5A-113: BRAIN MEASURES OF POSITIVE WELFARE IN PIGS

Final Report prepared for the Australasian Pork Research Institute Limited (APRIL)

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Executive Summary

"Quality of life" is a central concept in the welfare of livestock but remains difficult to assess because of the lack of markers of the experiences that an animal has during its life. The current indicators of animal welfare are poorly suited to assess the full spectrum of animal welfare states from negative to positive. This pilot project aimed to identify novel markers of negative and positive experience in pigs. One-hundred-and-forty-four 12-week-old pigs were used for the entire project, with sex-balanced pigs randomly assigned into three environmental treatments (Neutral, Positive, and Negative) for 4 weeks. From the 144 pigs, 36 pigs were randomly selected for biological sampling and 86 of the remaining 108 pigs were used for the analysis of meat quality at 22 weeks of age. The subset of 36 pigs (at 12 weeks of age) had baseline blood and saliva collected before commencing the environmental treatment. After 4 weeks of those treatments, half of the subset pigs received an aversive stimulus (snout snare restraint), and the other half did not (no snare), to stimulate an acute stress response. One hour after stimulation, a second saliva and blood sample were collected. The pigs were then humanely euthanised, and different regions of the brain were dissected and snap-frozen for later analysis. The prefrontal cortex was fixed before it was analysed using infrared spectroscopy. The plasma concentration of cortisol was measured in the plasma samples. A novel protocol to measure the expression of NEAT1, a non-coding RNA marker of cellular stress, was developed for the pig samples. The expression of NEAT1_total and NEAT1_long which are the two isoforms of NEAT1 gene, was measured in saliva and blood samples and hypothalamic tissues.

The pigs exposed to the negative environment had a lower average daily weight gain than did the pigs in the neutral and positive environment. The experiential response of the pigs to the different environmental challenges might not be strong enough to have induced differences in the concentration of cortisol in the plasma, or the expression of NEAT1_long in the blood, saliva, or the ventromedial region of the hypothalamus of pigs. The decrease in the expression of NEAT1 Total in saliva samples, that forms paraspeckles, after the acute stress suggests that it may be a useful indicator for acute mental stress in pigs. Mid Infrared spectroscopy (MIR), combined with machine learning, identified changes in the biochemical constitution of the prefrontal cortex that were associated with acute stress, but not with environmental conditions. This pilot investigation has demonstrated that NEAT1, a non-coding RNA, can be measured in blood, saliva, and brain tissue in pigs. NEAT1 in saliva could be investigated in more extreme conditions such as during lairage or transport. Non-Coding RNAs are promising practical markers of welfare.

This pilot study strongly suggests that MIR could be a technology to assess welfare post-mortem, but further work is required to validate this concept. This is very

promising and should be explored further to apply the technology to biological samples obtained from live animals over time as a potential method to assess the quality of life of food-producing animals.

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1. Introduction

“Quality of life” is a central concept in the welfare of livestock. Progress in animal welfare science has laid the foundation for several frameworks that can be used to assess the welfare of animals that are under human care (Duncan, 2019). The nature of the experience that an animal has is central in all of these frameworks with diverse levels of importance (Appleby et al., 2018). The quality of life is reflected by the level of adequacy of the biological functions and by the affective states in animals (Mellor and Beausoleil, 2015). Several behavioural and physiological indicators have been developed that are informative in the assessment of animal welfare (Appleby et al., 2018). While they are useful to assess the response of an animal to an experience, these indicators provide little information on the experiential process itself. At present, animal-based indicators can be divided into behavioural, physiological, and neurobiological indicators. They all provide valuable information on the mental state of an animal. However, the interpretation of any change in a physiological marker can be challenging because most physiological indicators respond to both positive and negative stimuli (Manteuffel et al., 2021). Importantly, physiological indicators can reflect negative or neutral welfare states, but rarely a positive welfare state, which is a major limitation when trying to understand the full spectrum of experiential states of an animal.

This project aimed at investigating novel biomarkers and methodologies to identify means to assess the positive experiential state in pigs. We have used pigs kept in a commercial facility. The quality of life was manipulated on a long term scale by exposing the pigs to environmental conditions that are known to induce either positive, neutral, or negative long-term experiences (Hemsworth et al., 1986; Terry et al., 2021), and on a short-term scale by exposure to an acute stressor (application of a snout snare or not). For this pilot study, production parameters, including meat quality, were measured and we focused on three biomarkers, cortisol as a classical marker of the stress response, a non-coding molecular marker with the potential to be integrative and specific to positive experience, and near-infrared spectroscopy which could offer an integrative approach to the assessment of quality of life. We used different biological media including saliva, blood, and brain tissue to explore the validity of these new approaches. The following sections introduce the potential of the non-coding molecular markers and near-infrared spectroscopy as tools to assess quality of life in pigs.

Nuclear paraspeckles are small cellular bodies that are found within the interchromatin space of the cell nucleus (Bond and Fox, 2009). The structural core of a nuclear paraspeckle is nuclear paraspeckle assembly transcript 1

(called NEAT1), a long noncoding RNA that binds together the other proteins that make up a paraspeckle (Fox et al., 2018). As well as acting as a scaffold for the paraspeckle, NEAT1 can alter the expression of many genes by impacting on the translation, transcription, and maturation of the microRNAs of those genes (Bond and Fox, 2009; Fox et al., 2018). The NEAT1 plays a role in cellular defense mechanisms by contributing to the maintenance of mitochondrion homeostasis (Wang et al., 2018). Observations in NEAT1 knockout mice suggest that changes in NEAT1 could reflect the capacity of an animal to cope with a psychological challenge and thus it may hold potential in the assessment of the associated mental state. Interestingly, the level of NEAT1 increases in the peripheral blood of patients with Parkinson's disease compared to non-affected patients (Boros et al., 2020) and could reflect the overexpression of NEAT1 in the subtantia nigra of patients with Parkinson's disease (Simchovitz et al., 2019). It seems possible then that levels of NEAT1 in blood could serve as a biomarker of the experiential state in pigs.

Measuring the quality of life of animals that are raised in either extensive or intensive systems is difficult, time-consuming, and expensive. Assessment of the quality of life relies on the frequent recording of many observations as well as the measurement of behavioural and physiological indicators, on every single animal (Manteuffel et al., 2021). In recent years, such assessment of complex systems has become more manageable with the advancement of digital technologies and mathematics, such as different types of sensors (e.g. biosensors, optical devices, radio frequency), machine learning (ML) and artificial intelligence (AI) algorithms in systems that are designed to collect and interpret extensive data as well as develop models that can be used in both research and in field applications (Manteuffel et al., 2021). But in general, the concept of a "life worth living" remains a theoretical construct.

Even in humans, the peripheral indicators of experience are not well identified. A way around this problem would be a technique that could measure biochemical changes in brain regions that are involved in the mechanisms of perception and / or expression of an experiential state, such as emotion. Among the several brain structures that are part of the "emotional brain", in humans, the biochemistry and organisation of the prefrontal cortex is impacted by the perception of life events, the processing of information, the formation of emotions, and decision-making (Dixon et al., 2017; Chafee and Heilbronner, 2022). For example, the prefrontal cortex undergoes biochemical and structural changes when an animal (human and non-human) is exposed to stressors (McEwen and Morrison, 2013). Another example is that two cell populations in the prefrontal cortex that respond to experiences of both positive and negative-valence differ in their

connectivity, profile of gene expression, and their impact on behaviour, at least in mice (Ye et al., 2016). An integrative measure of such changes could be a reliable indicator of the experiential status of an animal. Interrogating the central nervous system of an animal during its life is not an easy task. Methodologies that are commonly used in humans to assess these changes, such as surface electrodes, functional magnetic resonance imaging (fMRI), and more recently functional near-infrared (fNIR) spectroscopy, are considered state-of-the art. However, these methods are impractical for use in the field or in large animals such as those utilized by the meat industries (Blache and Maloney, 2017). These techniques have led to valuable insights in human neuroscience, revealing that exposure to either chronic or acute stressful events is associated with changes in the neurochemical balance, the anatomical structure, and the metabolic activity of the prefrontal cortex (McEwen, 2016). While some of these changes in the brain tissue, like neurotransmission, are short-lived, others, such as structural changes, are long-lasting (McEwen, 2016).

Infra-red spectroscopy is sometimes called vibrational spectroscopy because it is a way to measure the vibrations of atoms and their bonds. Stronger bonds vibrate at a higher frequency than weaker bonds, permitting the determination of the functional groups that are present in a sample. Techniques such as near-infrared (NIR) and mid-infrared (MIR) spectroscopy use light to measure molecular vibrations that are associated with changes in chemical bonds between atoms as well as variations in the structure of a sample (Workman and Weyer, 2012; Talari et al., 2017). Absorption bands in the NIR region (13,000 – 4,000 cm^{-1}) can identify chemical components that are present in a sample (Workman and Weyer, 2012). In addition, a narrow band within the MIR region (1,800 – 900 cm^{-1}) is known as the fingerprint region because it provides a signal that is related to the unique fundamental vibrations of many molecules of biological and biochemical interest, including amide groups derived from protein, lipids and fatty acids, ester groups, and carbohydrates (Talari et al., 2017). Therefore, spectroscopy in both the NIR and MIR regions could reflect structural changes that are induced by the experience that an animal has, either in the short- or long-term.

In this project, we hypothesised that:

1. Negative environmental conditions will negatively affect parameters of production and meat quality and these effects might be amplified by exposure to the acute stressor.

2. There will be an interaction between environmental conditions and the acute stressor on the concentration of plasma cortisol. Negative

environmental conditions will increase cortisol in plasma and these effects will be amplified by exposure to the acute stressor.

3. There will be an interaction between environmental conditions and the acute stressor on the expression of NEAT1 and its concentration in plasma, saliva, and brain tissue. The direction of changes cannot be hypothesised because NEAT1 has never been measured under psychological stress conditions.

4. The near infrared spectrum from the prefrontal cortex region will be different between pigs exposed to different environmental conditions or exposed, or not, to an acute stressor.

This project will contribute to the assessment and improvement of pig welfare by providing quantitative biological measures (biomarkers) of brain function during positive and negative experiences. The project will enable the development of non-invasive biomarkers, which the Australian pork industry can use to inform day-to-day management decisions and continuously improve the welfare of pigs.

2. Methodology

2.1 Animals and experimental design

The animal experiment was conducted at the CHM Westbrook Research facility with the experimental protocol approved by CHM Alliance Animal Ethics Committee and ratified through The University of Queensland Animal Ethics Committee (CHM PP 151/22).

A total of 144 (72 immunocastrated males, referred to as male/s hereafter, and 72 females) 12-week-old grower pigs were randomly allocated to sex-balanced pens of 24 pigs each that were assigned one of three environmental treatments for a 4-week period.

The three environmental treatments were:

- Neutral – pigs were housed under normal husbandry conditions, which involved twice daily health checks and twice weekly hosing of pens.
- Positive – pigs were housed under normal husbandry conditions with the addition of positive human contact for five minutes per workday following a similar protocol to Hemsworth et al. (1986). The pigs were also given hay twice daily in a hayrack and enrichment toys were provided and changed weekly.

- Negative – pigs were housed under normal husbandry conditions but with weekly changing and remixing of pen mates, which is a known chronic stressor, as described by Hemsworth (2018).

After the 4 weeks of the environmental treatment, a subset of 12 pigs (6 male and 6 female) per treatment was individually removed from the home pen and moved to an isolated area in which half of the pigs received an acute standardised aversive stimulus consisting in the application of a snout snare for 5 minutes, which is a known acute stressor (Terry et al., 2021). The other half of pigs were not subjected to the acute stressor and were removed from their home pen and placed in isolation. This resulted in a 3 x 2 factorial design shown in Figure 1. An hour after the acute stressor was imposed, the 36 subset pigs were humanely killed for tissue collection (details provided in 2.3 sample collection). The treatments were ceased once the 36 animals were removed, however, the remaining 108 pigs stayed in the pens until they reached slaughter weight at approximately 22 weeks of age, but only 86 were used for the analysis of meat.

The facility consisted of 24 pens of identical configuration (2.6 m x 3.3 m) within which 6 pens were utilised for this experiment. Penning was open galvanised steel panelling with partially slatted concrete floors. Water was supplied *ad libitum* via three nipple drinkers per pen. Feed was offered to each pen via a multi-space adjustable plastic feeder. Male pigs were administered the first dose of Improvac® (2 ml, Zoetis Australia Pty Ltd, Rhodes, NSW) at approximately 11 weeks of age and the second dose at approximately 17 weeks of age.

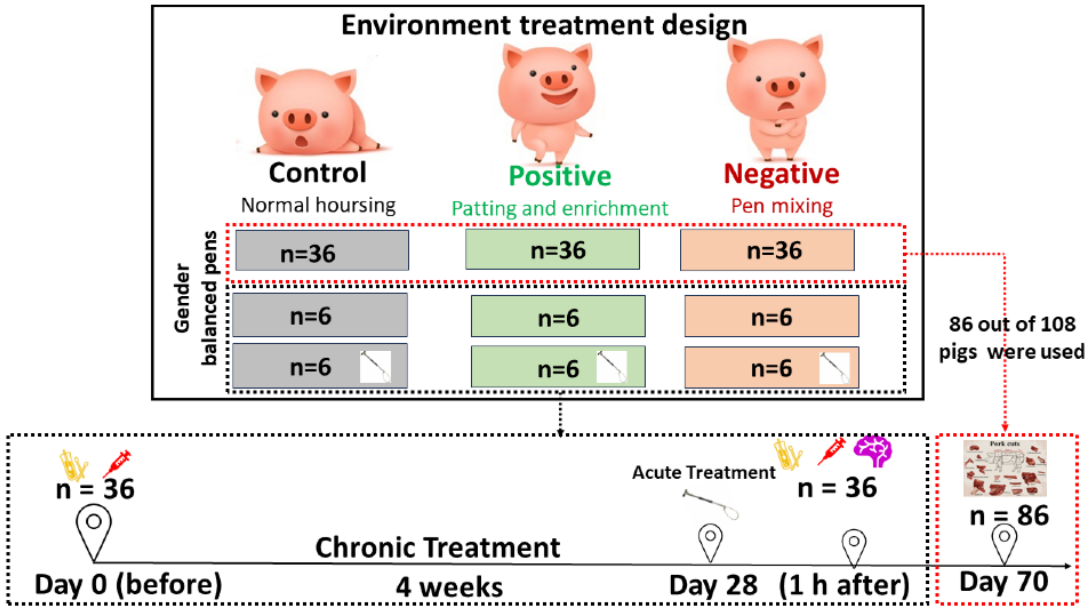

Figure 1. An overview of the experiment, animals and design. One-hundred-and-forty-four pigs were used for the entire project, with sex balanced pigs randomly assigned into three environmental treatments (Neutral, Positive, and Negative) for four weeks. Thirty-six pigs were randomly selected for biological sampling and of the remaining 108 pigs, 86 were used for the analysis of meat quality at 22 weeks of age. The subset of 36 pigs (age: 12 weeks) had baseline blood and saliva collected prior to commencing treatment. After 4 weeks of environmental treatment, half of the subset pigs received an aversive stimulus (snout snare restraint), and the other half did not (no snare) to stimulate an acute stress response. One hour after stimulation, a second saliva and blood sample were collected. Pigs were then humanely killed, and different regions of the brain were dissected and snap frozen for later analysis. The prefrontal cortex was fixed before analysis using infrared spectroscopy.

2.2 Measurement of average daily gain

The weight of each individual pig was measured at the beginning and endpoint of treatment application. The average daily gain (ADG) was calculated as the change in weight (final weight minus starting weight) divided by the days between weight recordings.

2.3 Measurements of slaughter performance and pork quality

Pork quality measurements were conducted on the 86 pigs following slaughter at 22 weeks of age. The 86 pigs were individually weighed the day prior to slaughter. The remaining 22 pigs were slaughtered the following week, but no measurements of meat quality were taken.

2.3.1 Slaughter performance

At the abattoir, carcases were dressed and graded individually with hot standard carcass weight (HSCW; AUS-MEAT Trim 1, AUS-MEAT Ltd, Murrarie, Qld) and subcutaneous fat depth at the P2 site (located 65 mm from the midline of the carcase at the head of the last thoracic rib, Autofom III, Frontmatec, Denmark) recorded pre-evisceration (post the carcase steam scalding). The HSCW was divided by the liveweight at 22 weeks to calculate dressing percentage. Muscle pH and temperature decline post-slaughter were measured in the *Longissimus thoracis et lumborum* (LTL) between the 12-13th rib, adjacent to the P2 site at 45 (before chilling) and 180 mins (post exit from the blast chill) using a portable pH / mV / temperature meter (TPS WP-80M fitted with IJ44 glass spear sensor and stab type temperature / ATC sensor, TPS Pty Ltd, Brendale, Qld).

Priorto boning of the carcases at approximately 24 h post-slaughter, ultimate *Longissimus thoracis et lumborum* pH (pHu) and temperature were recorded along with cold carcase weight, which along with HSCW was used to calculate chill loss. A 10 cm section of the loin primal from each left carcase side was removed (chine bone removed, plate boned ribs removed) with rind attached. Samples were collected within approximately 30 mins from first to last loin primal, weighed and sealed in individual plastic vacuum sealed bags before being frozen at -20 °C for further analysis of drip loss, colour, cook loss, and shear force.

2.3.2 Pork quality

Samples were thawed for 24 h at 4°C, prior to the objective pork quality assessment. Purge loss was assessed using the method described by Kauffman et al. (1986), in which a 2.5 cm slice of the thawed sample was weighed and then a pre-weighed filter paper was placed on the cut surface such that all portions were touching the surface and removed immediately and weighed to determine 24 h fluid weight of the filter paper. From there, % 48 h purge loss was calculated as follows:

$$\%\ 48h\ purge\ loss = -0.1 + 0.06 \times mg\ fluid + 0.9\%$$

Objective colour measurement was recorded after a 30 min blooming time using a Minolta Colour Chromameter (CR-400, Konica-Minolta, Tokyo, Japan) with 8 mm aperture that was calibrated against a white tile in accordance with the manufacturer's recommendations (D65 illuminant, 10° observer degree). The colour was recorded in triplicate on each sample. Colour

readings were averaged for each measurement of L* (lightness), a* (redness) and b* (yellowness) reading.

Cook loss and Warner-Bratzler shear force (WBSF) were measured, similarly to Thompson et al. (2005), in which samples were trimmed to approximately 70 g (5 cm × 5 cm × 2.5 cm) and cooked in a water bath at 70°C for 35 min. Once cooled in an ice bath for 30 min, they were patted dry, weighed to determine cook loss as a percentage of pre-cooking weight, and then refrigerated at 4°C for 24 h. Using a coring action, samples were cored with 1.27 cm diameter, with six cores per sample and then run through a Warner-Bratzler Meat Shear (GR-151, Tallgrass Solutions Inc., Manhattan KY, USA) to measure peak force.

2.4 Sample collection

Saliva and blood samples were collected prior to when environmental treatments commenced from each of the 36 pigs (at 12 week of age) that were used for the biological sampling study. At the end of the environmental treatment, at 16 weeks of age, half of the 36 pigs received an acute stressor in the form of a snout snare restraint and the other half were removed from their home pen but did not have the snout snare applied. One hour after the acute stress treatment was applied, saliva and blood were collected again.

Saliva samples were collected using a saliva swab [Salimetrics SalivaBio's Children's Swab (Startech, Sydney)] which was provided to the individual pig to chew. Samples were immediately snap frozen in liquid nitrogen. Blood samples were collected via jugular venipuncture into an EDTA vacutainer. A total of 2 mL of whole blood was aliquoted into cryovials and was snap frozen in liquid nitrogen immediately for the measurement of gene expression. The blood sample left in the EDTA tube was refrigerated immediately and centrifuged to separate and collect the plasma. At least two aliquots of 1.5 mL of plasma were stored at -20°C until being processed.

The pigs were sedated using a mixture of ketamine (4.4 mg/kg, Troy Animal Healthcare, NSW) and xylazine (2.2 mg/kg, Ceva Animal Health, NSW) then humanly killed by intravenous injection of an overdose of pentobarbitone (*Lethabarb*®, Virbac, Australia). Within 15 minutes of when the pigs were confirmed dead, brain samples were collected. The grey matter of the ventromedial prefrontal cortex, grey matter of the dorsolateral cortex, thalamus, lateral hypothalamus, ventromedial region of the hypothalamus, dorsomedial region of the hypothalamus, basal ganglia, dorsal hippocampus ventral hippocampus, amygdala, midbrain, and brain stem were dissected and snap frozen with liquid nitrogen for further analysis including the

analyses of expression of genes encoding for NEAT1 in the ventromedial region of the hypothalamus.

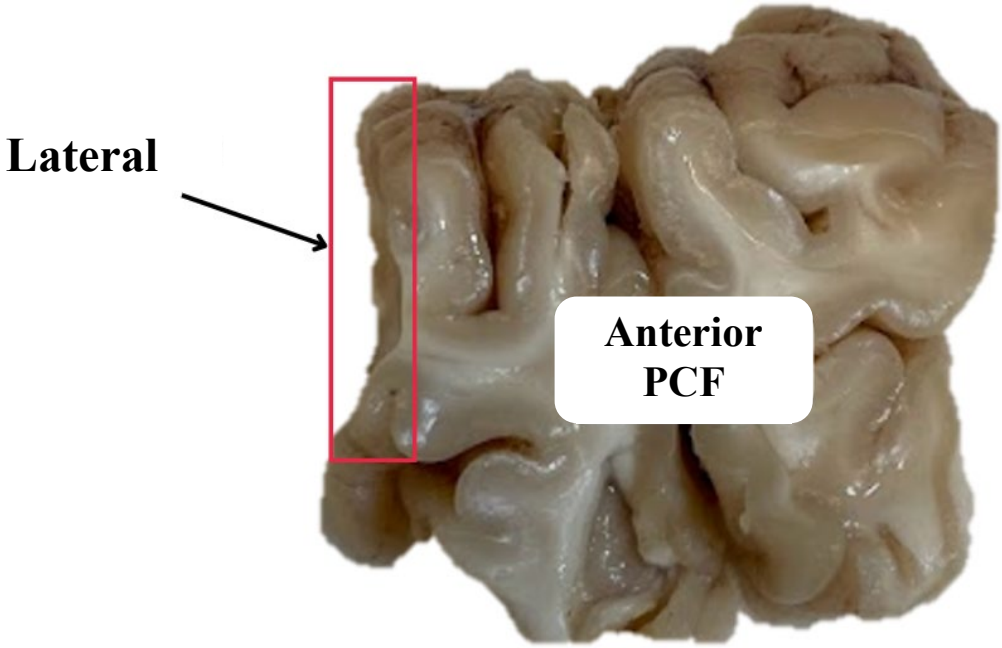

Figure 2. Diagram showing the two brain sections that were scanned to collect the near infrared spectra, namely the anterior and lateral prefrontal cortex (PCF).

A coronal slice of about 15 mm of the frontal lobes was cut and immersed in 10% paraformaldehyde in phosphate buffered saline (PBS) within 15 min after humane killing (Figure 2). The segment containing the prefrontal cortex was kept in the fixative at 4°C for 1 week and was then transferred to PBS until analysis using infrared spectroscopy. Any large blood vessels or pieces of dura mater or pia mater were removed from the brain before scanning. The samples were postfixed in 10% paraformaldehyde for 48 h at 4°C and then stored in PBS at 4°C before analysis using infrared spectroscopy.

2.5 Infrared spectroscopy

The NIRS and MIRS scans were performed approximately one month after the brains had been collected.

Near infrared and mid infrared analysis

The NIR spectra of the brain samples was collected from two anatomical regions (Figure 2). The first scan was taken on the anterior prefrontal cortex (APCF) while the second scan was taken at the side of the block in the lateral prefrontal cortex (LPCF).

The NIR spectra of the two brain sections was obtained using a Fourier transform (FT) NIR Bruker Tango-R spectrophotometer (Bruker Optics GmbH, Ettlingen, Germany). An integrating sphere with a gold-coating (diffuse reflection) was used to collect the FT-NIR spectra. The spectral data were collected in reflectance mode and recorded using the OPUS software (version 8.5, Bruker Optics GmbH, Ettlingen, Germany) with 64 interferograms at a resolution of 4 cm^{-1} in the wavenumber range between 11,550 and 3,950 cm^{-1}. The samples were scanned using a borosilicate cuvette that was cleaned between samples with 70% (v/v) water-ethanol and dried with laboratory Kimwipes® (Kimberly Clark, Acacia Ridge, QLD, Australia) between samples.

The MIR spectra of the brain samples were obtained using slice samples that were cut into 1 mm thick sections starting from the lateral part of the PCF (LPCF). Sections that contained the white and grey matter were separated using a sterilized razor blade (Wilkinson Sword) and scanned separately. The MIR spectra were recorded using a Bruker Alpha instrument fitted with an attenuated total reflectance (ATR) platinum diamond, single reflection, module (Bruker Optics GmbH, Ettlingen, Germany). The spectra of the brain samples were recorded in the spectral region between 4,000 and 400 cm^{-1} (average of 24 interferograms, resolution 4 cm^{-1}) (OPUS software, version 8.5, Bruker Optics GmbH, Ettlingen, Germany). The region between 2,400 to 1,900 cm^{-1} was removed from the analysis due to the interference of water vapour and CO_2. Air was used as the reference background spectra and collected every 10 samples. The ATR cell was cleaned with a mixture of 70% ethanol in water (v/v) and dried with laboratory Kimwipes® before the measurement of each sample.

2.6 Measurement of cortisol concentration in the plasma samples

The plasma concentration of cortisol was measured in duplicate using the manufacturer's protocol and reagents from the MP Biomedical I125 RIA cortisol Kit (#07-221106, Australian Bioresearch, Perth). The kit comprised Anti-Cortisol coated tubes, tracer (radioactive cortisol Iodine 125), and cortisol standards. The limit of detection was 0.25 $ng.ml^{-1}$ and the intraassay variability was 6.5% (1.4 $ng.ml^{-1}$) and 1.8% (3.2 $ng.ml^{-1}$).

2.7 Measurement of the expression of NEAT1

2.7.1 RNA extraction from the blood, the saliva and the VMH samples

Total RNA from the blood, the saliva, and the VMH samples was extracted using Mini NucleoSpin® RNA Blood kit (740200.50), Monarch Total RNA Miniprep Kit (NEB #T2010), and QIAzol lysis reagent (79306), respectively. All

procedures were performed in an RNase-free environment, with protocols as per the manufacturer's recommendation.

2.7.2 Reverse transcription

The concentration and quality of total RNA that was extracted from the VMH was measured with a Nanodrop spectrophotometer (ThermoFisher Scientific). The concentration and quality of total RNA that was extracted from the saliva and the blood samples was measured with a Qubit 4 Fluorometer (ThermoFisher Scientific). A total of 2,000, 400, and 200 µg total RNA from the VMH, the blood, and the saliva samples from each experimental pig was quantified, respectively, for the reverse transcription using QuantiTect reverse transcription (RT) protocol.

2.7.3 RT-PCR analysis

The RT-PCR analysis was performed using the AccessQuick™ Master Mix (2X) (Cat. No. A170C, Promega, Madison, USA) with Rotor-Gene Q (Qiagen, Chadstone, Australia). For this experiment, 1 µL of diluted cDNA template together with 0.8 µL of 10 µM forward and reverse primers (Table 1), 5 µL 2 × Master Mix, and 2.4 µL of RNase-free water was used for each reaction. Each reaction was run in triplicate using the following protocol: 2 min at 95°C; 10 seconds at 95°C, 30 seconds at annealing temperature (Table 1), and 5 min at 72°C for 40 cycles. An equal amount of RNAase-free water was used as a negative control. There are two isoforms of the NEAT1 gene, which are NEAT1 Total and NEAT1 Long. The expression of NEAT1 gene was measured by measuring the NEAT1 Total and NEAT1 Long using a standard curve method. The results were calibrated with the expression of the RPL4, GAPDH, and PPIA genes that have ranked to be the proper reference genes.

Table 1. Primers for reference and target genes.

Gene Name	Primer sequence (5'→3')	Amplicon length, bp	Annealing temperature, °C
RPL4	F: AGGAGGCTGTTCTGCTTCTG R: TCCAGGGATGTTTCTGAAGG	185	55.6°C
GAPDH	F: TCGGAGTGAACGGATTTGG R: GTGGAGGTCAATGAAGGGGTC	107	57.6°C
PPIA	F: CACAAACGGTTCCCAGTTTT R: TGTCCACAGTCAGCAATGGT	171	55.6°C
NEAT1 Total	F: CTGTTCGGGGGTGGAGGTTT R: ACCAGGCATCACTCTGCGTT	145	60°C
NEAT1 Long	F: CGTTGCGCTTACACTGTGGG R: ACATGCTCAGTGACCAGGCA	103	60°C

2.8 Statistical analysis

IBM SPSS Version 29.0 (IBM, US) was used to analyse the effect of treatments. GraphPad Prism 8.4 was used for all correlation analyses and generation of figures.

Normality checks and statistical models
Normality was assessed using a Shapiro-Wilk Test and Levene's Test. Data showing non-normality were transformed where possible. Outliers were removed when greater than 2.69 x σ (Qn ± 1.5 × IQR). Variables with two time points were analysed with Repeated Measures General Linear Mixed Model, and one time point was analysed with Mixed Design ANOVA. Individual pigs were set as random factors in the repeated measures analysis. Sex was set as a covariate but when main effects or interactions were found for sex, sex was made a fixed factor. Pen was included as a random factor, with fixed factors of "environment" and "acute stress [present / absent]" statistically nested in the model within pen. Bonferroni pairwise analysis was used to discriminate differences between groups when main effects or interaction was found. When normality through transformation was not achieved, the data were analysed using the non-parametric Kruskal-Wallis H Test. A P-value that was lower than 0.05 was taken as a significant difference, and a P-value greater than 0.05 but lower than 0.10 was taken as a trend of difference.

Due to non-normality of NEAT1 Total in the first sample, NEAT1 Total was analysed using change in NEAT1 Total from first to second sampling. Percentage of NEAT1 Long was calculated by dividing NEAT1 Long expression by NEAT1 Total.

All results are expressed as mean ± standard deviation unless otherwise specified.

Infrared spectroscopy analysis
Interpretation of the spectra and the development of a principal component analysis (PCA) was performed using Solo v9.2 (Eigenvector Research, Inc., WA, USA). Linear discriminant analysis (LDA) classification models were performed using Unscramble X (version 11, Oslo, Norway) and figures were plotted in R using the *ggplot* package.

Pre-processing of the NIR and MIR spectra was applied to remove, or at least minimize, background noise using the second-order derivative Savitzky-Golay filtering with second degree of polynomial and 11 smoothing window points (Rinnan et al., 2009). The MIR spectra were removed (in the region between 2,400 to 1,900 cm^{-1}) and pre-processed using detrend, standard normal

variate (SNV) and second-order derivative Savitzky-Golay filtering with second degree of polynomial and 11 smoothing window points (Rinnan et al., 2009).

The PCA is one of the most widely used techniques for data reduction during the exploratory analysis of spectral data. The PCA can reduce a large spectral dataset into a few dimensions (principal components) by identifying orthogonal components that capture most of the variance between samples in the data, allowing for easier visualisation, interpretation, and understanding of the underlying patterns and relationships between samples (Bro and Smilde, 2014). The PCA was used on all pre-processed spectra (NIR and MIR) to compare the two scanning locations. The effects of sex, environmental condition, and exposure (or not) to an acute aversive stimulus were assessed by identifying distinct sample groupings. The samples size and the statistical approach to analyse the IR spectrum did not allow for 2 factors analysis.

Samples were classified using a supervised classification method (LDA) to distinguish between pre-defined groups or classes (Ruiz-Perez et al., 2020). In this study, LDA was used to classify the brain samples into different categories or groups such as the type of tissue (e.g. grey and white matter), the sex of the animal, the three environmental conditions that the pigs were exposed to, and the presence or absence of exposure to an acute aversive stimulus before death. The models attempted to build a linear relationship between the dependent variable (categories or group) and the independent variable (NIR or MIR spectra) (Brereton and Lloyd, 2014). To construct a classification model, the NIR and MIR spectra of the brain samples were assigned as male or female, environmental exposure as positive, negative, or control, and acute aversive stimulus as yes or no. The NIR classification models were based on the spectra from the prefrontal cortex and the lateral region of the prefrontal cortex, while the MIR classification models were based on the spectra from the grey and white matter samples. The PCA and LDA models were validated using cross-validation (Venetian blinds with 10 data splits and one sample thickness) (Rinnan et al., 2009).

3. Outcomes

3.1 The effect of environment on average daily weight gain in the subsample of 36 pigs

In the 36 pigs that were used for biological sampling, we found no effect of environmental treatment or sex on ADG (Figure 3, P = 0.10 and P = 0.40, respectively).

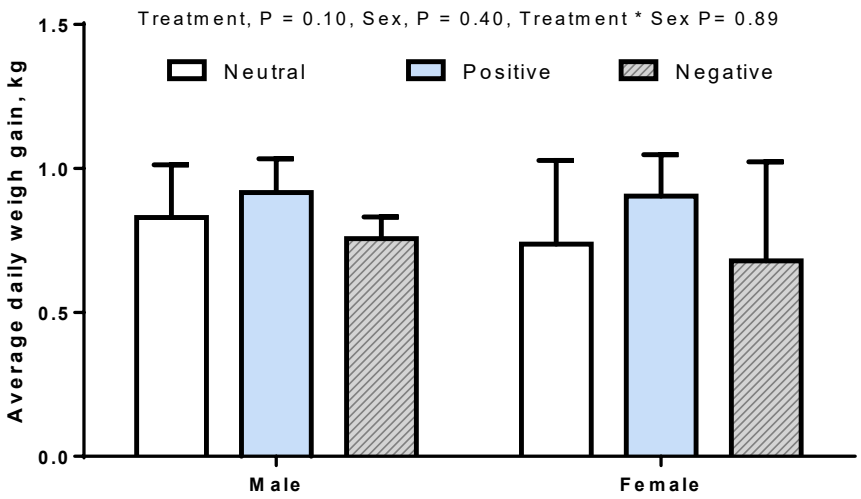

Figure 3. The 4-week ADG of pigs exposed to conditions of normal husbandry (neutral), positive enrichment (positive), and weekly pen mixing (negative). The ADG was calculated from 36 pigs that were used for biological sample collection. Data are shown as mean +/- SD.

3.1.2 The effect of environment on average daily weight gain in the 144 experimental pigs

The environmental treatments tended to have an effect on ADG when data obtained from all 144 experimental pigs were analysed (P = 0.067). There was no difference in ADG between the male and female pigs (P = 0.23). The pigs that received the negative treatment by weekly pen mixing (0.85 ± 0.09 kg) had lower ADG than those that received normal husbandry (0.91 ± 0.17 kg) (P = 0.052), or positive enrichment (0.92 ± 0.14 kg) (P = 0.034), respectively (Figure 4). No difference in ADG was found between the pigs that received positive enrichment and normal husbandry conditions (P = 0.882). There was a trend for an interaction between environmental treatment and sex (P = 0.05).

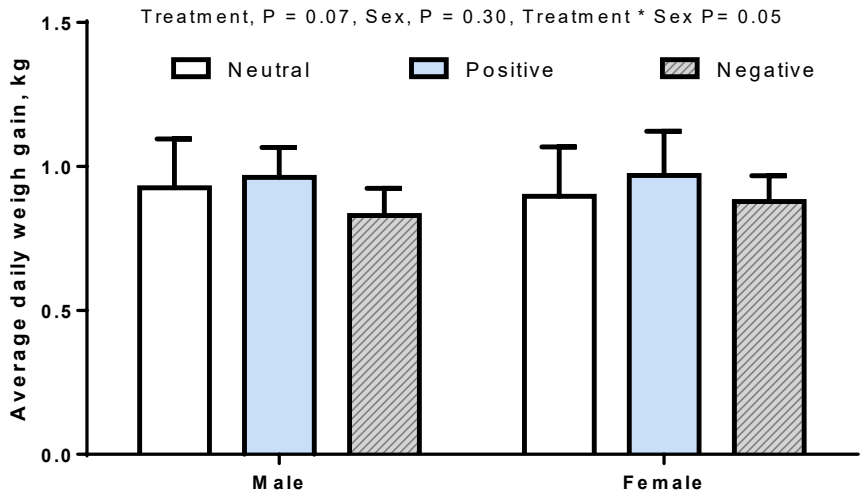

Figure 4. The 4-week ADG of pigs exposed to conditions of normal husbandry (neutral), positive enrichment (positive), and weekly pen mixing (negative). The ADG was calculated from 132 pigs after removing 12 outliers and analysed with ANOVA. Data are shown as mean +/- SD.

3.2 The effect of environment on slaughter traits and pork quality

There was no significant effect of environmental treatment on either carcass or pork quality measurements ($P > 0.05$, Table 2). Male pigs were heavier than females prior to slaughter ($P < 0.001$, Table 2) and tended to have heavier HSCW ($P = 0.063$). Females had a higher ($P < 0.001$) dressing percentage and in turn had a lower chill loss % ($P = 0.002$). Females had a higher ($P < 0.001$) initial and ultimate temperature compared to males. Males also had increased drip ($P < 0.001$) and cook loss ($P = 0.005$) compared to female pigs. There was no significant sex effect on P2 backfat depth, cold carcass weight, pH, temperature upon exiting the blast chiller, L*, or shear force.

There was an interaction between environment treatment and sex on the HSCW ($P < 0.05$) and a trend for an interaction between environment and sex treatment on the slaughter liveweight ($P = 0.067$) and cold carcass weight ($P = 0.053$). The pairwise comparison did not detect a difference in the hot standard carcass weight between any of the three groups. There was no interaction between treatment and sex on any other parameters collected at slaughter.

Table 2. Effect of environmental treatment and sex on carcass and pork quality parameters.

	Treatment				Sex			*P*-Value		
	Positive	**Neutral**	**Negative**	**SED**	**Female**	**Male**	**SED**	**Treatment**	**Sex**	**Treatment x Sex**
Slaughter live weight (kg)	106.0	108.9	107.5	5.68	102.3	112.7	2.28	0.88	<0.001	0.069
Hot carcass weight (kg)	88.8	92.2	88.3	2.68	88.2	91.3	1.59	0.48	0.063	0.046
P2 backfat depth (mm)	13.0	12.5	11.8	0.92	11.9	12.9	0.74	0.42	0.19	0.83
Dressing percentage (%)	80.5	80.7	80.3	1.5	81.9	79.1	0.4	0.97	<0.001	0.31
Cold carcass weight (kg)	81.2	84.6	81.0	2.4	81.0	83.5	1.5	0.45	0.11	0.053
Chill loss (%)	8.5	8.3	8.3	0.1	8.2	8.5	0.1	0.23	0.002	0.77
pH 1 [a]	6.05	6.01	6.11	0.077	6.08	6.03	0.056	0.47	0.40	0.86
pH 2 [b]	5.98	5.96	6.08	0.115	6.02	6.00	0.058	0.61	0.75	0.81
pHu [c]	5.51	5.53	5.56	0.066	5.52	5.55	0.036	0.77	0.45	0.10
Temp 1 (°C) [a]	10.12	10.57	9.45	0.588	11.04	9.06	0.475	0.17	<0.001	0.85
Temp 2 (°C) [b]	6.32	6.60	5.86	0.407	6.44	6.07	0.331	0.19	0.267	0.89
Temp ultimate (°C) [c]	3.33	3.35	3.14	0.096	3.61	2.95	0.078	0.06	<0.001	0.37
Drip loss (%)	2.0	1.9	1.9	0.19	1.6	2.2	0.15	0.96	<0.001	0.87
Cook loss (%)	23.7	22.9	24.0	0.73	22.7	24.4	0.59	0.30	0.005	0.20
L*	51.6	52.1	52.4	0.78	52.0	52.0	0.53	0.59	0.99	0.35
a*	6.3	6.3	5.9	0.31	5.8	6.5	0.25	0.52	0.010	0.58
b*	3.7	3.7	3.8	0.34	3.5	3.9	0.16	0.94	0.024	0.19
WBSF (N)	23.9	23.6	24.0	0.88	23.6	24.1	0.71	0.89	0.44	0.31

[a] pH and temperature recorded at approximately 45 minutes post slaughter, prior to entry to the blast chiller.

[b] pH and temperature recorded at approximately 180 minutes post slaughter, upon exit from the blast chiller.

[c] pH and temperature recorded at approximately 24 hours post slaughter, prior to boning.

3.3 Results of infrared spectroscopy

Analysis and interpretation of NIR spectra

The first two principal components (PC) of the FT-NIR spectroscopy accounted for more than 85% of the total variance between the NIR spectra of the two brain locations that were analysed (anterior prefrontal cortex and the lateral region of the prefrontal cortex), with PC1 explaining 82.4% and PC2 explaining 3.1% of the total variance, respectively (Figure 5).

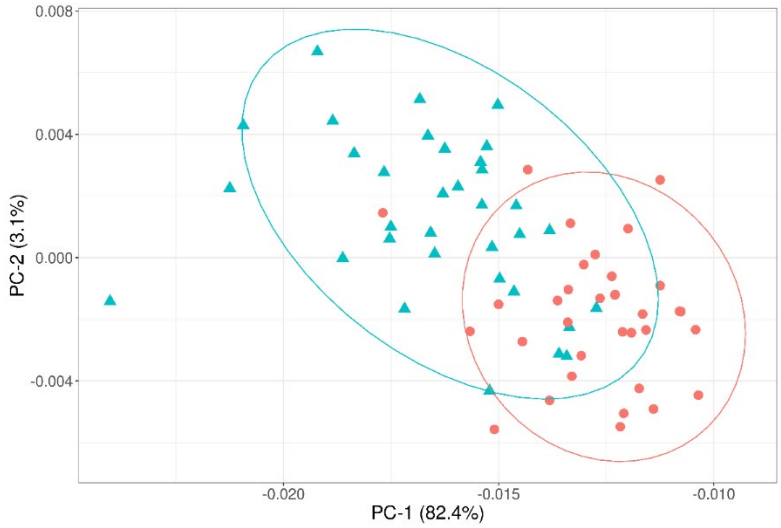

Figure 5. Principal component scores from the pre-processed FT-NIR spectra of pig brains that were scanned in the anterior region of the anterior prefrontal cortex (blue triangle) or in the lateral region of the prefrontal cortex (red circle).

The loadings derived from the PCA were investigated further as they provided the relevant information that was used by an algorithm to separate the two brain locations (Figure 6). The highest loadings in both PC1 and PC2 were observed at wavelengths around 7,200 cm^{-1} (O-H bonds), a region that is associated mainly with water, and around 5,400 – 5,800 cm^{-1} (C-H, C-H_2 and C-H_3) a region that is associated mainly with lipids, fatty acids, and proteins (Workman and Weyer, 2012). A high negative loading at around 4,200 cm^{-1} was observed in PC1 (C-H combination tones and C-C stretching combinations), and was likely associated with different chemical groups such as lipids, fatty acids, or amino acids (Workman and Weyer, 2012). In addition, other shifts were observed at wavelengths associated with the water region (O-H) as well as opposite loadings in the absorbance values in the region around 5,300 – 5,340 cm^{-1} (Workman and Weyer, 2012). The region around 5,300 cm^{-1} is associated mainly with the second overtone of O-H stretching and C=O vibrations in water and other compounds that contain carbon and oxygen (e.g. lipids, fatty acids) (Serdyukov et al., 2009).

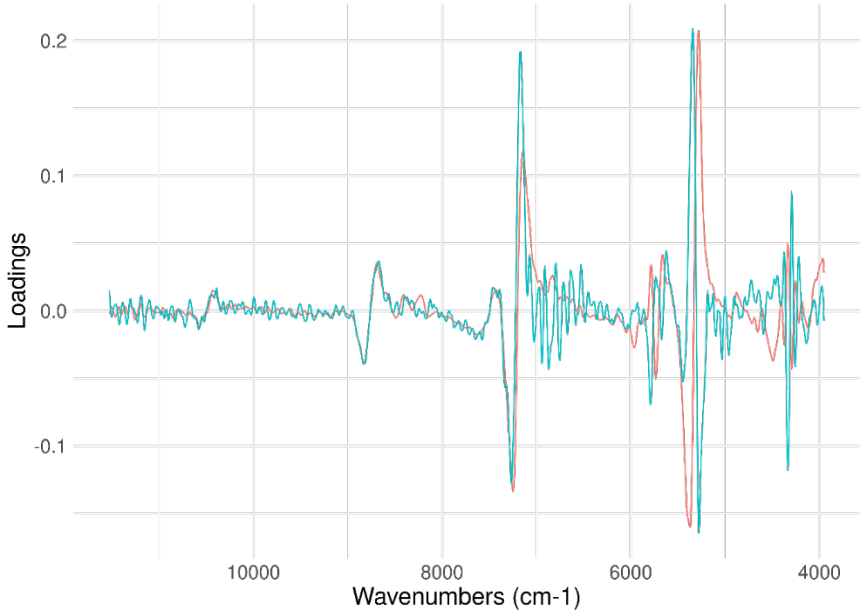

Figure 6. Loading plots for the principal components, PC-1 (red line) and PC-2 (blue line) of the pre-processed spectra (Savitzky-Golay filtering with the 2nd order derivatives) obtained from FT-NIR spectra of both anterior and lateral regions of the prefrontal cortex of pig.

The brain samples were classified according to anatomical location, animal sex, environmental conditions, and exposure or not to an acute stressor just prior to death (Table 3). Brain samples obtained from the lateral prefrontal cortex were 78% correctly classified as females, while 74% of the brain samples were classified as males. Sixty-five percent of the brain samples obtained from the anterior prefrontal cortex were classified correctly as female and 60% were classified correctly as male samples. The classification models correctly identified the environmental condition in fewer than 50% of the samples from either the anterior or the lateral prefrontal cortex. The samples obtained from the anterior prefrontal cortex were 58%, 60%, and 45% correctly classified as positive, control, or negative, respectively. The models did better at identifying the brains from the pigs that had undergone an acute stimulus just prior to death than they did at identifying differences in brains due to long-tern environmental conditions, correctly identifying at least 56% of the brains correctly from the pigs that had received the acute stimulus or not, from both the anterior and lateral regions of the prefrontal cortex.

Table 3. Confusion matrix of the classification of samples of lateral and anterior prefrontal cortex using linear discriminant analysis (LDA) according to animal sex (male, female), stimulus (Yes vs No), environmental conditions (positive, negative, control) in the grey and white brain tissue samples analysed using near infrared spectroscopy. In brackets is the percentage of correct classification.

Factor	PCF region	Grouping	Correct classification: count, (%)		
Sex			Female	Male	
	Lateral	Female	14 (78%)	4	
		Male	4	11 (73%)	
	Anterior	Female	11 (65%)	6	
		Male	7	9 (60%)	
Acute Stimulus			Yes	No	
	Lateral	Yes	10 (56%)	8	
		No	6	9 (60%)	33
	Anterior	Yes	10 (62%)	6	
		No	6	11 (65%)	33
Environmental condition			Positive	Negative	Control
	Lateral	Positive	4 (36%)	4	3
		Negative	3	2 (29%)	2
		Control	4	4	7 (47%)
	Anterior	Positive	7 (58%)	3	2
		Negative	2	5 (45%)	4
		Control	2	2	6 (60%)

Analysis and interpretation of MIR spectra

The first two PCs from the MIR analysis of the samples of grey and white brain matter explained more than 98% of the total variance, where PC1 explained 93% and PC2 explained 5% of the variance, respectively. A clear separation between the grey and white matter samples that were sourced from the lateral prefrontal cortex (Figure 7) was observed along PC2. Neither animal sex, environmental condition, or exposure to an acute stimulus had any influence on the score (no patterns were associated with these variables).

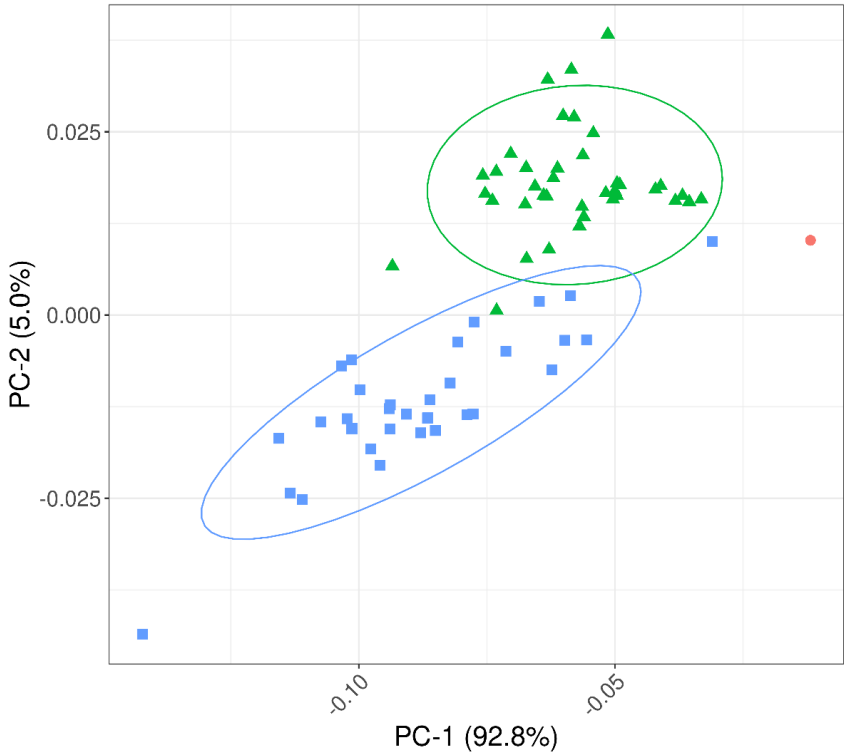

Figure 7. Principal component scores of pre-processed mid-infrared spectra of grey (green triangle) and white matter (blue square) from the lateral prefrontal cortex of pigs (n = 36). The red circle is for a reading from the buffer that the pig brains were stored in, given here as a control sample.

Analysis of the PCA loadings revealed that the highest loading in PC1 (98% variance) was at frequencies around 2,840 – 3,000 cm^{-1} (Figure 8). This region is associated with the presence of C-H_2 and C-H_3 groups related to lipids and fatty acids (Talari et al., 2017). High loadings were also observed at frequencies around 1,600 – 1,760 cm^{-1}, a region related to the amide I band (protein or compounds containing nitrogen) and the C=O stretching bond of fatty acid ester (Dovbeshko et al., 2000; Mossoba et al., 2005). The highest loadings in the region between 980 – 1,270 cm^{-1} can be associated with different compounds such as carbohydrates, lipoproteins, and proteins (Talari et al., 2017). The highest loadings in PC2 showed similar patterns, representing the mirror image of the loadings derived from the PC1. However, a shift in the region between 3,000 – 2,850 cm^{-1} from those reported in PC1 was observed. That region is mainly associated with fatty acids (Talari et al., 2017).

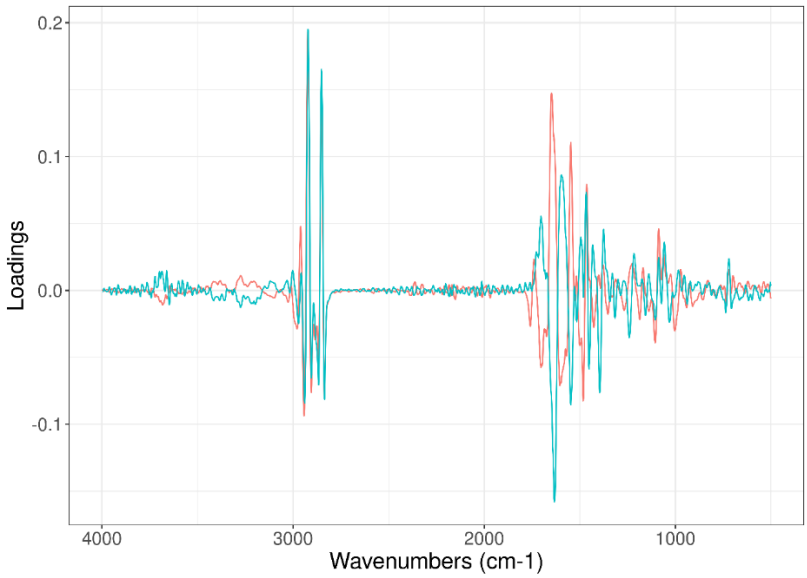

Figure 8. Loading plots for the principal components, PC-1 (red line) and PC-2 (blue line) of the pre-processed mid-infrared (MIR) spectra obtained from MIR spectroscopy of the grey and white matter of the lateral prefrontal cortex of pigs. The pre-processing method consisted of detrend, standard normal variant (SNV), and second derivatives order (Savitzky-Golay) filtering with 11 moving windows.

The results from the LDA classification for the samples sourced from either grey or white brain tissues and analysed using MIR spectroscopy are shown in Table 4. In the grey brain tissue, 62.5% of samples from female pigs were correctly classified as female, and 61% of the males were correctly identified. In the white matter tissue 57% and 61% of the samples were correctly classified as female and male, respectively. The grey matter samples from pigs that had been exposed to the acute stimulus, or not, just prior to death were classified correctly 68% (for presence of the stimulus) and 65% (for absence of the stimulus) of the time. The classification rate for the acute stimulus in the white matter was slightly higher at 74% and 70% for presence and absence, respectively. For exposure to the three environment conditions in the white brain tissue, the LDA classification rates were correct for 60% of the samples from pigs in the positive treatment, 44% for pigs in the negative treatment, and 50% for pigs in the control treatment. The rate of correct classification was lower than 45% for the samples from the grey matter tissue.

Table 4. Confusion matrix of the classification of samples of lateral prefrontal cortex using linear discriminant analysis (LDA) according to animal sex, stimulus (Yes vs No), and environmental conditions (positive, negative, control) in the grey and white brain tissue samples analysed using mid infrared spectroscopy. In brackets is the percentage of correct classification.

Factor	Tissue	Grouping	Correct classification: count, (%)		
Sex			Female	Male	
	Grey	Female	10 (62.5%)	6	39
		Male	9	14 (61%)	
	White	Female	12 (57%)	9	39
		Male	7	11 (61%)	
Stimulus			Yes	No	
	Grey	Yes	13 (68%)	6	39
		No	7	13 (65%)	
	White	Yes	14 (74%)	5	39
		No	6	14 (70%)	
Environmental conditions			Positive	Negative	Control
	Grey	Positive	5 (45%)	3	3
39		Negative	4	5 (36%)	5
		Control	5	5	4 (29%)
	White	Positive	9 (60%)	4	2
39		Negative	5	7 (44%)	4
		Control	1	3	4 (50%)

3.4 Plasma concentration of cortisol in response to environmental and acute stress

At the start of the study, the concentration of cortisol in plasma was not different between the groups in the different environmental treatments (P = 0.054) or between the groups that were late subjected, or not, to acute stress (P = 0.14; Figure 9A). There was no interaction between environmental treatment and acute stress (P = 0.52). At the end of the study, there was

again no significant effect of acute stress (P = 0.66) on the concentration of cortisol in plasma (Figure 9B). Similarly, there was no interaction between environmental treatment and acute stress (P = 0.45). However, the environmental treatments tended to affect the plasma cortisol concentration before (P = 0.054) and after the treatments (P = 0.08).

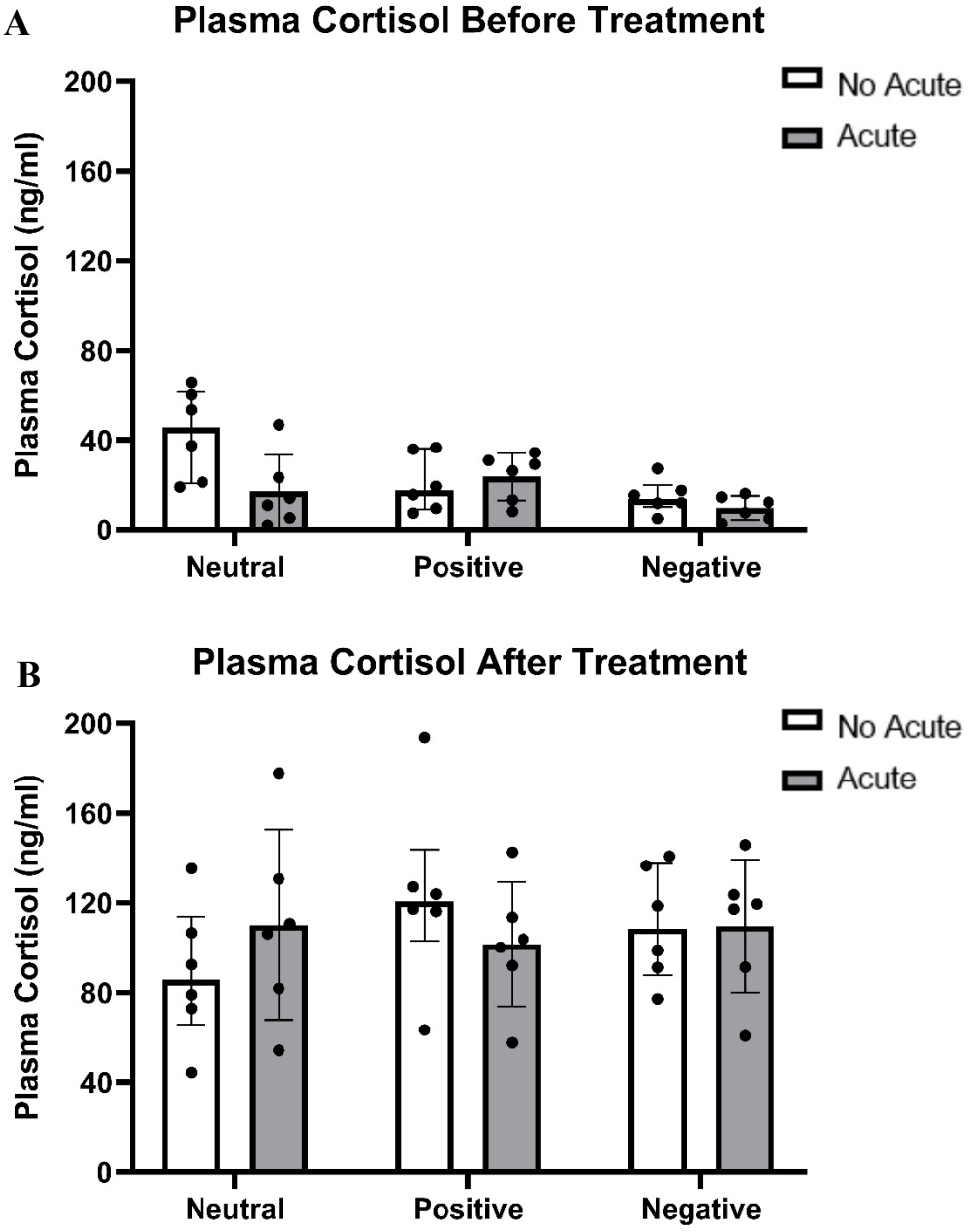

Figure 9. Effect of environmental treatment and acute stress on the concentration of cortisol in the plasma of pigs, at the start (A) and the end of the experimental period (B).

There was an interaction between time of sampling and environmental treatment on the concentration of cortisol in the plasma (Table 5, P = 0.038). The baseline samples taken before the start of environmental treatment showed a trend towards a difference between groups (P = 0.054), with pigs

that were to be placed in the positive enrichment (22.19 ± 11.26 ng/ml) and the pigs that were to be placed in normal husbandry conditions (29.90 ± 21.99 ng/ml) having higher cortisol levels than the pigs that were to be placed in the negative treatment (12.21 ± 6.70 ng/ml) (P = 0.062 and P = 0.059 respectively, Bonferroni pairwise analysis) (Figure 9A). However, at the end of treatment, the pigs receiving the negative treatment tended to have higher plasma cortisol compared to those that were kept in normal husbandry conditions (P = 0.08).

Table 5. Main effects and interactions between environmental treatments, acute stress, and time of sampling on the level of plasma cortisol in pigs

Source	Type III Sum of Squares	df	Mean Square	F	P value
Environmental treatments	0.304	2	0.152	3.289	0.054
Acute stress	0.109	1	0.109	2.351	0.66
Time of sampling	1.911	1	1.911	41.338	<.001
Environmental treatments * Acute stress	0.063	2	0.031	0.676	0.45
Environmental treatments * Time of sampling	0.17	2	0.17	4.783	0.038
Acute stress * Time of sampling	0.256	1	0.128	3.616	0.052
Environmental treatments * Acute stress * Time of sampling	0.025	1	0.025	0.71	0.407

At the end of the treatments, the cortisol concentrations were higher in all of the groups than they were prior to treatment, with the normal husbandry conditions (99.33 ± 37.22 ng/ml), positive enrichment (112.57 ± 35.65 ng/ml) and negative treatment (110.07 ± 26.44 ng/ml), all being significantly higher than their respective baseline levels (P < 0.001, Bonferroni pairwise analysis) (Figure 10A).

Acute stress had no effect on the concentration of plasma cortisol (P = 0.14; Figure 10B). However, the concentration of cortisol was higher at the second sampling than it was at the baseline (P < 0.001).

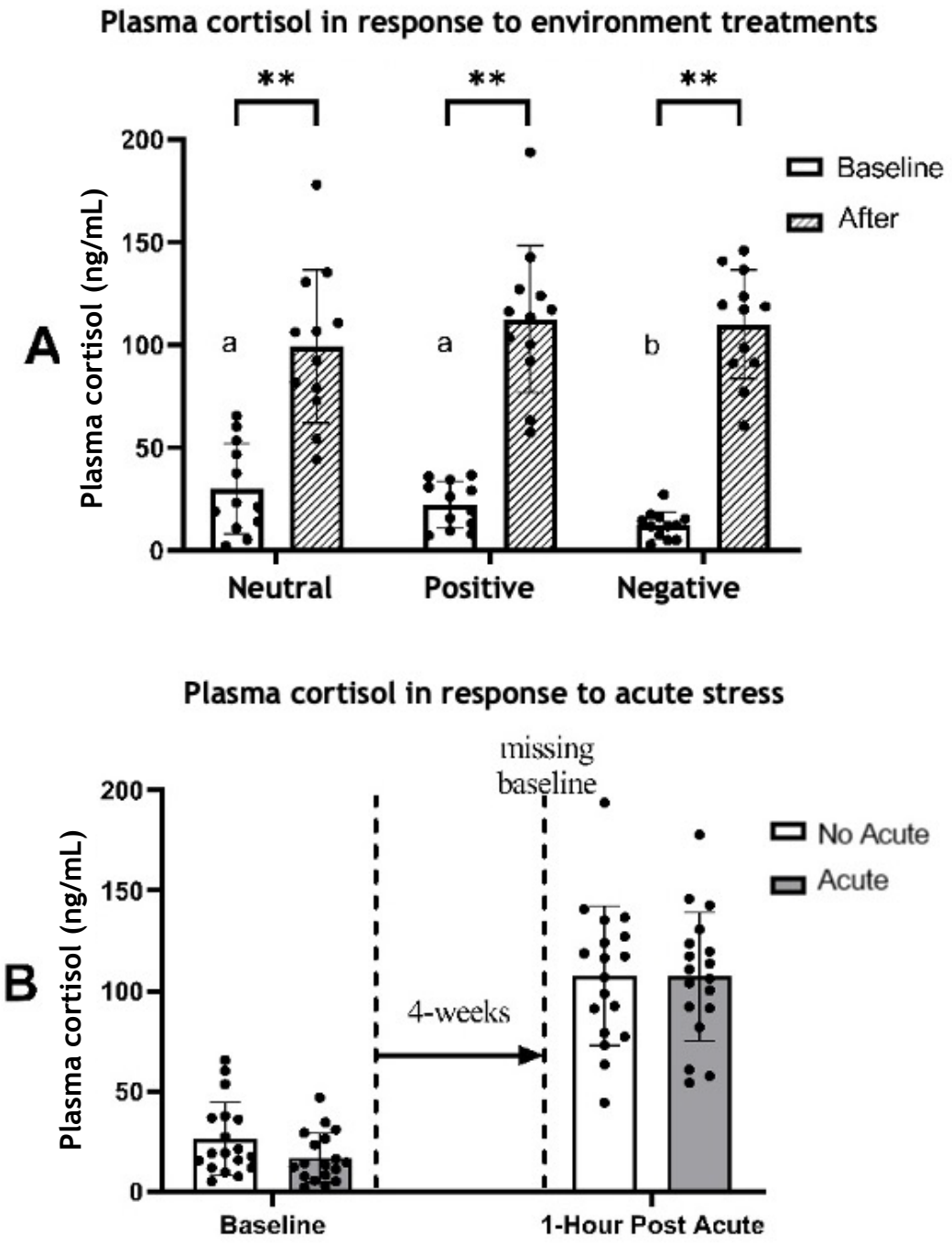

Figure 10. Plasma cortisol of pigs before entering treatment and after 4-weeks exposed to the three environmental treatments (panel A) or a 1-hour post-acute stress (or not) (B). A: pigs in normal husbandry conditions (neutral), with enrichment (positive), or exposed to weekly pen mixing (negative). B: pigs exposed to snare (acute) or no snare (no acute). Cortisol was analysed from 33 pigs after the removal of outliers with repeated measures general linear mixed model. ** $P < 0.001$ and a,b = trend towards a difference between groups. Data shown as mean ± SD.

There was an interaction between time of sampling and sex on plasma cortisol (Figure 11, $P = 0.046$). Before entering the treatments, the female

pigs (24.37 ± 15.92 ng/ml) had a higher plasma concentration of cortisol than did the male pigs (18.50 ± 16.22 ng/ml) (P = 0.041, Bonferroni pairwise analysis). In both the female (105.65 ± 36.15 ng/ml) and male (108.99 ± 30.46 ng/ml) pigs the plasma cortisol level was elevated at the second biological sampling point (P < 0.001, Bonferroni pairwise analysis).

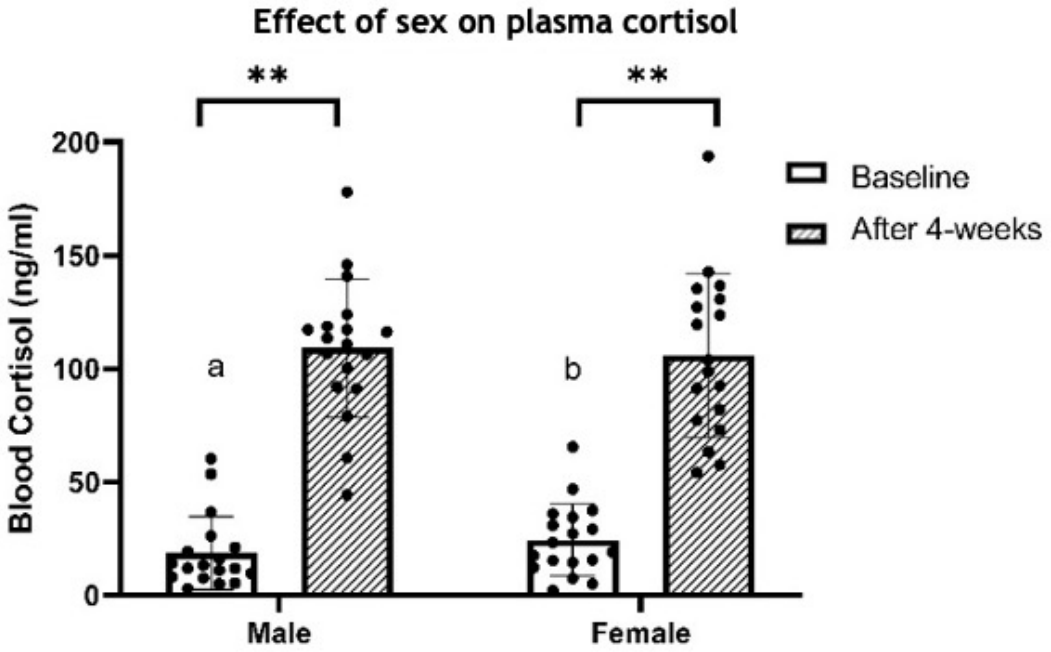

Figure 11. Plasma cortisol in female (n = 18) and male (n = 18) pigs at 12-weeks of age before entering treatments and after four weeks of exposure to the environmental treatments. Data were analysed from 33 pigs after the removal of outliers with repeated measures general linear mixed model. ** P < 0.001 and a,b = P < 0.05 between groups. Data are shown as mean +/- SD.

3.5 Expression of NEAT1 in ventromedial hypothalamus in response to environmental and acute stress

There was no main effect of environment or acute treatment on the expression of total NEAT1 in the VMH (P = 0.17 and P = 0.27 respectively) (Figure 12). There was no interaction between environmental and acute treatment (P = 0.25).

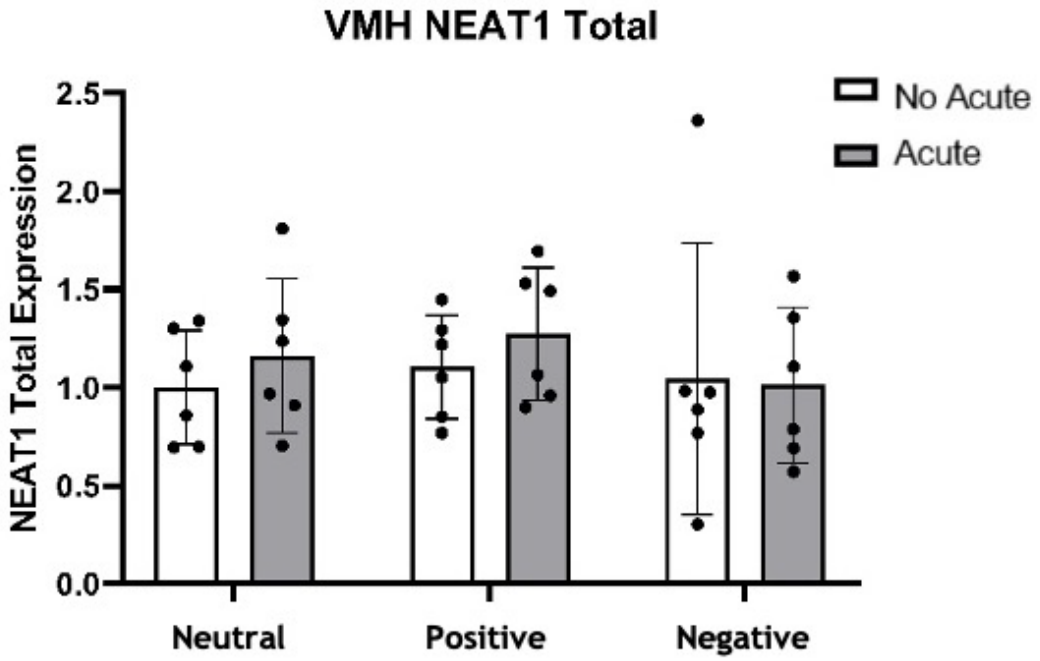

Figure 12. Relative expression of total NEAT1 (both NEAT1 Total and NEAT1 Long) in the VMH of pigs after 4 weeks of normal husbandry conditions (neutral), enrichment (positive) or pen mixing (negative) with half in each treatment exposed to a snout snare (acute). Data were analysed using mixed model ANOVA with 35 pigs after removing an outlier, and are shown as mean ± SD.

Primers targeting NEAT1 Long were used to compare the expression of the longer isoform only. There was no main effect of environment or acute treatment on the expression of NEAT1 Long in the VMH (Figure 13, P = 0.293 and P = 0.517 respectively).

The percentage of NEAT1 Long represents the change in isoform, as the change in preferential isoform expression between NEAT1 Total and NEAT1 Long may be how NEAT1 expression in the pig responds to the environmental or acute stress. No main effect of environment or acute treatment on the expression percentage of NEAT1 Long in the VMH was found (Figure 14, P = 0.995 and P = 0.762 respectively).

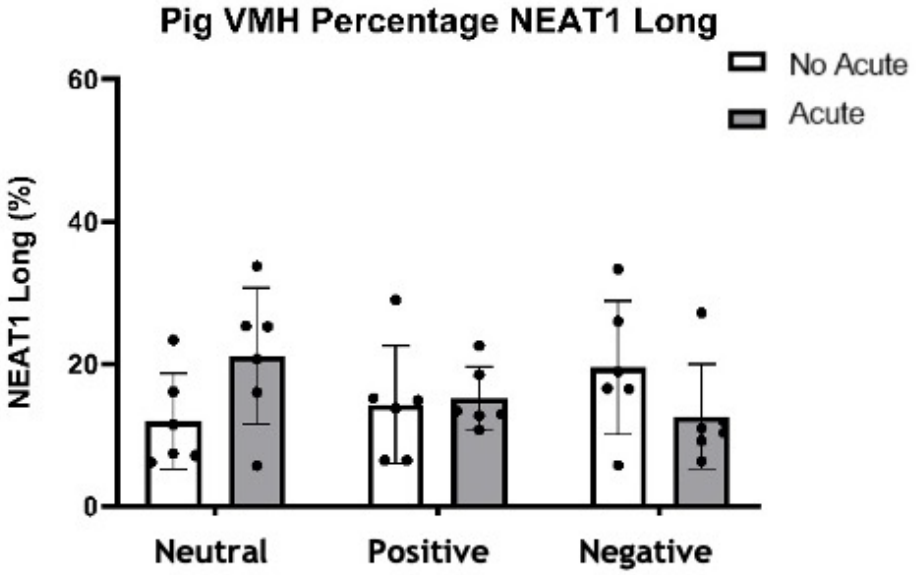

Figure 13. Relative expression of NEAT1 Long in the VMH of pigs after 4 weeks of normal husbandry conditions (neutral), enrichment (positive) or pen mixing (negative) with half in each treatment exposed to a snout snare restraint (acute). Data were analysed with Kruskal-Wallis H Test (36 pigs), and are shown as mean ± SD.

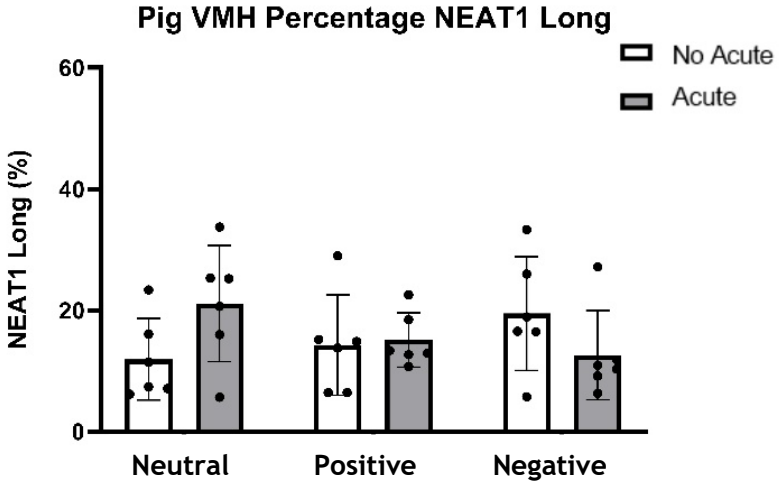

Figure 14. Percentage of NEAT1 Long in the VMH of pigs after 4 weeks of normal husbandry conditions (neutral), enrichment (positive) or pen mixing (negative) with half in each treatment exposed to a snout snare (acute) or no snare (no acute). Data were analysed with mixed model ANOVA using log-transformed expression data (36 pigs), and are shown as mean ± SD.

3.6 Expression of NEAT1 in blood in response to environmental and acute stress

There was no interaction between the environmental or acute stress on the expression of NEAT1 Total in the blood of pigs before (P = 0.70) of after (P = 0.95) the experimental period (Figure 15). There was no significant change in the relative expression of NEAT1 Total in the blood from the start to the end of the environmental and acute stress treatments (P = 0.13).

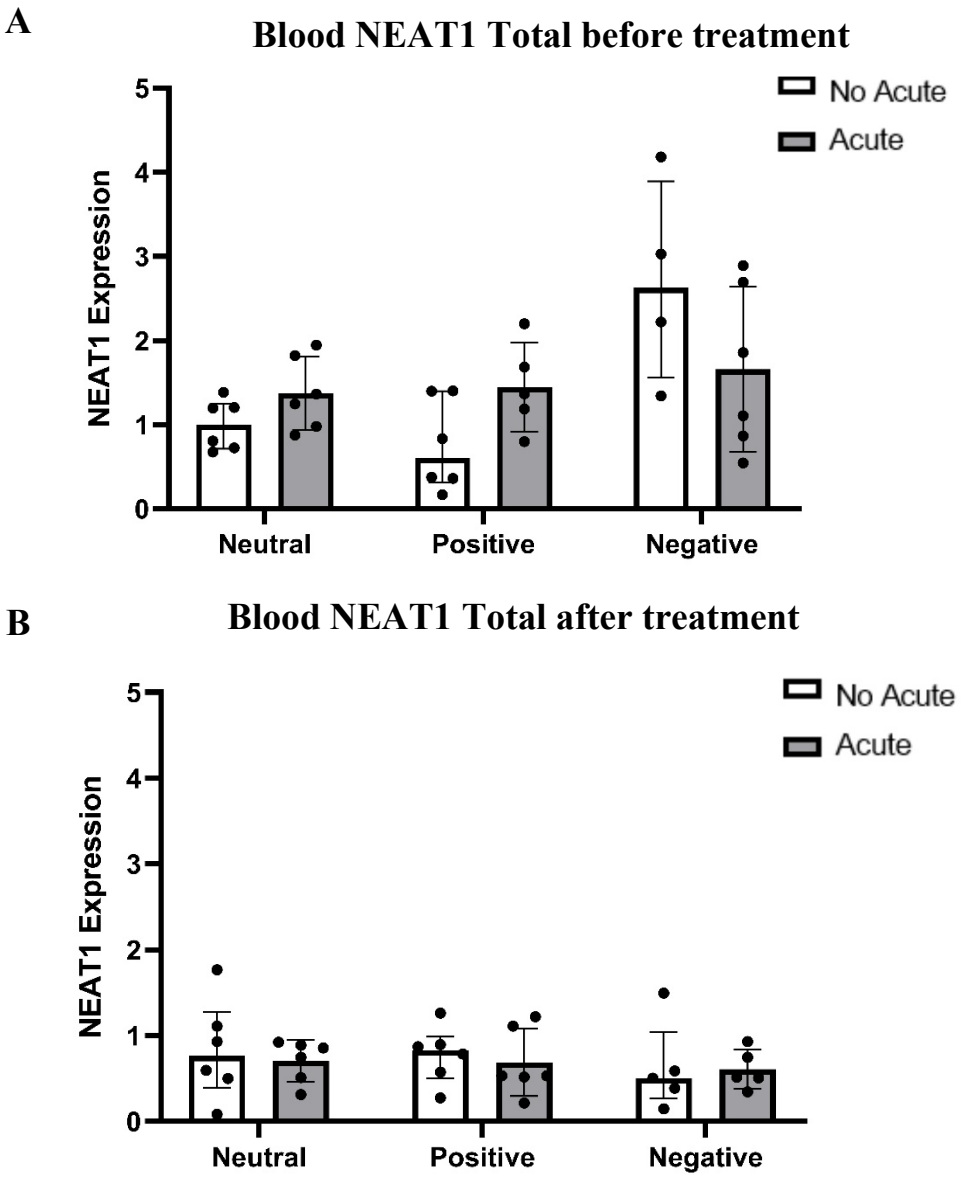

Figure 15. Relative expression of NEAT1 Total (both NEAT1 Total and NEAT1 Long) in the blood of pigs before they were placed in the various environmental treatments (A) and after 4-week of environmental treatments (B). After removing outliers, NEAT1 Total in blood was analysed with a mixed model ANOVA in 28 pigs. Data are shown as mean ± SD.

At the start of the current study, there was no significant effect of environmental treatment (P = 0.09; trend) or acute stress (P = 0.34) on the relative expression of NEAT1 Total in blood (Figure 15A). However, the expression of NEAT1 Total trended to be greater in pigs from the Negative group than that in pigs from the Neutral group. At the end of the study, there was no significant effect of environmental treatment (P = 0.10), or acute stress (P = 0.07) on the relative expression of NEAT1 Total in blood (Figure 15B). While the expression of NEAT1 Total trended to be lower in pigs from the Negative group than that in pigs from the Neutral group.

The environmental treatment tended to affect the expression of NEAT1 Total in blood (P = 0.058), while there was no main effect of acute stress on the expression of NEAT1 Total in pig blood (P = 0.421) (Figure 16). The expression of NEAT1 Total in pig blood was significantly higher before the environmental treatment than it was at the end of environmental treatment (P = 0.023).

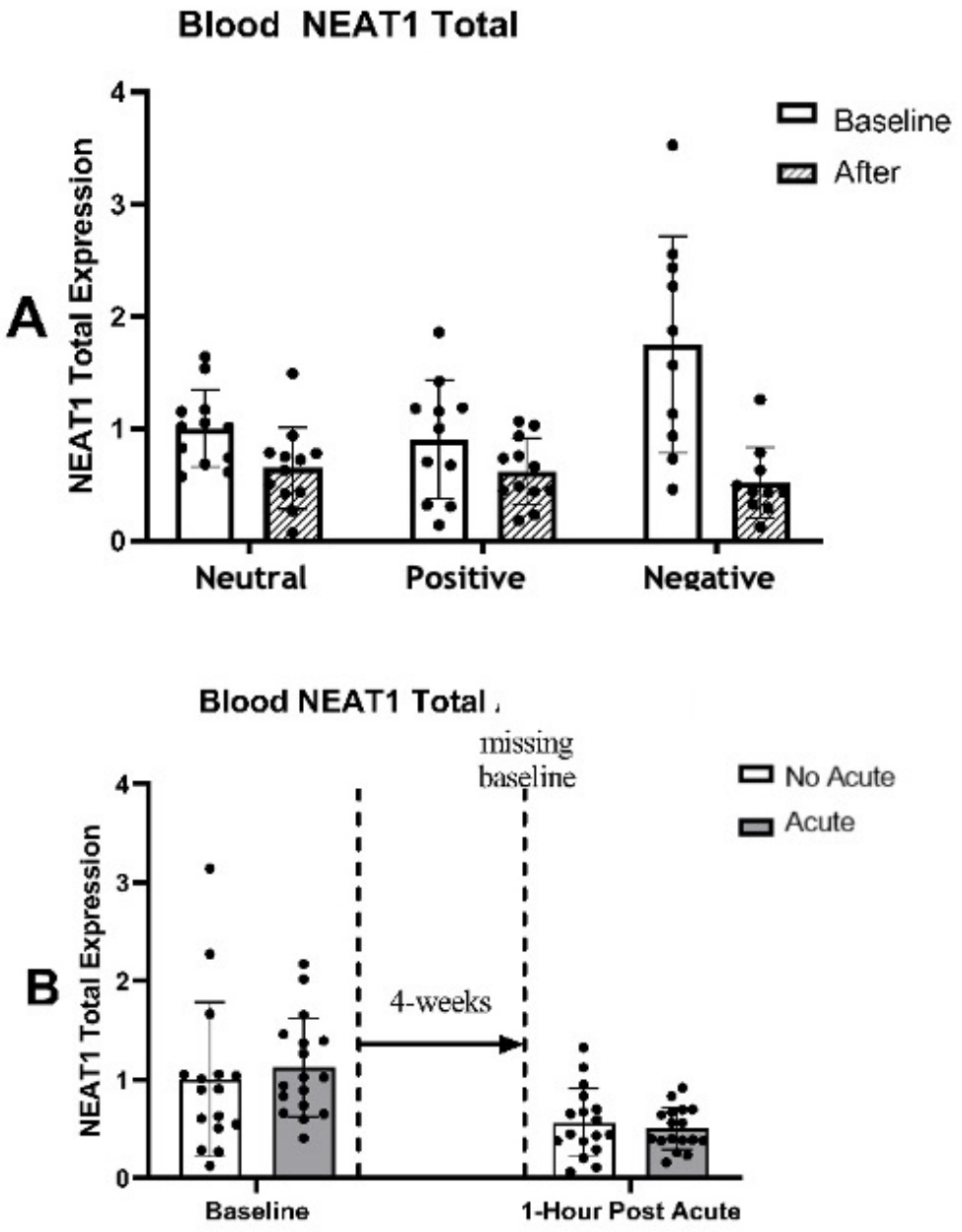

Figure 16. Blood NEAT1 Total (both NEAT1 Total and NEAT1 Long) relative expression in pigs before entering treatment and after 4-week environmental treatment (A) or 1-hour post-acute stress (B). A: pigs in normal husbandry conditions (neutral), enrichment (positive), and pen mixing (negative). B: pigs exposed to snout snare (acute) or no snare (no acute). After removing outliers, NEAT1 Total in blood was analysed with a mixed model ANOVA in 28 pigs. Data are shown as mean +/- SD.

There was no interaction between the environmental and acute stress on the expression of NEAT1 Long in the blood of pigs collected before ($P = 0.70$) or after the experimental period ($P = 0.48$) (Figure 17).

There was no significant difference in the relative expression of NEAT1 Long in blood between the start and end of the environmental treatments and the application of the acute stress treatments after the environmental treatment (P = 0.11). At the start of the study, there was no significant effect of environmental treatment (P = 0.90) or acute stress (P = 0.82) on the relative expression of NEAT1 Long in blood (Figure 17A). At the end of the study, there was no significant effect of environmental treatment (P = 0.97), or acute stress (P = 0.75) on the relative expression of NEAT1 Total in blood (Figure 17B).

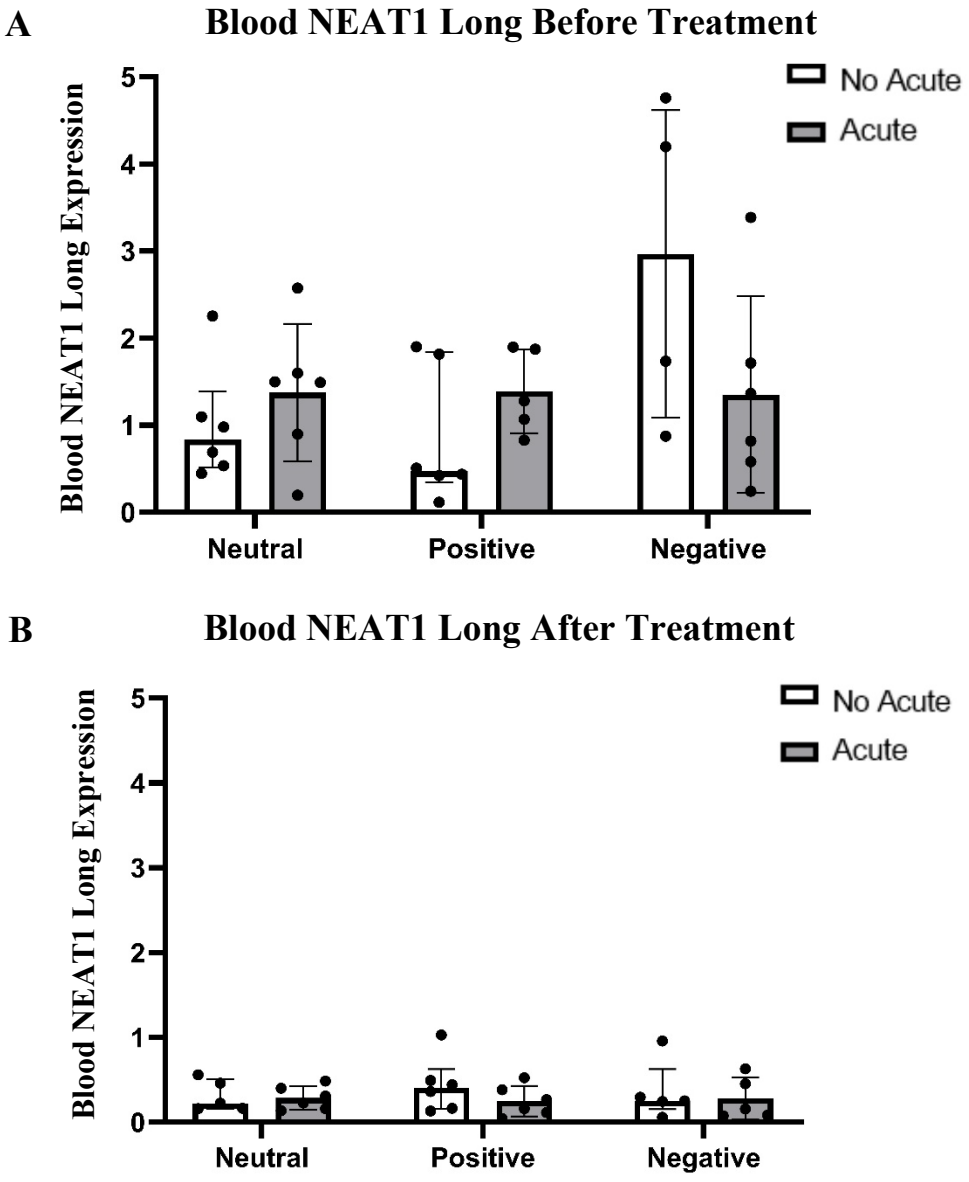

Figure 17. Blood NEAT1 Long relative expression in pigs before entering treatment (A) and after 4-weeks of the experimental treatments (B). After removing outliers, NEAT1 Total in blood was analysed with a mixed model ANOVA using 30 pigs. Data are shown as mean +/- SD.

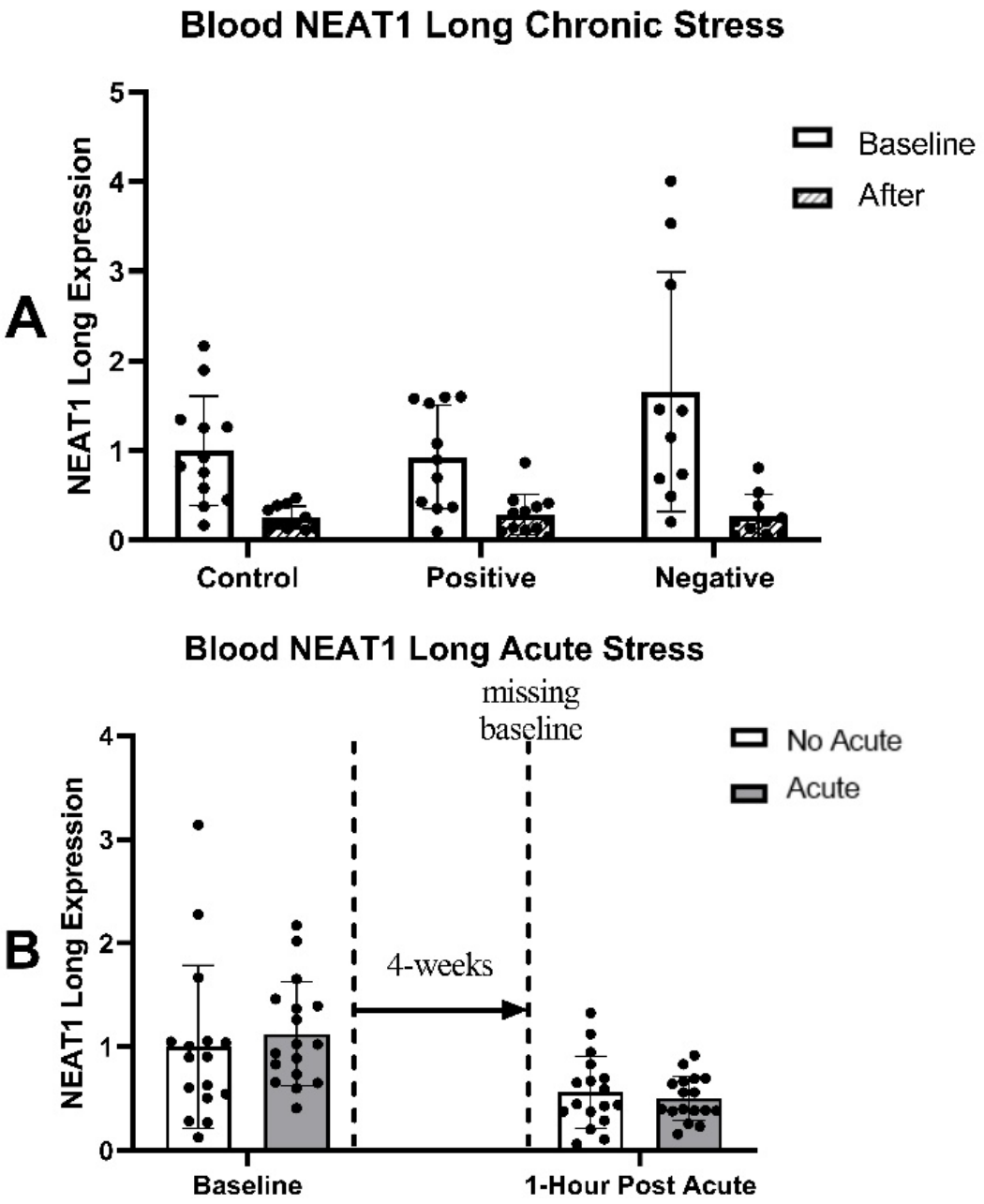

Figure 18. Blood NEAT1 Long expression in pigs before entering treatment and after 4-week environmental stress (A) or 1-hour post-acute stress (B). A: pigs in normal husbandry conditions (neutral), enrichment (positive), and pen mixing (negative). B: pigs elicited to snout snare restraint (acute) or no snare (no acute). NEAT1 Long was analysed with repeated measures general linear mixed model using 30 pigs after the removal of outliers. Data are shown as mean +/- SD.

Overall, there was no main effect of environmental treatment or acute treatment on the expression of NEAT1 Long in pig blood (P = 0.90 and P = 0.81 respectively) (Figure 18). However, the expression of NEAT1 Long in pig

blood before the environmental treatments was significantly higher than at the end of the environmental treatments (P = 0.013).

There was a significant interaction between time of sampling and sex on the expression of NEAT1 Long in pig blood (P = 0.025). Before entering the treatments, the female pigs (1.33 ± 0.90) had higher NEAT1 Long expression than did the male pigs (0.76 ± 0.63) (P = 0.033, Bonferroni pairwise analysis). The NEAT1 expression was lower in both female (0.19 ± 0.19) and male (0.29 ± 0.16) pigs at 16 weeks of age than it was at the baseline measures at 12 weeks of age (Figure 19, P < 0.001 and P = 0.01 respectively).

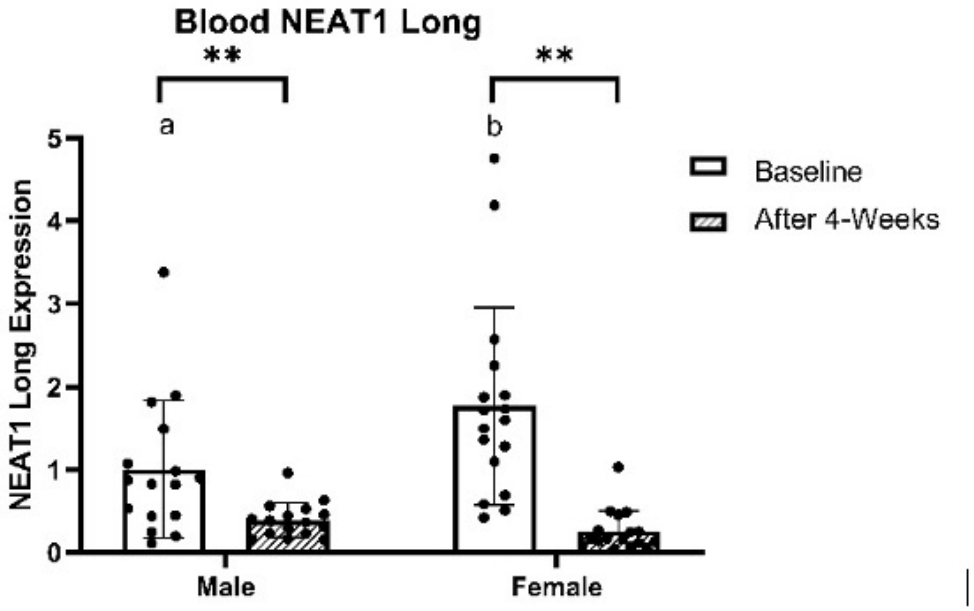

Figure 19. Blood NEAT1 Long expression of female (n = 16) and male (n = 14) pigs at 12-weeks of age before entering treatments and at 24 days proceeding. Data were analysed with repeated measures general linear mixed model using 30 pigs after the removal of outliers. ** P < 0.001 and a, b P < 0.05 between groups. Data are shown as mean +/- SD.

There was no main effect of environment or acute treatment on the percentage expression of NEAT1 Long in pig blood (Figure 20, P = 0.65 and P = 0.19 respectively). The percentage of expression of NEAT1 Long in pig blood before the environmental treatments was significantly higher than it was at the end of environmental treatments (P = 0.02).

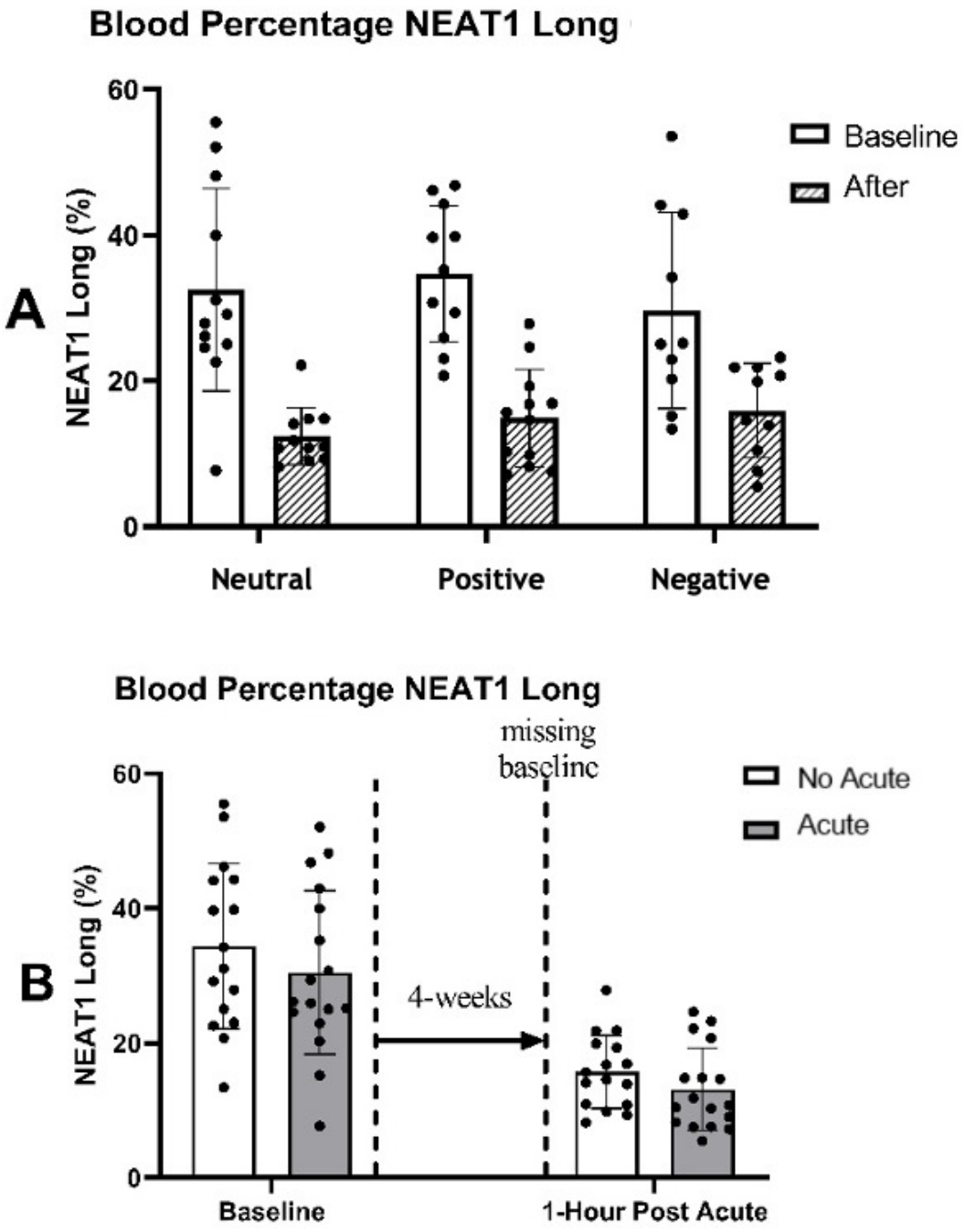

Figure 20. Blood percentage of NEAT1 Long in pigs before entering treatment and after 4-week environmental stress (A) or 1-hour post-acute stress (B). A: pigs in normal husbandry conditions (neutral), enrichment (positive), and pen mixing (negative). B: pigs elicited to snout snare (acute) or no snare (no acute). Data were analysed with repeated measures general linear mixed model using 30 pigs after the removal of outliers, and are shown as mean +/- SD.

3.7 NEAT1 expression in pig saliva in response to environmental and acute stress

The total RNA in the saliva swab that was collected at the start of the environmental treatments was degraded, thus no expression data were obtained prior to the environment treatments. After the environment treatments, there was no effect of environmental treatment on the expression of NEAT1 Total in the saliva of the experimental pigs (P = 0.15, Figure 21). The pigs that received acute stress had significantly lower expression of NEAT1 Total in the saliva compared to pigs that received no acute stress (P = 0.01). There was no effect of environmental stress or acute stress on the expression of NEAT1 Long (P = 0.90 and P = 0.54, respectively) or the percentage of NEAT1 Long (P = 0.73 and P = 0.53, respectively) in the saliva of experimental pigs.

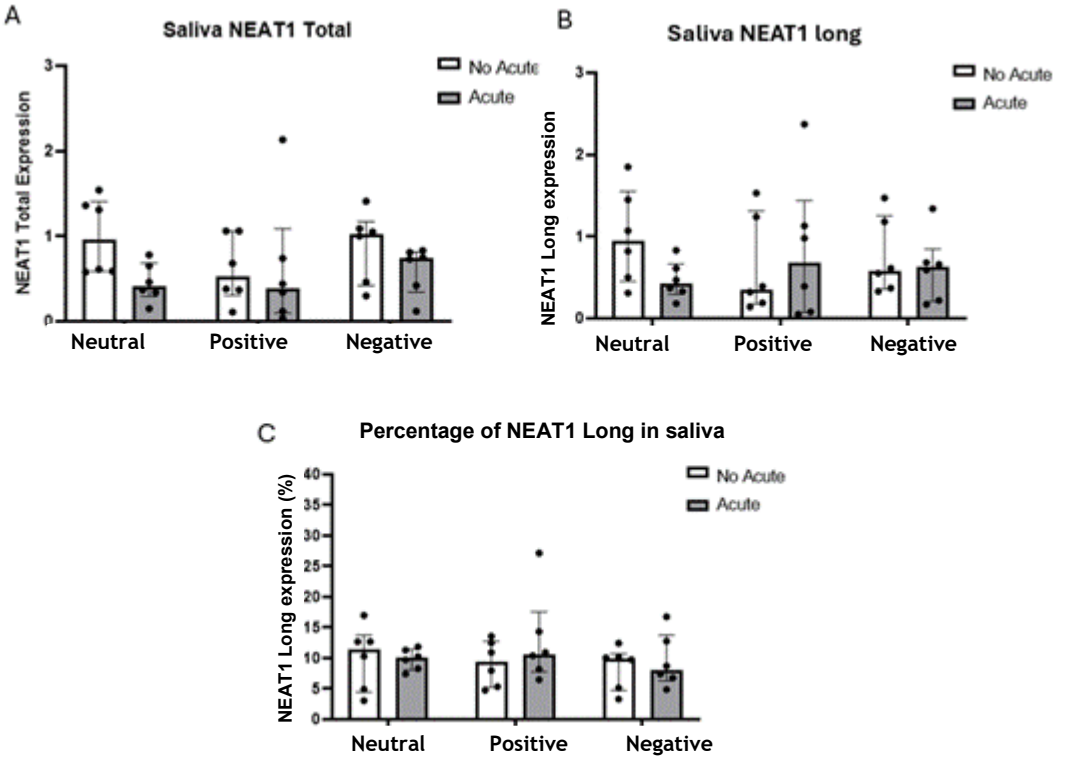

Figure 21. Expression of NEAT1 Total, NEAT1 Long and NEAT1 Long percentage in the saliva of pigs after 4-week environmental stress.

3.8 Correlation between NEAT1 expression in different tissue media

3.8.1 NEAT1 expression in the ventral medial hypothalamus and blood

We investigated if there was a correlation between NEAT1 expression in the VMH and in the blood for each individual pig (Figure 22). There was no evidence of a correlation between NEAT1 Total expression (R^2 = 0.015, P = 0.49), NEAT1 Long expression (R^2 = 0.011, P = 0.57), or percentage of NEAT1 Long (R^2 = 0.003, P = 0.77).

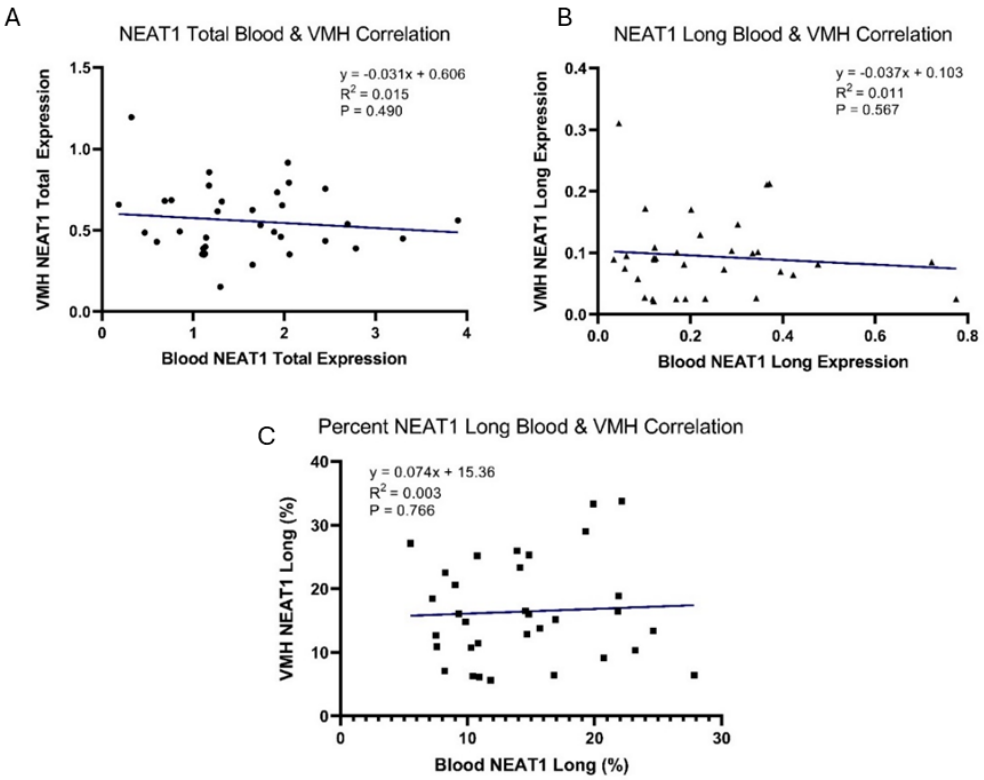

Figure 22. Correlation between expression in the VMH and in the blood of NEAT1 Total (34 pigs, A), NEAT1 Long (33 pigs, B), and percentage of NEAT1 Long (33 pigs, C). Data were analysed using Pearson's correlation.

3.8.2 NEAT1 expression in the ventral medial hypothalamus and the saliva

There was no evidence of a correlation for NEAT1 Total expression (P = 0.82, R2 = 0.002), NEAT1 Long expression (P = 0.49, R^2 = 0.01) or percentage of NEAT1 Long (P = 0.06, R^2 = 0.10) between the saliva and VMH (Figure 23).

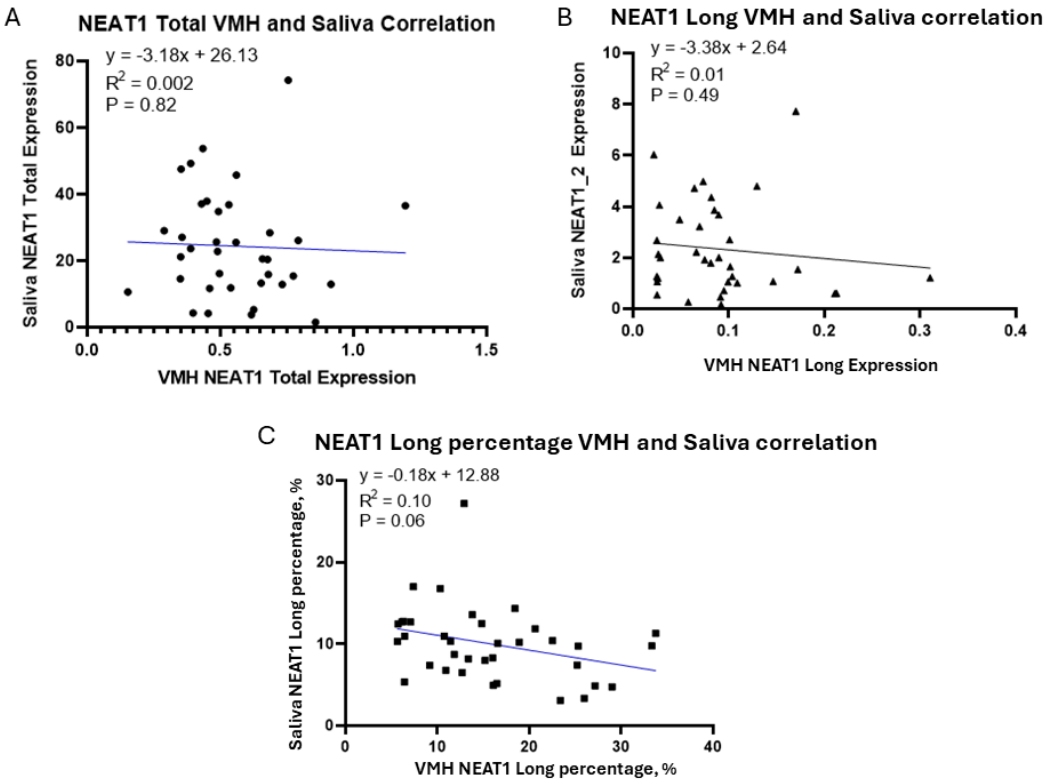

Figure 23. Correlation of VMH with saliva in NEAT1 Total expression (34 pigs, A), NEAT1 Long expression (33 pigs, B), and percentage of NEAT1 Long (33 pigs, C). Data were analysed using Pearson's correlation.

3.8.3 NEAT1 expression in the saliva and the blood

There was no evidence of a correlation in NEAT1 Total expression (P = 0.20, R^2 = 0.05), NEAT1 Long expression (P = 0.51, R^2 = 0.01) or percentage of NEAT1 Long (P = 0.75, R^2 = 0.003) between the saliva and the blood (Figure 24).

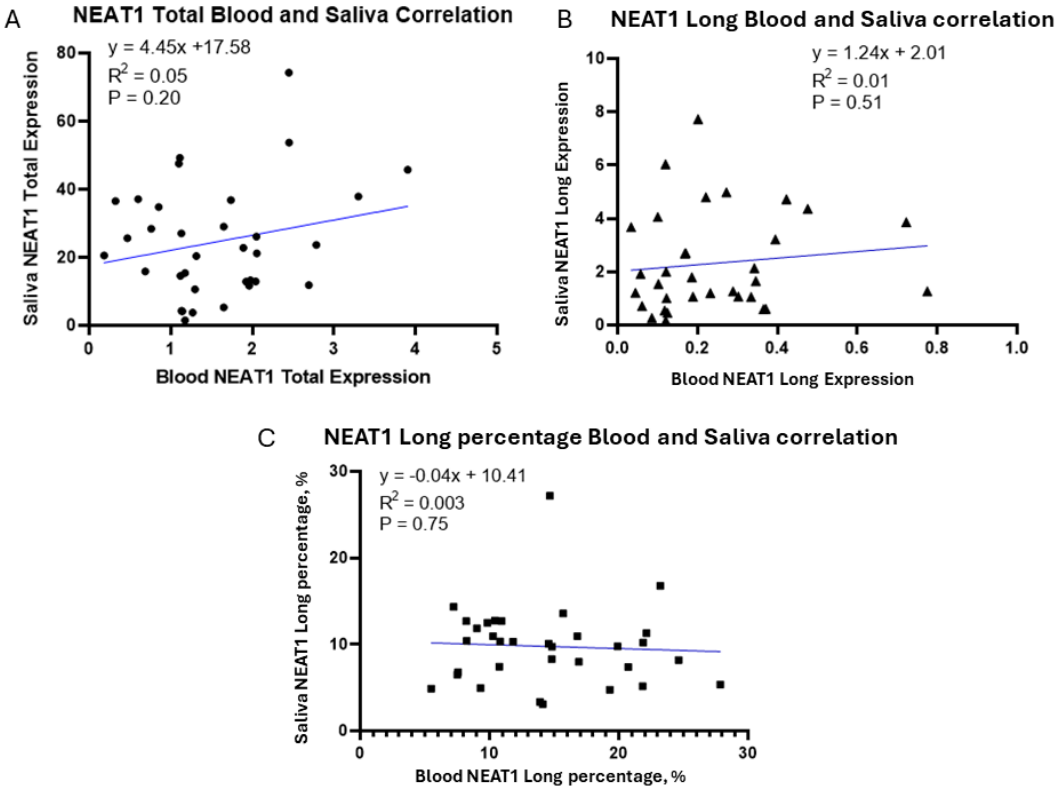

Figure 24. Correlation between saliva and blood in NEAT1 Total expression (34 pigs, A), NEAT1 Long expression (33 pigs, B), and percentage of NEAT1 Long (33 pigs, C). Data were analysed using Pearson's correlation.

4. Application of Research

This pilot study aimed to explore potential biomarkers that may be related to positive and negative experiential state, and ultimately assess the quality of life of commercial pigs.

This pilot investigation has uncovered that:

1. NEAT1, a non-coding RNA, can be measured in blood, saliva, and brain tissue in pigs. NEAT1 in saliva could be investigated in more extreme conditions such as during lairage or transport. Non-Coding RNAs are promising practical markers of welfare with the development of hand-held apparatus able to detect RNA in real-time.

2. Using MIR provides some promise as a welfare assessment tool post-mortem, but further work is required to validate this concept, and to demonstrate that this technology can be used on materials harvested from the live animal, such as saliva or blood. The identification of MIR as a welfare assessment tool takes us one step closer to real time, objective welfare assessment for the pork industry.

The experimental model used in this project included negative, neutral, and positive welfare states. This chronic level of imposition on the quality of life influenced some production parameters. We observed a reduction in growth in the negative environmental treatment. However, the positive treatment did not seem to induce a strong positive experiential state in pigs, and we did not see any change in production parameters. It is possible that the positive environmental treatment was not sufficiently different from the neutral treatment because the pigs receiving the normal husbandry practices in the commercial farm were already experiencing good welfare. The absence of a difference in plasma cortisol between the different treatments supports the hypothesis that the pigs were familiarised in the commercial farm and, there, even the negative treatment was not that stressful. Alternatively, the novelty of the environment, as the pigs were moved in a new shed at the start of the environmental treatment, which might have induced the increase in cortisol in all the pigs, could have masked any effect of the different human-pig interactions. These limitations in the intensity of the negative or positive environmental treatment need to be kept in mind when considering our results on the identification of biomarkers of positive experiential states in pigs.

The NEAT1 results represent a very novel discovery as NEAT1 has never previously been identified in livestock. The biological role of NEAT has been investigated mainly in cell culture or in relation to very damaging diseases such as cancer or neurodegenerative diseases rather than in the whole animal exposed to different psychological sources of stress, and relatively mild sources. There is a need to conduct further investigation to measure NEAT1 in saliva and blood in pigs exposed to more extreme environmental challenges such as, for example, transport and pre-slaughter conditions. Importantly, we validated quantitative PCR techniques for measuring the relative expression of NEAT1 in saliva, blood, and brain samples in pigs. We also reported the use of pig blood (geomean of RPL4 and PPIA), pig saliva (GAPDH and RPL4), and VMH (geomean of RPL4 and GAPDH) specific reference genes for the normalization of the expression of target genes, given that stability of housekeeping genes can be variable amongst species, tissue, and treatment. Lastly, we also investigated the processing of gene

expression data from qPCR that considers the reaction-specific amplification efficiency, as described by Pfaffl (2001). The work on optimisation that was done and the development of a protocol to measure NEAT1 in different biological media will allow the smooth running of future projects using these qPCR techniques.

Our results from the classification of the NIR and MIR spectra indicate that the MIR spectra captured sex-specific characteristics in the prefrontal cortex with successful classification rates between 57 to 65% which, with the sample size, can be considered as a good classification rate. Exposure to the chronic environmental conditions was not well predicted by the MIR spectra (with classification rates below 45% in most tissues). By contrast, we obtained quite good classification rates for the classification of samples according to the presence or absence of the aversive stimulus a few hours before death, with rates higher than 65% in all cases. The prediction level is not as high as those previously reported for the identification of more simple biological outcomes like foodborne pathogens or bloodstains (Pan et al., 2015, Pereira et al., 2017). Our values for the predictability need to be considered in the light of the complexity of nervous tissue in the brain and a number of other factors that could affect the activity and the structural composition of the prefrontal cortex of each animal. Given the relative simplicity of discriminating spectra based on bloodstains or foodborne pathogens, we submit that a 65% success rate for the brain is encouraging for the future of MIR in the assessment of animal welfare. It must also be noted that the signal measured by NIRS and MIRs could have been attenuated because the brain tissue was fixed, and fixatives such as formaldehyde can denature some molecules such as DNA and RNA (Srinivasan et al., 2002).

The NIRS study was opportunistic and shows that information in the infrared spectrum (that are indicative of for e.g. biochemical and chemical signatures) can be used to identify changes in the brain tissue that are associated with different stimuli. The study has also shown that the IR spectrum has the potential to detect structural change in tissue from the prefrontal cortex of the brain after the exposure of an animal to an acute stressor.

Large-scale studies involving more animals, as well as the scanning of fresh tissue collected post-mortem rather than fixed brains, should be considered to further validate the predictive power of MRI. Then, if the positive results obtained in the present study were replicated, we would investigate the use of this technique to a practical application (e.g. to evaluate the effect of stunning methods on animal stress). In addition, the same technology needs

to be validated to develop a practical method to assess welfare (positive or negative) using blood or saliva samples.

An Honours student, a PhD student, and a post-doctoral research fellow were dedicated to this project. To date, the team has written and submitted a manuscript on the IR spectroscopy which is under review. A second manuscript on findings reported above is being drafted.

5. Conclusion

This pilot project was designed to try to find new methodologies or new biomarkers of quality of life in pigs. We subjected pigs to long-term and short-term positive or negative challenges. We have validated methodologies to measure a marker of cellular stress, NEAT1, at the whole organism level and in different biological media. It seems that the experiential response to the different environmental challenges might not have been strong enough to induce differences in the concentration of cortisol in the plasma, or the expression of NEAT1_long in the blood, saliva or the ventromedial region of the hypothalamus of pigs. The decrease in the expression of NEAT1 Total, that encodes paraspeckles, in saliva samples after the acute stress suggests it may be a useful indicator for acute mental stress in pigs. Mid Infrared spectroscopy (MIR), combined with machine learning, identified changes in the biochemical constitution of the prefrontal cortex that were associated with acute stress but not with environmental conditions. This is very promising and should be explored further to apply this technology to biological samples obtained from live animals over time, so that the technology can be validated as a reliable method to assess the quality of life of food-producing animals.

6. Limitations / Risks

For this pilot study, the timing of sampling and the number of samples were chosen based on the best of our knowledge before conducting the experiment. It was challenging to select the timing because we were targeting biomarkers, such as NEAT-1 or NIRS of brain tissue, for which very little information was available in whole animals. The dynamic of changes in NEAT1 in whole animals has never been studied previously. It is possible that the dynamic of the response in the expression of NEAT1 to both long- and short-term challenges used in the present study could have affected the assessment of NEAT1 in the biological media collected.

We took the opportunity to study some animals post-slaughter to gain extra information on productivity and aspects of pork meat quality. While this offers promise, the lack of observed impact of the environmental treatments is likely due to the timing of these treatments in relation to when the slaughter measurements were taken. Extending the environmental treatments until the pigs were sent to slaughter, rather than stopping when the 36 pigs were removed at 16 weeks of age, would have been more beneficial to investigate post-farm gate impacts.

7. Recommendations

As a result of the outcomes in this study the following recommendations are made:

- While our model of environmental challenge seems to be efficient to induce a negative welfare state, it might have been less efficient in inducing a strong positive welfare state that could be contrasted with the neutral state in pigs receiving normal husbandry management. Therefore, the development of a strong positive welfare or experiential state should be considered, possibly by increasing the variety of enrichment or rotating enrichment within the pens.
- The potential of NEAT-1 as a biomarker of welfare still needs to be explored more closely with negative or positive events or in response to the above "very positive experiential state".
- The most promising outcome of the project is the use of NIR to assess quality of life. Large-scale studies involving more animals, as well as the scanning of fresh tissue collected post-mortem rather than fixed brains, should be considered and evaluated before we extend the use of this technique to a practical application (e.g. to evaluate the effect of stunning methods on animal stress).

8. Acknowledgements

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