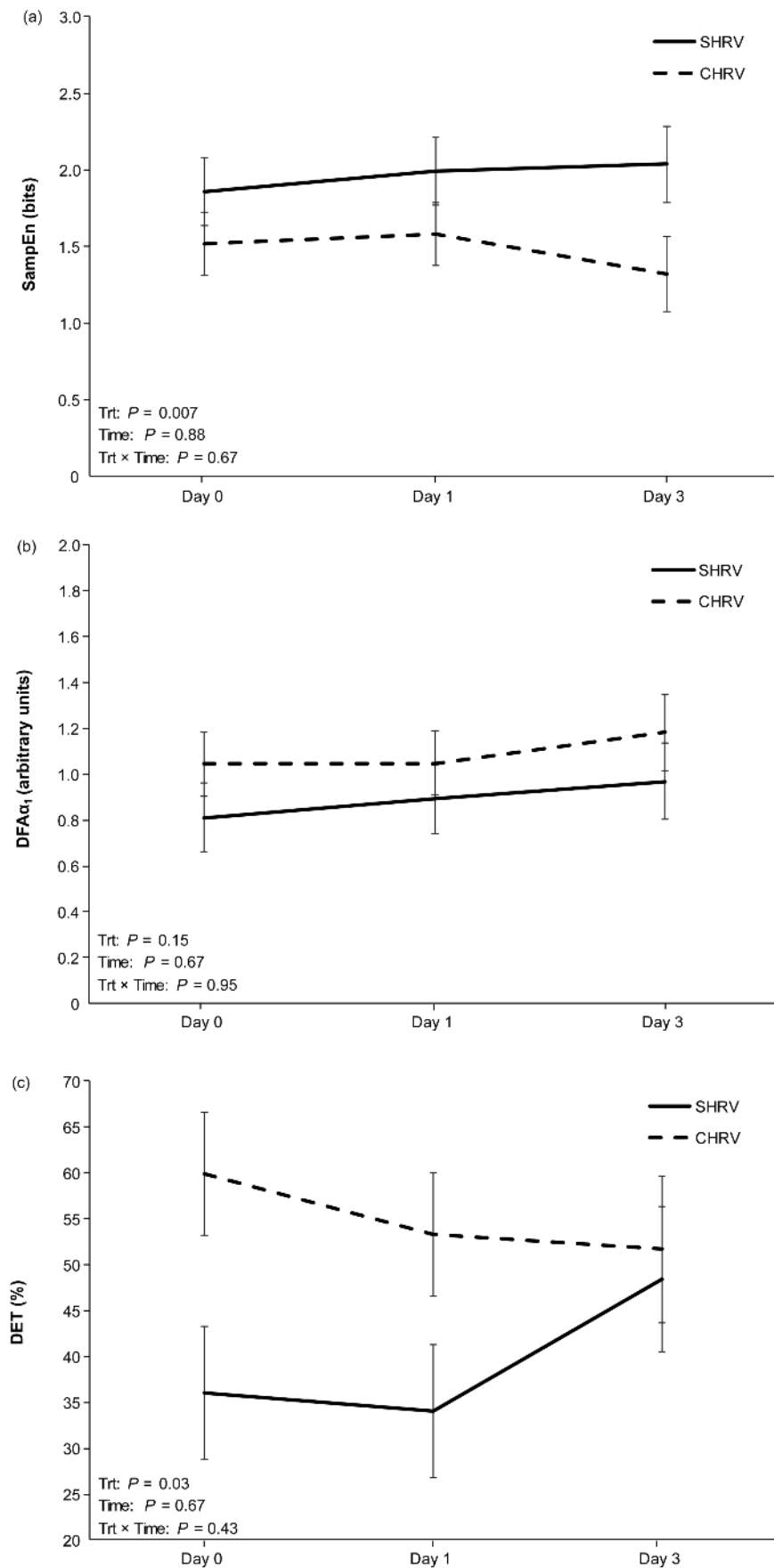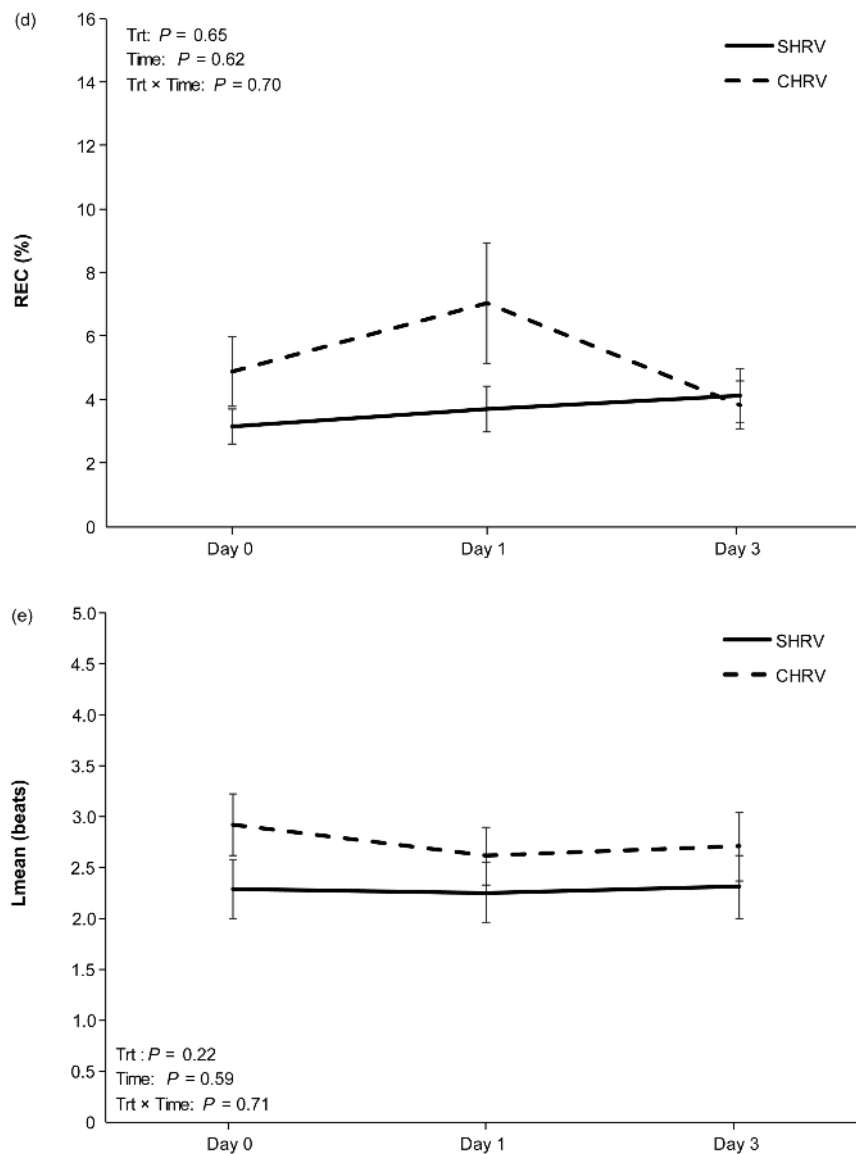
Figure 5

Figure 5 (cont)

Temporal changes to non-linear HRV parameters over the three-day experimental period organised by treatment for (a) least squares means (± SEM) of sample entropy (SampEn), (b) least squares means (± SEM) of detrended fluctuation analysis ($DFA_{\alpha 1}$), (c) back-transformed least squares means (± approximated SEM) of percent determinism (%DET), (d) back-transformed least squares means (± approximated SEM) of percent recurrence (%REC) and (e) back-transformed least squares means (± approximated SEM) of mean length of diagonal lines in a recurrence plot (Lmean). Piglets in the CHRV treatment group were castrated on day 0. Piglets in the SHRV treatment group underwent simulated castration but were not castrated until after the experiment ended.

In addition to the likelihood that posture is not a consistently reliable measure of pain, it is also possible that the performance of postural behaviours 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 behaviour for both treatments, making them indistinguishable from one another. This concern has been raised previously in animal studies that utilise automated data collection equipment attached to the experimental animal (Buijs *et al* 2018).

Castrated piglets exhibited greater cortisol concentrations 1 h post-castration compared to SBC piglets. 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). The results reported here are in agreement with previous studies, which have indicated that serum cortisol of surgically castrated piglets reaches maximal concentration approximately 0.5 to 1 h post-castration (Prunier *et al* 2005; 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, likely due to additional factors that may lead to cortisol secretion, such as general arousal or handling (Hay *et al* 2003; Prunier *et al* 2005; Moya *et al* 2008; Marchant Forde *et al* 2009; Rault *et al* 2011). Regardless, the results shown here demonstrate that castrated pigs experienced greater pain-related stress due to the castration procedure.

Linear measures of HRV were largely unchanged in CHRV 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, however, an effect of castration on LF/HF, which potentially indicates increased sympathetic activity in response to the procedure. The low 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 spectral power can largely be removed by the administration of anti-cholinergic drugs (eg 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 as representing 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 is possible that sympathetic activity was increased in CHRV 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 non-linear HRV measures that complement traditional linear HRV measures and may improve HRV methodology for distinguishing between treatment groups in response to stressors. Heart rate is affected by a number of physiological processes that interact and exert a non-linear 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 non-linear HRV measures in swine (outside of biomedical research; eg Batchinsky *et al* 2007) has been conducted. In previous research with dairy cattle, non-linear 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 piglets, 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 piglets on each day of the three-day post-castration period. This result is particularly evident on day three, where SampEn continued to decrease for CHRV piglets compared to SHRV piglets. Therefore, it is possible that the pain-related stress resulting from castration did not completely diminish prior to the end of the three-day experimental period. This is in agreement with previous studies evaluating piglet behaviour, where castrated piglets exhibited altered scratching and tail wagging behaviours for three and five days, post-castration, respectively (Hay *et al* 2003).

Percent determinism is obtained using RQA, which allows multi-dimensional data to be represented in a two-dimensional plot for quantification of recurring data points, patterns, and trajectories that, in many cases, go undetected by other non-linear measures (Eckmann *et al* 1987). Specifically, an 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 as a result of 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 stress following the castration procedure (Hagen *et al* 2005).

Animal welfare implications and conclusion

Castrated piglets exhibited greater cortisol than sham castrated piglets at 1 h post-castration. However, no other differences in serum cortisol were observed. Piglets exhibited lower overall SampEn and greater overall %DET than SHRV piglets, suggesting greater pain-related stress as a result of the procedure. Postural behaviour 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 non-linear HRV measures may be valuable for evaluating surgical castration pain-related stress in future studies. Additional work should evaluate new HR monitoring technologies and any potential effect they may have on behaviour and physiology when worn by the piglet.

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