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Exploration of microRNAs as biomarkers of affective state in the pig

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Abstract

The assessment of animal welfare has advanced to include measures of emotions. A variety of behavioural and physiological markers are commonly used to infer affective states in animals; however, these often lack specificity, or are simply not suitable for production environments. Therefore, there is a need to develop novel measures of animal emotions. Recently, microRNA (miRNA), have emerged as diagnostic markers for a number of neurological conditions and have been implicated in human affective disorders including anxiety and depression. Thus, these molecules have the potential to become markers of the activity associated with emotional processes. In this research, I have explored the efficacy of miRNA as biomarkers for both positive and negative emotional states in pigs. I investigated how miRNA activity changes in brain during different affective states, and explored if these changes could be corroborated with changes in the blood, thus providing a peripheral measure of emotion in pigs.

In chapter 3 I hypothesized that 1) pain in response to tail docking would result in differentially expressed miRNAs in both the blood and brain of piglets, and that this expression would be modulated by local anaesthetic, 2) changes in miRNA expressions would be similar between the blood and the brain, thus providing a reliable proxy marker of brain miRNA expression associated with pain processes. MiR-412 and miR-7a were differentially expressed in the Periaqueductal-grey (PAG) brain region following tail-docking, but the response was not mitigated by analgesic. MiRNA expression was not altered in either the Amygdala (AMY) region of the brain or in blood following treatment, when compared with control animals, suggesting the observed change in expression may not reflect the animal's underlying emotional state but perhaps another biological response to tissue damage, such as inflammation. Furthermore, the sampling time-points selected in this study may have resulted in missed peaks of miRNA up- or down-regulation. Consequently, under these experimental conditions, the effectiveness of circulating miRNA as a biomarker of acute pain in pigs is questionable. Nonetheless, there may be some benefit in their application as biomarkers of inflammation in response to tissue damage.

Chapter 4 tested the validity of measuring circulating cortisol as an indicator for effective delivery of a single intravenous dose of the antidepressant fluoxetine hydrochloride to the pig brain. Previously, pharmacological agents that manipulate the affective state have been used to evaluate novel biomarkers for assessment of emotional states. Antidepressant treatment resulted in increased mean plasma cortisol levels 15-165 minutes following treatment compared with saline controls, suggesting that, similar to other species, plasma cortisol is an indicator of fluoxetine hydrochloride efficacy. However, individual cortisol profiles of pigs treated with the antidepressant were highly variable with either the expected, an unorthodox, or no response. In humans, individual variation in patient responses to anti-depressant treatment has been observed previously. Therefore, I propose that variability in individual cortisol responses following anti-depressant treatment in pigs may be attributed to the following; (1) inherent pharmacological differences, including disparity in receptor numbers, structures or functions, or (2) variations in HPA axis responsiveness to the antidepressant, or (3) a combination of these. For these reasons, I conclude that the inter-individual variations in cortisol responses observed currently preclude the use of cortisol as a reporter for fluoxetine hydrochloride efficacy in pigs.

In chapter 5 I exposed pigs to husbandry practices known to impact emotional states, namely barren housing to induce a negative emotional state, or highly enriched housing conditions to create a relatively more positive state. Differential expression patterns of miRNA in the brain and blood were analysed, and concentrations of the neurotransmitter's serotonin (5-HT), and dopamine (DA), and their retrospective metabolites (5-HIAA and DOPAC), as well as Judgement Bias were used as corroborating measures to infer the emotional status in the pigs. Dopamine was altered in the brain following enriched housing when compared with barren housing. No change was observed in the miRNA, behaviour or brain serotonin. The results of this experiment imply that either A) miRNAs are not likely valid biomarkers of affective states, at least under the type of conditions employed in this study, or B) the experimental paradigm used with housing as a modifier of emotional state was not sufficient to create differential emotional states, and therefore establish the validity of miRNA as biomarkers.

The outcomes of this dissertation suggest that, for the most part, the experimental conditions employed in these trials were insufficient in altering miRNA signatures in brain and blood parameters. The application of miRNA as biomarkers of emotion may not be valid for environmental and/or husbandry conditions where physiological and tissue responses are not elicited, but there may be some benefit in their application as markers of pain or inflammation. However, with regards to the latter, further investigation is required in relation to the types of tissues to be sampled, the time at which sampling occurs and the ability to effectively conclude that the measures taken are specific to the negative emotional experience of pain.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and, where applicable, any partner institution responsible for the joint-award of this degree.

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Date: October 20th, 2021

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Finally, I would like to thank my children, Esme and Eadie. You have been both patient and understanding when I have not always been as present as I would have liked to. I hope this encourages you to reach high in your own endeavours, and know that with hard work and a belief in yourself, anything is possible.

~I dedicate this thesis to my family, but most of all my children~

List of publications and presentations

Published journal papers:

Marsh L, Terry R, Whittaker A, Hiendleder S, Ralph C. (2020) Pronounced Inter-Individual Variation in Plasma Cortisol Response to Fluoxetine Hydrochloride in the Pig. *Animals*. 10(3):504.

Marsh L, Hutchinson M, McLaughlan C, Musolino S, Hebart M, Terry R, Verma P, Hiendleder S and Whittaker A. (2021) Evaluation of miRNA as biomarkers of emotional valence in pig. *Animals*. 11(7): 2054.

Chapters not yet under review:

Marsh L, Whittaker A, Verma P, Royle J, Nattrass G, Ralph C and Hiendleder S (2021) MicroRNA as a biomarker of pain processing in piglets following tail-docking. To be submitted to *Front. Vet. Sci*.

Supporting journal papers:

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Conference presentations:

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Thesis Structure

This thesis is presented as a combination of the 'Conventional' and 'Thesis by publication' formats. The experimental chapters are either published journal articles or manuscripts currently under review. The manuscripts are formatted according to the journal specifications. Consequently, the format of references differs throughout this dissertation. A consolidated list of references is provided at the end of the main body of the thesis.

General Introduction

The ability to improve the welfare of animals is critically dependent on being able to assess their welfare accurately. In other words, measures of welfare must be robust and repeatable in order to provide the basis from which to develop further the means to improve welfare. The challenge to develop meaningful assessments of the welfare of animals used in agriculture is exacerbated by the need to not simply identify when welfare is deleterious, but be able to know when the welfare of animals is good (Boissy et al., 2007b). This is because there is now a determined drive by the livestock industries to continuously improve the welfare of animals, with the ultimate objective of providing as many opportunities for the achievement of positive welfare as possible. Not surprisingly, this has resulted in substantial research efforts to develop tools to assess the welfare of livestock (Boissy et al., 2007b; Mellor, 2012; Tilbrook and Ralph, 2018). While considerable progress has been made, limitations remain in terms of the practical application of these tools, and in their ability to provide a holistic assessment of welfare (Barrell, 2019). With respect to the latter, the most meaningful measures of welfare encompass a vast range of physiological and psychological functions of the animal. These functions include those classically associated with stress systems, and include biological responses (i.e., behavioural, neuroendocrine and immunological), likely attributed to assist the animal to cope with challenges or threats to homeostasis. It is common to refer to these measures collectively as biological markers of welfare, or biomarkers (Martinez-Miro et al., 2016).

Research to develop novel measures of animal welfare has advanced to include measures of animal emotions, instigated by a general recognition that animals are sentient, meaning that they have sufficient functional brain sophistication to transduce sensory and nerve impulses into experienced sensations (Hemsworth et al., 2014). Furthermore, for an animal to be sentient it must be conscious. Sensory and neural inputs are processed and interpreted by the animal, are suggested to be species-specific and dependent on the animals individual nature and past experiences (Hemsworth et al., 2015).

Although animal sentience has, for the most part, been solely referred to in relation to mammalian vertebrate species (Proctor et al., 2013), discrepancies in the literature regarding what other animals species are capable of being sentient remain. For example, evidence suggesting some invertebrate may experience at least some level of sentience, likely attributed to similar neural anatomy, functionality and behaviour observed in humans (Boyle, 2009). For the purpose of this thesis, when discussing animals and sentience, I refer to animals as both human and non-human vertebrate species, including livestock. The emotional state of an animal is referred to as its affective state (Paul et al., 2005; Mendl et al., 2009), and includes the 'feeling' of emotions (Nettle and Bateson, 2012a). Affective states are regarded as comprising subjective, behavioural, physiological and cognitive components that can be characterized along two-dimensional levels; 1) arousal (i.e. high vs low), and 2) valence or direction of emotion (i.e. positive vs negative) (Whittaker and Marsh, 2019). Although there is no way of knowing if animals experience emotions in the same way as humans do, their similar brain sophistication and neuroanatomy (Davidson et al., 2002; Boissy et al., 2007b) imply that animals are likely to experience negative emotional states such as thirst, hunger, nausea, pain and fear, and positive emotional experiences such as satiety, contentment, companionship, curiosity and playfulness (Hemsworth, 2018). Currently, the assessment of animal emotions has relied on physiological or behavioural indices to infer the emotional state of the animals. Physiological measures, including those classically associated with stress systems (i.e., activation of the sympatho-adrenal medullary system and the hypothalamo–pituitary adrenal axis associated with catecholamine and glucocorticoid synthesis respectively), can be effective in determining the emotional arousal of animals (Arroyo and Bassols, 2017; Neethirajan et al., 2021); however, these are often unable to distinguish between the valence of emotions. Conversely, behavioural measures, including cognitive bias tests, have successfully identified emotional valence in a range of species (Mendl et al., 2009; Baciadonna and McElligott, 2015; Roelofs et al., 2016), but these measures can be time consuming and thus not suitable for production environments. Therefore, the need to develop novel biomarkers for assessment of animal affect are required. These measures must be robust, not only

having the ability to differentiate valence of affect, but also including markers that take into account the biological functioning of the animal.

There are various biomarkers that can be measured peripherally and have the potential to quantify biological functioning and affective states. Recently, microRNA (miRNA) have emerged as diagnostic markers for a number of neurological conditions, including those associated with emotional processes (Malan-Müller et al., 2013; Wingo et al., 2017). These small (approximately 22-24 nucleotides long), single stranded, non-protein coding RNA molecules are involved in the regulation of genes post-transcriptually. miRNAs are Synthesised throughout the body, including the central nervous system (CNS), where they contribute to the complex functioning of the brain, including neurodevelopment, neurogenesis and neurological disease (De Strooper, 2009; Bredy et al., 2011; Codocedo and Inestrosa, 2016). In human psychological research, miRNA have been implicated in a number of affective disorders including, but not limited to, anxiety (Haramati et al., 2011; Fonken et al., 2016), major depressive disorder (MDD) (Lopez et al., 2014; Wang et al., 2017; Chen et al., 2021), schizophrenia (Lai et al., 2011; Du et al., 2019b; Santarelli et al., 2019), post-traumatic stress disorder (PTSD) (Balakathiresan et al., 2014; Snijders et al., 2017; Linnstaedt et al., 2020), bipolar-mania disorder (Rong et al., 2011; Camkurt et al., 2020), and obsessive-compulsive disorder (OCD) (Yue et al., 2020; Lin et al., 2021). In animal welfare research, miRNA have been shown to be differentially expressed during stress in various livestock (Miretti et al., 2020). Therefore, they have the potential to become markers of the activities associated with emotional processes. In this body of research, the efficacy of miRNA as biomarkers for both positive and negative emotional states in the pig was explored. The outcomes of this research are to provide industry with a suite of miRNA biomarkers that can accurately and objectively assess the emotional states of pigs, where this assessment is essential for continued improvement to animal welfare in farming systems.

Hypothesis and aims

Here I hypothesise that in pigs, miRNA expression would be differentially expressed in the brain and blood during both positive and negative affective states. The principal aim of this work was to gain a greater understanding of how miRNA activity changes in the brain during different affective states, and determine if changes in brain miRNA could then be corroborated with changes in the blood, thus providing a peripheral measure of emotion in pigs that could be employed on farms. In order to investigate the principal aim, subsequent individual aims were developed, as follows:

Aim 1: To investigate miRNA expression in the brain and blood of piglets following tail-docking with and without anaesthetic (**Chapter 3**).

Aim 2: To determine if miRNA expression in the brain is similar to miRNA expression in the blood of piglets exposed to pain from tail-docking (**Chapter 3**).

Aim 3: To explore the efficacy of measuring circulating cortisol as an indicator for effective delivery of a single intravenous dose of the SSRI fluoxetine hydrochloride to the pig brain (**Chapter 4**).

Aim 4: To investigate miRNA expression in the brain and blood of pigs exposed to enriched or barren housing conditions (**Chapter 5**).

Aim 5: To determine if miRNA expression in the brain was similar to miRNA expression in the blood of pigs exposed to enriched or barren housing (**Chapter 5**)

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Chapter 1: Review of the Literature

Introduction

The study of animal welfare has evolved from the integration of multidisciplinary and multi-faceted fields of research (Millman et al., 2004). The origins of farm animal welfare science probably dates to the 1960s, when the Brambell Committee, established by the British Government, defined 'welfare' as a wide term that embraces not only the physical, but also the mental wellbeing of the animal (Brambell et al., 1965). The concept introduced the notion that when attempting to evaluate the welfare state of an animal, the scientific evidence concerning the feelings of animals must be taken into account, and these must be derived from the structure, function and behaviour of the animal (Hemsworth et al., 2014). Since this time, the field of animal welfare science has continued to expand and diversify to meet challenges and opportunities within diverse animal industries (Blokhuis et al., 2013). As a consequence, research incorporating measures of animal emotion (or affective state), have gained momentum. In alignment with this change in research direction, increased importance has been placed on the assessment and promotion of positive affective states in animals, which are now considered fundamental components of animal welfare thinking (Mellor, 2012). Despite this research effort, the challenge remains in developing tools that can accurately and objectively assess the emotional states of animals.

To date, the assessment of animal affect has tended to focus on markers derived from the biological functioning of the animal, where biological functioning refers to the biological activity arising when an animal attempts to cope with its environment, and involves physiological systems classically associated with stress, incorporating a range of neurological, immunological, endocrinological and behavioural responses (Hemsworth et al., 2014; Tilbrook and Ralph, 2018). For example, biological products derived from activation of the sympatho-adrenal medullary (SAM), and hypothalomo–pituitary-adrenal (HPA), systems are commonly applied, including measurement of catecholamines (i.e., dopamine, epinephrine, nor-epinephrine (Kiseleva, 1992; Korte et al., 1997; Loudon et al., 2019)) and glucocorticoids (i.e., cortisol or corticosterone in pigs (Barnett et al., 1996; Pol et al., 2002; Reimert et al., 2013; Loudon et al., 2019; Wiechers et al., 2021)), respectively. Measures derived from autonomic functioning are also used and

include alterations in cardiac function (i.e., heart rate, heart-rate variability and respiration rate (Mohr et al., 2002; von Borell et al., 2007; Dos Reis et al., 2014; Crestani, 2016)), immunological activation (i.e., measurement of lymphocyte counts, IgG concentration, pro-and anti-inflammatory cytokines (Tuchscherer et al., 1998)), circulating neuropeptides (i.e., endogenous endorphins or oxytocin (Loijens et al., 2002; Odendaal and Meintjes, 2003; Marcet Rius et al., 2018), and changes in body temperature (i.e., infrared thermography techniques (Cannas et al., 2018; Telkanranta et al., 2018; Heintz et al., 2019)), among others. Behavioural indices used to infer emotional state are common, and can include whole-body or discrete body part behaviours (i.e., startle response, locomotion, approach and freezing, social, play, grimace, facial expressions, and tail or ear movements (Désiré et al., 2002; Boissy et al., 2011; Held and Špinka, 2011; Proctor and Carder, 2014; Paul et al., 2018; Marcet-Rius et al., 2019)), as well as cognitive behavioural tests (i.e., judgement bias tests (Mendl et al., 2009; Baciadonna and McElligott, 2015; Roelofs et al., 2016; Roelofs et al., 2017)). It is common to refer to these biological markers collectively as biomarkers.

Although these biomarkers are commonly used to infer the affective state of animals used in livestock, these often lack specificity or are simply not suitable for a production environment. For example, physiological measures, including those classically associated with stress systems, can be effective in determining the emotional arousal of animals (Arroyo and Bassols, 2017; Neethirajan et al., 2021); however, they are often unable to distinguish between the valence of emotions. Conversely, behavioural measures, including cognitive bias tests, have successfully identified emotional valence in a range of species (Mendl et al., 2009; Baciadonna and McElligott, 2015; Roelofs et al., 2016), but these can be time consuming and thus not suited for production environments. Therefore, there is a need to develop novel biomarkers of emotional states in animals. These biomarkers must be robust, able to be rapidly assessed on farm, have the ability to differentiate between positive and negative affect, and require validation in order to complement or replace existing measures.

Recently, microRNA (miRNA) have emerged as diagnostic markers for a number of pathological conditions (de Planell-Saguer and Rodicio, 2011). Some of these conditions include various cancer types (Keshavarzi et al., 2017; Peng et al., 2017; Qiu et al., 2018; Zhai et al., 2018), cardiovascular disease (Wojciechowska et al., 2017; Eyileten et al., 2018; Kaur et al., 2020), rheumatic diseases (Huang et al., 2019; Nziza et al., 2019; Ren et al., 2021), as well as neurological conditions (Quinlan et al., 2017; Bahlakeh et al., 2021). In human psychological research, miRNA have been shown to be involved in the development of neurological conditions, including those associated with emotional processes (Malan-Müller et al., 2013; Wingo et al., 2017). These single-stranded molecules are involved in the regulation of genes post-transcriptually. Dysregulation in miRNA functioning has implicated them in a number of affective disorders including, but not limited to, anxiety (Haramati et al., 2011; Fonken et al., 2016), major depressive disorder (MDD) (Lopez et al., 2014; Wang et al., 2017; Chen et al., 2021), schizophrenia (Lai et al., 2011; Du et al., 2019b; Santarelli et al., 2019), post-traumatic stress disorder (PTSD) (Balakathiresan et al., 2014; Snijders et al., 2017; Linnstaedt et al., 2020), bipolar-mania disorder (Rong et al., 2011; Camkurt et al., 2020), and obsessive-compulsive disorder (OCD) (Yue et al., 2020; Lin et al., 2021). Therefore, they have the potential to become markers of the activities associated with emotional processes.

In this review I explore the literature relating to miRNA as biomarkers of affective states in animals. Firstly, I discuss the neuroanatomy and neurophysiology of the brain during emotional processing. I briefly describe the current measures of emotions in animals, their limitations, as well as future directions required for the development of novel markers of emotion. I then provide an overview of miRNA, and the current literature pertaining to their potential application as biomarkers of emotional states.

Research Methods

A review of the literature was conducted electronically and derived from databases including Web of Science, PubMed and Google Scholar. Literature with a year of publication between 1965 and 2021 was included. Databases were searched using terms related to emotional states in animals and included the

following terms: miRNA, microRNA, animals, emotion, affect, neurology, physiology, welfare and assessment. Reference lists of sourced articles were also checked for articles of relevance. Only articles with the full text available in English language publications, or those with translations, were included. Following the database searches, articles were evaluated on the basis of the title and abstract content for relevance. The findings are reported in a narrative format.

Affective/emotional state

Over the last decade, the study of animal emotions has gained momentum within the fields of human psychology, neuroscience and animal welfare. Emotions are complex and can be defined as intense but short-lived physiological and psychological responses that are associated with specific bodily changes (Boissy et al., 2007b; Whittaker and Marsh, 2019). The term 'affect' has been used to describe the subjective *feeling* of emotions (Boissy et al., 2007b). The true nature of animals' emotional experiences remains controversial (Boissy et al., 2007b), along with whether or not their level of consciousness permits the 'feeling' of an emotion in the same way that humans might feel emotions (Burgdorf and Panksepp, 2006). In animal welfare assessment, scientists attempt to evaluate affective states in animals based on cognitive, motivational and physiological changes, triggered by environmental situations (Paul et al., 2005; Mendl et al., 2009; Nettle and Bateson, 2012b). Animal welfare is, therefore, an umbrella term referring to an ongoing state made up of the cumulative experiences of an individual (Mellor and Beausoleil, 2015). A welfare state is ascribed based on the nature of an animal's experienced affects, and their relative weighting over time (Mason and Mendl, 2013). Good welfare is defined when animals, on balance, experience positive states such as reward and contentment, and poor welfare when the contrary applies (Ahloy-Dallaire et al., 2018).

Affective states likely evolved from basic mechanisms that gave animals the ability to avoid harm or punishment and seek valuable resources relating to survival and reproductive success (Cardinal et al., 2002; Paul et al., 2005). Affective experiences vary along three main axes: 1) valence corresponding to bodily activation or excitation (positive versus negative) 2) arousal or emotional intensity (high versus

low), and motivational intensity to approach (high versus low) (Paul et al., 2005; Duncan and Barrett, 2007). For example, emotions such as pain and anxiety would exhibit negative valence, high arousal and high avoidance; whereas the emotion of reward would exhibit positive valence, high arousal and high approach (Mendl et al., 2009; Mendl et al., 2010; Nettle and Bateson, 2012b). Affective states may also vary in duration ranging from short-term states such as anticipatory joy, to more persistent states like grief. These longer-term states make up 'mood', which has an adaptive function in guiding an animal's allocation of behavioural effort based on its environment and current physical condition (Nettle and Bateson, 2012b). Emotions are likely elicited by appraisals, where emotions are produced based on an individual's evaluations of stimuli, their context and their personal significance (Moors et al., 2013).

The field of affective neuroscience is dedicated to understanding the processes within the brain that control and regulate emotional processes. Previously, animal models have been used in an attempt to understand emotions in human psychology. LeDoux (LeDoux et al., 1988; LeDoux, 1998), suggested that the most reliable way to assess affective states objectively is to look directly at the emotional processes occurring within the brain and, through advancements in biomedical research and neuroimaging technologies, the brain regions, cell types and physiological systems that control cognition, emotion, and behaviour have been identified (Davis and Whalen, 2001; Berridge and Kringelbach, 2013). Although there is no way of truly knowing if non-human animals experience emotions exactly as humans do, the similar behavioural patterns, neural anatomy and brain chemistry seen in a number of animal species could imply that they are likely to feel as humans do (Davidson et al., 2002; Boissy et al., 2007b). For example, between human and pig (*Sus scrofa*), similarities in brain growth rates, size and development, as well as neural anatomy including gyral patterns and the distribution of grey/white matter have been observed (Lind et al., 2007) It is therefore plausible that our understanding of emotional processing in humans could be applied to assess the emotional state of animals.

The affective brain

The brain is organised into discrete regions and neuronal systems that regulate emotional processes (Pawlina-Tyszko et al., 2020). Emotions are largely regulated by the limbic system, which includes several functionally and anatomically interconnected nuclei and cortical structures located within the telencephalon and diencephalon. These neuronal structures are responsible for the regulation of both positive and negative emotional processes, which, in response to emotional evoking stimuli, regulate autonomic and endocrine activation, memory formation, arousal, motivation and reinforcing behaviour (Boissy et al., 2007b).

Neurophysiology of emotions

There are two central neural pathways or networks that are involved in the regulation of emotions; 1) the mesolimbic dopaminergic pathway, or reward pathway, and 2) the serotonergic pathway. The mesolimbic pathway comprises the ventral tegmental area (VTA), ventral striatum, which includes both nucleus accumbens (NAc), and olfactory tubercle, amygdala, and prefrontal cortex (PFC) (Boissy et al., 2007b; Berridge and Kringelbach, 2013) (Figure 1). This pathway has been implicated in the pathophysiology of psychological disorders such as depression, anxiety and substance abuse disorders in humans (Greenwood et al., 2011). Drug induced or naturally rewarding stimuli alter neuronal plasticity within the reward pathway and are associated with the projection of catecholaminergic neurons, including dopamine (DA), as well as increased neurotransmission of opioidergic components within the reward circuity. These neuronal networks are highly organized and help to innovate the ventral and dorsal divisions of the striatum to regulate positive emotional experiences such as reward. These networks are also critical to motivated and reward seeking behaviour (Boissy et al., 2007b; Berridge and Kringelbach, 2013). Dopaminergic projection from the PFC results in the release of dopamine in the NAc and leads to motivated behaviours indicative of reward expectation, such as those of consumptive, exercise or sexual natures (Navratilova et al., 2016).

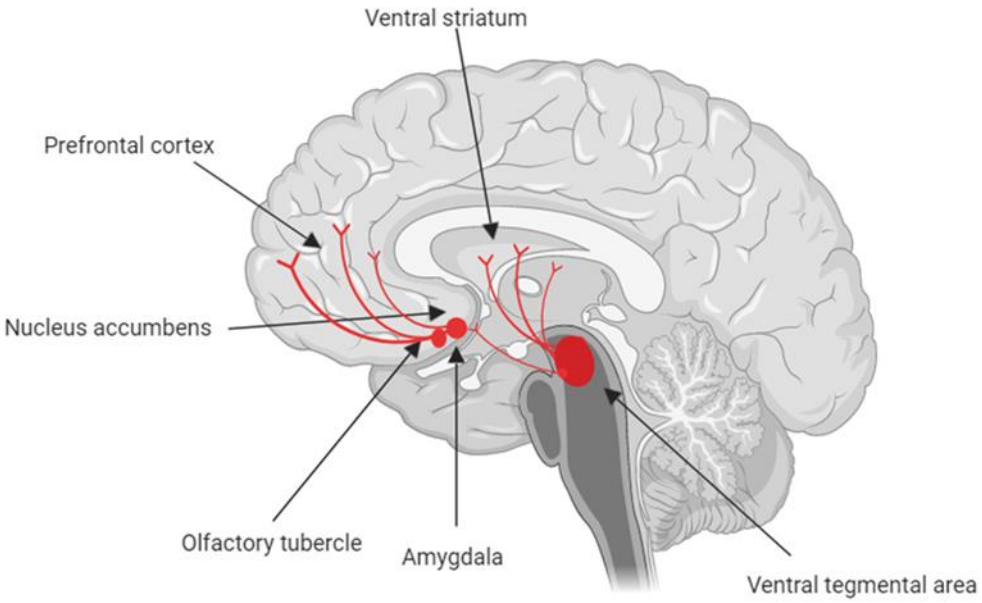

Figure 1: Indicates dopaminergic projections of the mesolimbic pathway originating from the Ventral tegmental area (VTA). Key structures in this pathway include the nucleus accumbens (NAc), olfactory tubercle, amygdala and prefrontal cortex (PFC).

Emotions are also associated with activity of the serotonergic pathway that originates from the raphe nuclei in the brain stem (LeDoux, 1998; Rolls, 2000; Davis and Whalen, 2001). The anatomical organization of the serotonergic pathway include the amygdala, hippocampus, NAc (ventral striatum) and ventral diencephalon (ventral tegmentum, basal forebrain, thalamus and hypothalamus) (Gray, 1982; Panksepp, 1998; Bechara et al., 2000; Cardinal et al., 2002; Phillips et al., 2003; Makris et al., 2008) (Figure 2). Serotonin (5-hydroxytrytophan or 5-HT) functions as a neurotransmitter and is highly expressed within the mammalian brain. 5-HT neurons are primarily observed in raphe nuclei, and the thalamus and basal ganglion in vertebrate species (Niblock et al., 2004). Tryptophan (TRP) is the precursor of peripheral and central 5-HT, where 5-HT is produced from TRP via the rate-limiting enzyme tryptophan hydroxylase (TPH) (Faye et al., 2018). Dysregulation in serotonergic neurotransmission has been implicated in a number of human mood disorders, including generalised anxiety disorder (GAD) (Ren et al., 2018; Szeitz and Bandiera, 2018), major depressive disorder (MDD) (Jacobsen et al., 2012; Dell'Osso et al., 2016; Ren et al., 2018; Szeitz and Bandiera, 2018), and post-traumatic stress disorder

(PTSD) among others. The regions of the brain that regulate negative emotions like fear, pain and anxiety have also been identified. These include the hypothalamus, thalamus, ventral pallidum, amygdala, anterior cingulate cortex, peri-aqueductal grey (PAG), the hippocampus and pre-frontal cortex (Gray, 1982; LeDoux, 1998; Panksepp, 1998; Rolls, 1999; Bechara et al., 2000; Davis and Whalen, 2001; Cardinal et al., 2002; Phillips et al., 2003).

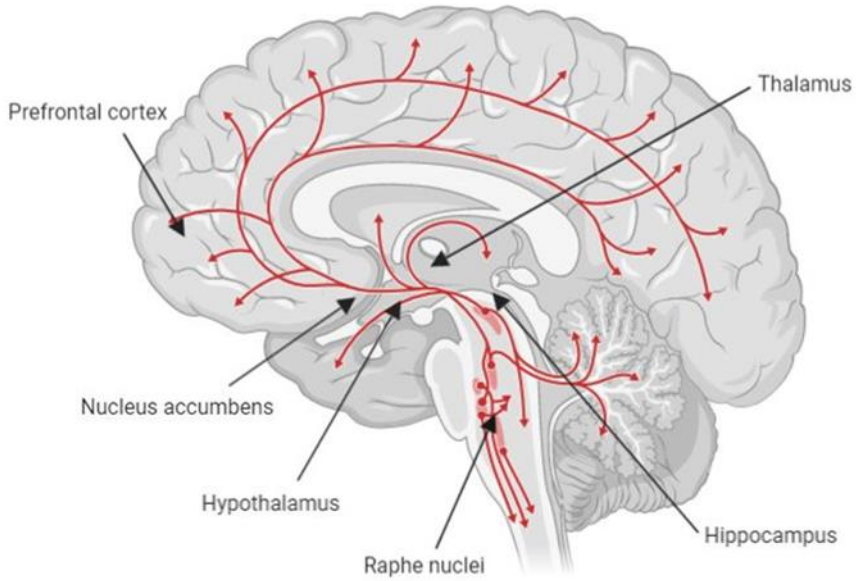

Figure 2: Indicates serotonergic projections within the reward pathway with key structures including the Raphe nucleus, Hippocampus, Hypothalamus, Thalamus, Nucleus accumbens and prefrontal cortex.

Pain

It is generally agreed that the assessment of animal welfare should consider quantification of pain. The perception and regulation of pain is complex, involving both central and peripheral nervous systems (Tilbrook and Ralph, 2018). The International Association for the Study of Pain (IASP) has defined pain as: 'An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage' (Raja *et al.,* 2020) Based on this definition, pain incorporates a conscious, subjective experience or perception of a feeling or sensation (Patel, 2010), that differs from

basic nociception (the neural process involving the transduction and transmission of a noxious stimulus to the brain via a pain pathway (Figure 3)). Thus, pain is a complex interplay between signalling systems, modulation from higher brain centres, as well as the unique perception of the individual (Steeds, 2009). Following noxious stimuli (resulting from mechanical, heat, cold or chemicals (Sneddon, 2018)), the location, intensity, and temporal pattern is transduced into a recognizable signal through unmyelinated nociceptors at the terminal end of sensory neurons (Bourne et al., 2014). The sensory afferent neurons then send this signal via action potentials and synapses with nerve cell bodies within the dorsal root ganglion (DRG) of the spinal cord (Lee and Neumeister, 2020). This signal is then transmitted to the brain via spinothalamic and spinoparabrachial tracts, where it synapses with various neurons within brain regions including the parabrachial medulla oblongata, periaqueductal gray (PAG), hypothalamus and thalamus, amygdala and limbic system, insular cingulate and somatosensory cortex (Sneddon, 2018; Lee and Neumeister, 2020). Signals running through the medial spinothalamic tract project to the medial thalamus, resulting in the autonomic and unpleasant emotional perception of pain (Yam et al., 2018; Lee and Neumeister, 2020). Descending pain pathways, including activation of midbrain and medullary areas, can reduce nociception by modulating its effects. For example, the PAG receives afferent signals from cortical and subcortical areas and can exert profound analgesic effects as well as control of descending pain pathways (Sneddon, 2018).

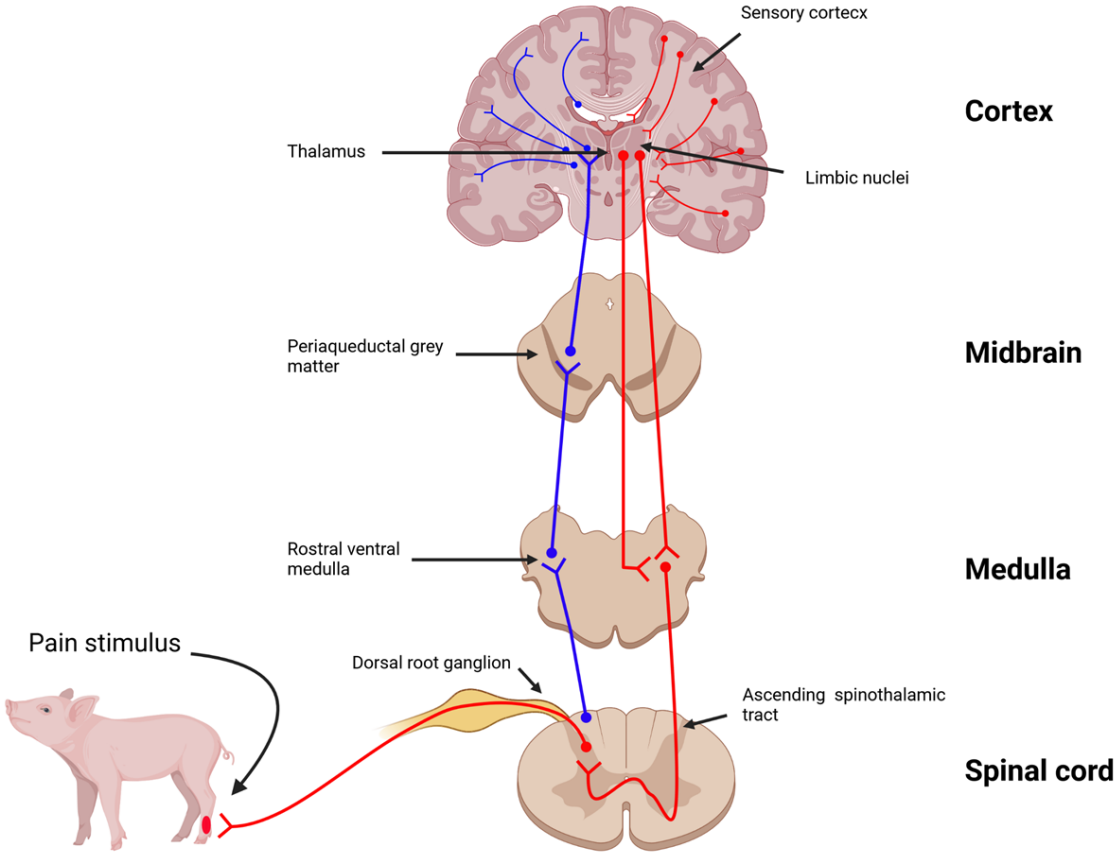

Figure 3: Indicates transduction and transmission of a noxious stimulus to the brain via a pain pathway. Sensory afferent neurons synapse with nerve cell bodies within the dorsal root ganglion (DRG), of the spinal cord and send pain signals to the brain via ascending spinothalamic and spinoparabrachial tracts (red arrows). The pain signal connects various regions within the limbic system and sensory cortex. Activation of midbrain and medullary areas can reduce nociception by modulating its effects via the descending pain pathway (blue arrows).

Despite our knowledge of the neuronal systems involved in the regulation of both positive and negative emotions, the challenge lies in developing ways in which we can measure these emotional processes in animals, on farm, in the home pen of the animal, without compromising the flow of production. Although the use of various imaging technologies (e.g., functional magnetic resonance imaging and electroencephalograms) can provide objective information on specific brain activity associated with emotional processes, these are not suitable for a production environment. Furthermore, it is difficult to assess these mechanisms in farm animals without extraction of brain tissue and complex laboratory analyses (Mendl et al., 2009). Consequently, the majority of the literature in relation to the assessment

of animal emotion has focussed on various physiological and behavioural indices. Below, I briefly discuss some of these methods and highlight limitations pertaining to their use.

Measuring emotions in animals

One of the most difficult aspects of the assessment of animal welfare is the ability to determine the affective state in non-verbal beings. In humans, verbal reports of emotion are considered the 'gold standard' indicator of affect (Paul et al., 2005); however, given animals are not capable of communication through verbal speech, a reliance on other measures that indirectly assess the affective experience of animals is needed. The majority of these methods tend to focus on physiological, behavioural and cognitive components. These measures are often derived from human literature and, when applied to animals, enable us to draw comparisons and identify similarities in their affective experience (Kremer et al., 2020). As stated earlier, the majority of biomarkers used to assess the emotional states of animals have primarily focussed on behavioural and physiological indices, with particular emphasis placed on their use for the assessment of negative emotional states.

Behavioural measures of affect

Some of the most significant advancements in the assessment of animal welfare have been made through animal behavioural research. Emotions comprise subjective, cognitive and behavioural components, where the behavioural component refers to how the subjective experience translates into a tangible action (Neethirajan et al., 2021). Spontaneous or 'whole-body' behavioural measures have been used to infer affective states in a range of livestock, including sheep (Boissy et al., 2007b; Marino and Merskin, 2019), goats (Bellegarde et al., 2017), cattle (Lambert and Carder, 2017; Battini et al., 2019; Green et al., 2021), chickens (Herborn et al., 2020) and pigs (Reimert et al., 2013; Paoli et al., 2016; Camerlink et al., 2018; Rius et al., 2018b; Czycholl et al., 2020). Some of these behaviours include approach and avoidance (Paul et al., 2018), freezing and locomotion (Boissy et al., 2001; Paul et al., 2005), play (Boissy et al., 2007b; Held and Špinka, 2011; Ahloy-Dallaire et al., 2018), anticipatory (Spruijt et al., 2001), social (Boissy et al., 2007b), aggression and defence (Kremer et al., 2020), discrete body postures (i.e., head, ear and tail movements) (Reefmann et al., 2009; de Oliveira and Keeling, 2018;

Tamioso et al., 2018; Battini et al., 2019; Camerlink and Ursinus, 2020), vocalisations (Briefer et al., 2015; Briefer, 2018; Green et al., 2021; Laurijs et al., 2021), facial expressions (Hintze et al., 2016; Descovich et al., 2017; Camerlink et al., 2018) and grimace (Dalla Costa et al., 2014; Di Giminiani et al., 2016; McLennan et al., 2016). Some behavioural tests including open field, novel object, elevated plus maze, and light/dark have been used to investigate negative emotional states (Harding et al., 2004), and have been applied in a range of species, including rodents (Cardoso et al., 2009), sheep (Reefmann et al., 2009), laying hens (Nordquist et al., 2011), and pigs (Murphy et al., 2014). These behavioural tests measure the unconditioned responses (largely measures of locomotion and exploration) of an animal to an unfamiliar situation/environment that contains elements that the animal perceives as adverse/threatening (such as open spaces/or varying light intensities) (Roelofs et al., 2016). A major limitation of these tests is that any interpretation of emotions is based on the behavioural reactivity or arousal of the animal, and not necessarily on the valence or emotional direction (i.e., positive or negative). For example, Burman et al. (2008), highlighted the difficulty in interpreting spontaneous behaviour in rats during open field tests, suggesting the activity of an animal could be portrayed as an indicator of curiosity-motivated exploration as much as fear-motivated escape. Another impediment to the interpretation of these types of tests is that there can be bias towards the study of negative emotions and less on positive emotions (Paul et al., 2005; Boissy et al., 2007b; Burman et al., 2008). Furthermore, difficulty may arise in the ability to decipher what behaviours are in response to emotions compared with those that are not (Kremer et al., 2020). A corollary to this is specific behaviours (i.e., locomotive or body posture) can be displayed across various affective contexts, thus making it difficult to determine the valence of affect (Paul et al., 2005; Kremer et al., 2020). Additionally, behaviours elicited from high arousal situations may be more easily observed compared with behaviours displayed during low arousal situations (Kremer et al., 2020).

Cognitive measures of affect

Cognition can be defined as "the mental actions or processes that enable the acquisition, processing, storage, and use of information" (Shettleworth, 2009; Franks, 2018), and the study of animal cognition is

becoming an increasingly important pillar of research in many disciplines, including psychology, behavioural ecology, and animal welfare (Franks, 2018). Emotions comprise cognitive elements, where an individual's cognitive processes are influenced during various emotional states (Paul et al., 2005; Boissy et al., 2007a; Boissy and Lee, 2014). Specific emotions produce particular patterns in information processing (Désiré et al., 2002), and these patterns of cognition can be measured empirically (Franks, 2018), thus providing the ability to infer back to the emotional states of animals. Cognitive bias is a term used to describe how an individual processes information in a particular way as a result of the underlying emotional state, and includes judgement, attention and memory biases (Kremer et al., 2020). One such method to investigate how emotions influence cognition in animals is the judgement bias test (JBT), which allows us to assess variations in emotional valence (Hintze et al., 2018).

Judgement bias tests

The use of the JBT was first developed in rats (Harding et al., 2004), and since has been applied in a number of livestock species including, but not limited to, chickens (Iyasere et al., 2017; Zidar et al., 2018; Ross et al., 2019; Pichová et al., 2021), sheep (Doyle et al., 2010a; Doyle et al., 2011; Destrez et al., 2012; Destrez et al., 2013; Monk et al., 2019), cattle (Neave et al., 2013; Daros et al., 2014; Bethell, 2021), and pigs (Douglas et al., 2012; Murphy et al., 2013; Scollo et al., 2014; Asher et al., 2016; Düpjan et al., 2017; Roelofs et al., 2017). These tests use the behavioural response of an animal as an indicator of its underlying affective state in response to an unknown or ambiguous stimulus (Roelofs et al., 2016). Animals first learn to discriminate between a positive stimulus, such as a high value reward, and an aversive stimulus, such as no reward or punishment (Bateson et al., 2015). Once animals have learnt to discriminate between positive and aversive stimuli, they are then tested on an ambiguous stimulus, intermediate between the two learned stimuli. These tests are based on the assumption that if, under ambiguity, the animal behaves in a manner normally associated with a positive reward, that animal has an enhanced expectation of a positive outcome and thus the reaction implies a positive emotional state (Whittaker and Barker, 2020). Conversely, if the animal displays behaviours consistent with an aversive outcome, that animal has reduced anticipation of a positive outcome, and this implies the animal is in a

negative affective state (Whittaker and Barker, 2020). Despite a similar basic concept, various study designs, within and across species, have been developed and include differences in cue modalities (i.e., visual, spatial, auditory, tactile and olfactory cues), numbers of ambiguous cues presented (e.g., graded vs single intermediate), numbers of trials where the animals are exposed to an ambiguous stimulus (e.g., repeated vs singular), reinforcer types (e.g., rewarder vs partial-reward vs no-reward), as well as differences in outcome measures (e.g., latency-based vs choice-based) (Burman et al., 2009; Mendl et al., 2009; Baciadonna and McElligott, 2015; Roelofs et al., 2016; Hintze et al., 2018).

The benefit of using cognitive judgement tests as an indicator of emotional states in animals is the ability to distinguish between the valence of affect (e.g., negative emotions such as anxiety or positive emotions such as reward), and potentially between states of the same valence (e.g., anxiety/ depression) (Paul et al., 2005; Burman et al., 2008; Burman et al., 2009). While the JBT is considered to have good validity (Lagisz et al., 2020), such tests are less suited to production environments due to the time it takes to train animals to perform the test (Roelofs et al., 2016). Furthermore, a number of criticisms have been identified pertaining to the test design, including loss of ambiguity towards the ambiguous stimulus (Doyle et al., 2010b; Roelofs et al., 2016), the 'reward' and 'punishment' are not perceived by the animal as equal in strength and thus may influence approach behaviour during the test (Mendl et al., 2009), and the motivation level across successive sessions may vary among and between individuals (Henry et al., 2017; Kremer et al., 2020).

As highlighted, there is a growing body of literature on behavioural methods to assess the affective state in animals. Measures used may include the quantification of spontaneous and whole-body behaviours, or by ascertaining responses to provoked tests such as cognitive biasing, for example the judgment bias test (Paul et al., 2005; Kremer et al., 2020). However, given the difficulties in teasing apart valence and arousal of emotional responses using spontaneous behaviour alone, as well as the un-befitting application of cognitive bias testing in a farm setting, many studies have relied on the assessment of physiological indices to infer emotional states in animals.

Physiological measures of affect

The autonomic nervous system

The autonomic nervous system (ANS) has been described as the body's general-purpose physiological system responsible for the modification of peripheral functions including, but not limited to, digestion, heart rate (HR), and respiration rate (RR), urination, and pupillary responses among others. (Mauss and Robinson, 2009). It comprises sympathetic and parasympathetic branches working in unison to maintain homeostasis. Increased sympathetic activity activates the body's "fight or flight" response following threat or perceived danger, while the parasympathetic branch controls the "rest and digest" response (Alshak and M., 2021).

Originally it was believed that differing emotional states involved specific patterns of ANS activation; however recent literature questions the validity of using specific ANS responses to predict discrete emotional experiences, especially when they are assessed in isolation. For example, heart rate has been shown to increase in response to fearful stimuli but also in situations of pleasure (Ralph and Tilbrook, 2016). Likewise, due to the general-purpose nature of ANS functioning, the challenge remains in differentiating between markers of ANS activity related to emotional processes and those that reflect other ANS functions, including digestion, homeostasis, attention and effort (Cacioppo et al., 2007; Mauss and Robinson, 2009). It is likely, though, that using an approach that incorporates multiple indices of ANS activity will provide greater capacity to assess aspects of animal welfare including animal emotions.

A number of physiological biomarkers are commonly used in the assessment of animal welfare and include those classically associated with stress systems. There are many definitions of stress, most of which have been focused around challenges to homeostasis. One definition developed by Tilbrook and Clarke (2006), defined stress as "a complex physiological state that embodies a range of integrative and behavioural processes when there is a real or perceived threat to homeostasis". A stressor has been defined as a chemical or biological agent, environmental condition, external stimulus or an event that causes stress to an animal and challenges an animal's ability to adapt to its environment (Gregory, 2008;

Hurcombe, 2011). However, despite its negative connotation, it is important to note that the adaptive purpose of the physiological stress response is to promote survival (Dhabhar, 2014). Although chronic stress has shown to be harmful to the body (Irwin et al., 1990; Glaser and Kiecolt-Glaser, 2005; Chrousos and Kino, 2007), short-term stress can be protective as it prepares the organism to deal with challenges (Dhabhar, 2014). Thus, when animals experience stressful stimuli, their body attempts to return to homeostasis by creating alterations in the biological activity of those systems that allow an animal to cope with stressors (Muir, 2006).

Heart rate, heart rate variability and respiration rate

Cardio-respiratory functioning including RR, HR, and heart rate variability (HRV), reflects the dynamic interaction between physiological and psychological factors that modulate overall heart rhythm, and are represented by variations in beat-to-beat intervals (Wu and Lee, 2009). Increases or decreases in HR are controlled by the sympathetic and vagal branches respectively (Baciadonna et al., 2019). Alterations to HR and RR can provide an indication of the body's reaction to emotional stimuli. However, these measures are more applicable as an indicator of emotional arousal, given the inability to differentiate the valence of affect (Boissy et al., 2007b; Briefer et al., 2015).

Fluctuations in HRV are a function of the ANS and depict the continuous interplay between sympathetic and para-sympathetic branches. As the sympathetic tone increases, HR and RR increase, but the heart's R-R interval (measured on an ECG) shortens, resulting in lowered HRV (Figure 4). Conversely, when the parasympathetic tone is increased the opposite occurs, resulting in wider R-R intervals in the heartbeat and overall increased HRV.

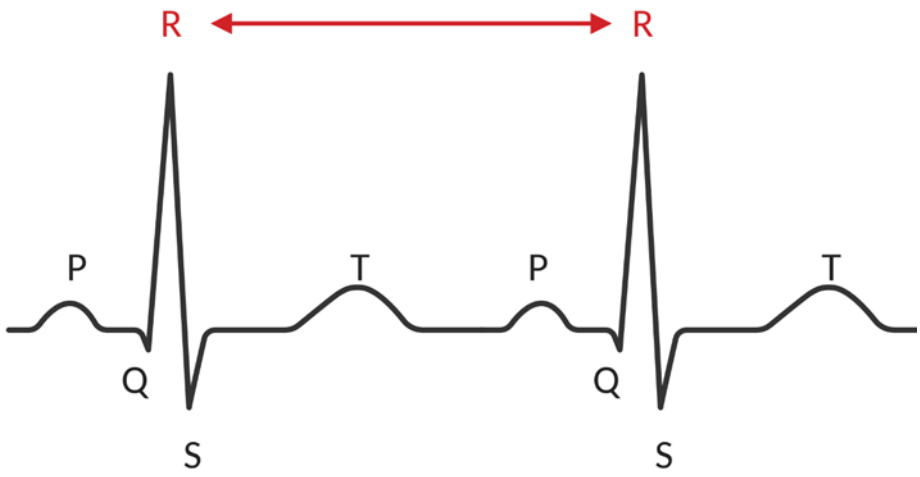

Figure 4: The heart's PQRST complex indicating the consecutive R-R intervals measured using ECG. When the sympathetic tone increases, the R-R intervals become shorter, resulting in lowered HRV. As the parasympathetic tone increases, the R-R interval becomes wider and increases HRV.

In humans, measures of HRV have been used to assess different emotional experiences and include correlations with both negative and positive emotional states. For example, a low HRV has been implicated with depression and PTSD (Baciadonna et al., 2019), and high HRV is associated with lower levels of anxiety (Dishman et al., 2000) and is an indicator of overall good heath (Thayer and Sternberg, 2006). Consequently, the measure of HRV has been proposed as a potential indicator of emotional valence in animals (von Borell et al., 2007; Coulon et al., 2015; Tamioso et al., 2018).

Despite a growing body of literature in the area, the majority of studies measure HRV primarily as an indicator of stress or poor welfare (Mohr et al., 2002; Abbott, 2005; von Borell et al., 2007; Kovács et al., 2015; Scopa et al., 2020) and pain (Stewart et al., 2010). There have been some studies, however, that have shown correlations between measures of HRV and positive emotional states in animals, including dogs (Katayama et al., 2016; Zupan et al., 2016a; Varga et al., 2018), sheep (Reefmann et al., 2009; Coulon et al., 2015; Tamioso et al., 2018), pigs (Imfeld-Mueller et al., 2011; Zebunke et al., 2011; Zupan et al., 2016b) goats (Briefer et al., 2015; Luigi et al., 2019) cattle (Lange et al., 2020), and horses (Léa et al., 2018). The majority of these studies used known food rewards, various housing conditions or positive

human interaction treatments to evoke positive emotional states in the animals, which are then used to correlate with HRV data.

Measurement of HRV is a non-invasive tool that can be used to investigate the functioning of the ANS, including the dynamic relationship between sympathetic and vagal tone. The current literature investigation suggests that HRV is an indicator of positive emotional states in animals. It is still in its infancy but has the potential to be a reliable tool for animal welfare assessment. Few studies have been able to draw adequate conclusions between HRV measures and emotional valence (see above); however, some major limitations remain. HRV is influenced by a variety of factors including age, sex, physical fitness, diurnal rhythm, respiration, posture and physical activity *(von Borell et al., 2007)*, and further incorporation of these factors under standardised experimental settings is required for validation purposes.

Infrared thermography

Measurement of surface temperatures, using infrared thermography (IRT), has been identified as a reliable non-invasive method to detect stress in animals (Valera et al., 2012; Bartolomé et al., 2013; Yarnell et al., 2013; Dai et al., 2015; Herborn et al., 2015; Pierard et al., 2015). Infrared thermography measures changes in peripheral blood flow (Travain et al., 2016) by sensing heat radiating from the skin (Kuraoka and Nakamura, 2011). The fairly novel technique of thermal imaging has recently been applied in a handful of studies to examine the affective state of animals. The presumptive basis of the method is that the temperature of peripheral regions is modulated via autonomic physiological reactions (Kuraoka and Nakamura, 2011). This may be under the amygdala, which is known to control electro-dermal activity (Bagshaw and Coppock, 1968) and skin conductance responses (Laine et al., 2009). Furthermore, in primates at least, sympathetic nerves are responsible for constricting arteriovenous anastomoses in the nasal skin region (Bergersen, 1993), and some research suggests that the amygdala is responsible for this vasoconstriction (Yu and Blessing, 2001). Like much of the research, it has mainly been investigated in conjunction with a stress response, or negative emotional state (see (Nakayama et al., 2005; Stewart

et al., 2007)). However, a few studies have examined temperature in peripheral tissues in the presence of an assumed positive emotional response (Moe et al., 2012; Proctor and Carder, 2015; Travain et al., 2016).

Animal thermography has been trialled on a range of body sites with somewhat variable results, dependant on the species tested, site used and the surface area of the region. Decreased eye temperature in cattle (Stewart et al., 2008) and decreased ear canal and ear pinna temperature in sheep (Beausoleil, 2004; Lowe et al., 2005) have been observed in response to fear, pain or stressful events, and are considered successful measures to detect negative emotional states (Proctor and Carder, 2016). Furthermore, IRT has been used to assess the relationship between facial surface temperatures and affective states in monkey, where it was concluded that decreased nasal temperatures correlate with a negative emotional state (Nakayama et al., 2005). Conversely, however, a reduction in nasal temperatures in cattle (Proctor and Carder, 2015), decreased comb temperatures in hens (Moe et al., 2012), as well as increased peri-orbital temperatures in dogs (Travain et al., 2016) were observed under assumed positive emotional states.

Given that variations in temperature have been shown to follow the same directional pattern in both positively and negatively valanced states (see (Moe et al., 2012; Travain et al., 2015; Travain et al., 2016)), the technique appears to be measuring emotional arousal rather than valence. Furthermore, there may be value when designing studies to measure temperature changes in response to a change in emotional valence, rather than assuming a simplistic interpretation that the absolute temperature will increase or decrease from a baseline value (Ioannou et al., 2015). Studies have also highlighted some potential confounding factors in interpretation; for example, inter-individual differences in temperature were observed in dogs (Travain et al., 2016), and diet-induced thermogenesis was implicated in comb temperature differences in hens (Moe et al., 2012). Furthermore, some limitations in terms of the ability to detect changes in surface temperature accurately have been identified. For example, environmental factors, including sunlight, wind and ambient temperature, may confound results (Stewart et al., 2005).

Additionally, differences in anatomical surfaces (noses, ears and heads), as well as variations in temperature associated with coat colour and coat thickness have been identified (Bartolomé et al., 2013).

At the current time, and based on the limited research available, this technique cannot be recommended as a sole measure of affective state, especially where determining emotional valence is of importance. However, given its relative ease of application, researchers should consider using this method alongside other established measures of affective state assessment. This could assist in bolstering evidence for other methods, build knowledge around the thermography technique, and provide an added objective measure of arousal.

Catecholamines and glucocorticoids

Activation of physiological stress systems almost always includes activation of the sympatho-adrenal-medullary system (SAM), and hypothalamic-pituitary-adrenal (HPA) axis, associated with catecholamine and glucocorticoid synthesis respectively (Möstl and Palme, 2002; Ashley et al., 2005; Ayala et al., 2012). The major catecholamines of the SAM system include epinephrine, (adrenalin), norepinephrine, (noradrenalin) and dopamine (DA), which function as neurotransmitters and hormones regulating the sympathetic branch of the ANS and the adrenal medulla. The main sites for catecholamine production are the brain, the adrenal medulla and sympathetic neurons, where plasma catecholamines display a half-life of 1-2 minutes (Peaston and Weinkove, 2004) In the periphery, noradrenaline appears to be involved predominantly in the sympathetic control of blood flow and pressure, while adrenaline is involved in metabolic processes including fat and glucose metabolism (Bupesh et al., 2014). These molecules can, and have been, readily measured in the blood and urine of animals (Miki and Sudo, 1998). Most research to date has investigated levels of catecholamines in animals in response to negative stress events (Sgoifo et al., 1996). Within the CNS, catecholamines released from neurons within the ventral tegmental area are known to modulate reward, motivation and emotions at the level of the amygdala and the nucleus accumbens (Bupesh et al., 2014). The use of EEG has enabled investigation of the role of norepinephrine in social play in rats, a highly rewarding and pleasurable activity, and found that the

enhancement of norepinephrine was negatively modulated by both motivation and expression of play behaviour (Ej Marijke et al., 2015). However, without the use of imaging technologies, there is limited practicality in using peripheral catecholamines as biomarkers for emotional states in animals.

The corticosteroids produced following activation of the HPA axis include a number of sex steroids, mineralocorticoids and glucocorticoids that are involved in a wide range of physiological processes, some of which include stress responses, immune and inflammatory function, neurobiological function, carbohydrate metabolism, blood electrolyte balance and changes in behaviour (Rangappa, 2015). Activation of the HPA axis increases production of vasopressin (arginine vasopressin in most species but lysine vasopressin in pigs), and corticotrophin releasing hormones (CRH) from the hypothalamus (Alexander et al., 1996). CRH and vasopressin stimulate the synthesis of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland, and circulating ACTH then stimulates the adrenal cortex to synthesise glucocorticoids including cortisol (Alexander et al., 1996; Ayala et al., 2012). The release of pituitary and adrenal hormones following an increase in HPA activity is likely modulated by emotional states through the neuroanatomical connections of the HPA axis with the subcortical regions responsible for emotional regulation (Figure 5).

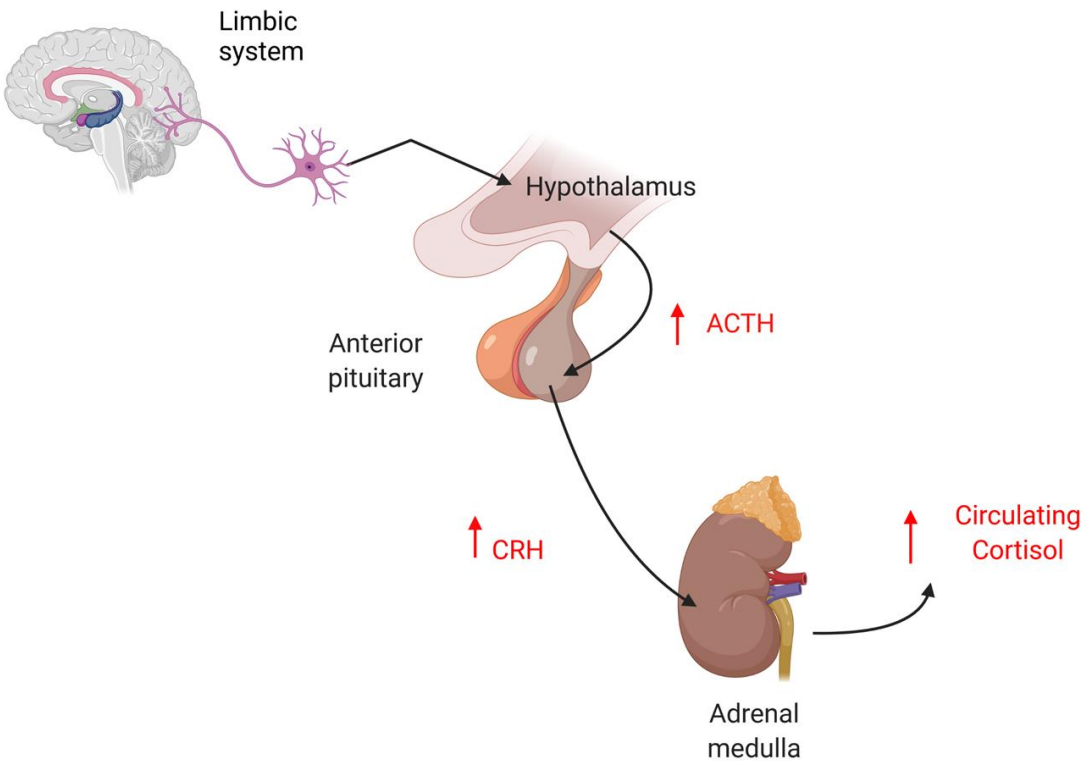

Figure 5: Indicates the release of cortisol following activation of the Hypothalomo-pituitary-adrenal (HPA) axis. Neuroanatomical connections from subcortical regions associated with emotions, including limbic structures, send signals via neurotransmitters to the hypothalamus, resulting in increased Adrenocorticotropic hormones (ACTH). The increase in ACTH stimulates the Anterior pituitary to release corticotropin-releasing hormones (CRH) into the periphery, resulting in increased circulating cortisol.

To date, the majority of the research using circulating cortisol as a marker of emotion tends to focus on its application during negative states such as fear, anxiety or pain. Limited research using cortisol as a marker of positive emotional states has been conducted, although it is generally assumed that lower levels of circulating cortisol indicate a more positive affective state (Andrew et al., 2005; Steptoe and Wardle, 2005; Steptoe et al., 2007). For example, in humans, circulating cortisol, both for the total output and the awakening response, is shown to be lower in individuals with greater levels of positive affect (Dockray and Steptoe, 2010). Despite this assumption, there are clear limitations in using circulating cortisol as a marker of emotions in animals. For example, it has been suggested that the synthesis of glucocorticoids can increase in response to both positive emotional states like reward, excitement and pleasure as well as negative emotions, including pain, anxiety and fear (Ralph and Tilbrook, 2016). For example, salivary cortisol was elevated in calves exposed to both positive (suckling) and negative (no

suckling) stimuli. Additionally, synthesis of glucocorticoids has been shown to be highly variable between and across individuals even when experimental designs control for factors known to influence cortisol concentrations, including age, sex, exercise, eating and diurnal rhythm (Marsh et al., 2020). Given these issues, there are clear limitations to using circulating cortisol as a marker of emotional states in animals: primarily in terms of the inability to differentiate between the valences of affect as well as implications with interpretations of the data.

Brain Neurotransmitters

The brain is comprised of various neurotransmitters that act as signalling molecules between neurons and their target cells. Neurotransmitters are used by the body for different functions and include acetylcholine, glutamate, GABA, glycine, dopamine, norepinephrine, and serotonin. These molecules are the primary chemical messengers released from neurons that relay, amplify and modulate signals to other cells (Marc et al., 2011). These neurological signals are not only the backbone of the majority of neurological functions, they also exert endocrine and immunological actions, and are now considered primary targets for detecting a number of psychological mood disorders and affective states.

Dopamine and metabolites

Dopaminergic neurons located in the midbrain are the primary source of dopamine (DA) in the CNS and function as neurotransmitters involved in the regulation of movement, prediction of reward, motivation, cognition and working memory processes (Arias-Carrion and Poppel, 2007). In humans, dysregulation of DA transmission plays a key role in the development of a number of diseases, including Parkinson's disease, schizophrenia, attention deficit disorders and drug and alcohol addiction (Bupesh et al., 2014). Dopamine is the driver of reward-seeking behaviour, where reward has previously been defined as an object an individual will acquire or seek out through the allocation of time, energy or effort (Arias-Carrión et al., 2010). Administration of psychostimulants, including cocaine and amphetamine, have been shown to increase brain dopamine in the ventral striatum and elicit a positive affective state in humans (Volkow and Swanson, 2003) and rats (Burgdorf et al., 2001). In mice, high levels of brain dopamine are associated with positive affective states (Ashby and Isen, 1999; Burgdorf and Panksepp,

2006). Furthermore, increased anxiety and depression-like behaviour were observed in DA transporter knock-out rats (Bahi and Dreyer, 2019). The results of these studies support the role of central DA involvement in emotional regulation. However, given that animals are required to be chemically/physically restrained prior to the utilization of brain imaging technology, the practicality of its application as a measure of emotional state in livestock species is arguable. Furthermore, quantification of DA in brain requires extraction of brain tissue from the animal, warranting this method ineffective from a productivity viewpoint. A number of studies have attempted to measure peripheral by-products of central DA, including the main metabolite of DA, homovanillic acid, in cerebral spinal fluid (SSF), plasma and urine (Kopin et al., 1988; Amin et al., 1992; Amin et al., 1995). The difficulty of this method, however, is the inability to differentiate what proportion of the homovanillic acid in the blood is attributed to either central DA activity or peripheral norepinephrine metabolism (Kopin et al., 1988).

Serotonin and metabolites

Serotonin (5-hydroxytryptophan or 5-HT), is a monoamine neurotransmitter abundant throughout the body. Most 5-HT is found outside the CNS where it is involved in a number of physiological processes including Serotonin regulates numerous biological processes including cardiovascular function, bowel motility, ejaculatory latency, and bladder control (Berger et al., 2009). Withing the brain, 5-HT is involved in the regulation of sleep-wake cycles, body temperature, pain perception, hormonal functions of the hypothalamus, as well psychological conditions, including anxiety and depression (Owens and Nemeroff, 1994; Berger et al., 2009; Suominen et al., 2013). Furthermore, brain 5-HT modulates virtually all behavioural and neuropsychological processes, including mood, perception, reward, anger, aggression, appetite, sexuality and attention (Berger et al., 2009). 5-HT has shown to aid in the regulation of affective states (Cools et al., 2008), and dysregulation of serotonergic activity has been linked with psychological conditions including, but not limited to, anxiety and depression (Jans et al., 2007). In pigs, depletion of brain 5-HT has been shown to induce pessimistic-like behaviour in a cognitive bias paradigm, suggesting animals were in a more negative affective state (Stracke et al., 2017). Like other neurotransmitters, including DA (mentioned above), without the use of neuroimaging techniques or extraction of brain tissue,

difficulty remains in developing reliable measures in the blood that can be used as markers of serotonergic activity in the brain. More recently, however, blood plasma 5-HT has been measured in piglets in response to the provision of enrichment (Lykhach et al., 2020). The study found that piglets whose play was enriched with toys had increased blood plasma 5-HT, reduced aggression and increased play behaviour (a rewarding experience indicative of positive emotions in mammals (Sommerville et al., 2017; Rius et al., 2018a)), compared with control animals. Based on these findings, there may be potential for correlations between brain-blood 5-HT levels and affect, but further investigation is required.

Oxytocin

Oxytocin (OT) is a neuropeptide Synthesised from magnocellular and parvocellular neurons of the paraventricular nucleus (PVN) and supra-optic nucleus (SON) of the hypothalamus (Matsuzaki et al., 2012). Magnocellular neurons secrete OT into capillaries within the posterior lobe of the pituitary, while parvocellular neurons transport OT to various areas of the brain (Lajtha and Lim, 2006). In the periphery, OT is primarily involved in parturition and lactation (Viero et al., 2010). The central OT, however, contributes to a wide range of functions, including social and maternal bonding, anxiety, fear conditioning and behaviour (Di Simplicio and Harmer, 2016; Jones et al., 2017). Various brain regions, including the amygdala, hypothalamus, nucleus accumbens and brain stem, contain OT receptors (Lee et al., 2009), where OT acts as a neurotransmitter and is distributed among these regions (Insel et al., 1997; Domes et al., 2007). It is implicated in homeostatic, neuroadaptive processes associated with stress responses via interactions with the hypothalamic-pituitary-adrenal (HPA) axis, as well as the dopamine mesolimbic reward stress system (Kovatsi and Nikolaou, 2019). OT is often proposed to reflect situations of positive valence (Rault et al., 2017) and therefore it has the potential to be a biomarker for the neural processes involved in positive emotions.

The potential of oxytocin (OT) to be an indicator of animal affect has been investigated in several species (Kremer et al., 2020). However, results on its efficacy are conflicted in the literature. For example, In pig, positive human contact has shown to increase OT levels in cerebrospinal fluid (CSF) (Rault, 2016),

and In dog, OT increases in urine following positive stroking from humans (Mitsui et al., 2011). Conversely however, social situations involving dogs indicated that following intranasal administration of OT reduced friendliness toward the owner (Hernádi et al., 2015). Another study pig found that OT administration reduced social contact in neutral or positive situations but increased it in negative situations (Camerlink et al., 2016). A recent review by Rault et al. (2017), suggested OT may not necessarily correlate with positive situations or outcomes, as there is evidence that negative events also elicit an OT response. Furthermore, the authors stated that the current literature may be bias towards investigating positive valence states due to a lack of controlled experimental settings that incorporate both valence and arousal (Rault et al., 2017). Another impediment to its application is that the measurement of central OT remains constrained by the sampling methods available (Lürzel et al., 2020), particularly in a livestock setting. Although endogenous OT can be readily measured in blood, urine, saliva and milk (Rault et al., 2017), it is not it is not clear if OT levels in these matrixes are linked with central OT. A number of studies have attempted to correlate peripheral OT with behavioural indices, however the majority of these reported inconsistent correlations between central and peripheral OT levels in humans (Kemeny and Shestyuk, 2008; Jokinen et al., 2012; Kagerbauer et al., 2013; Striepens et al., 2013), and monkeys (James et al., 2003). McEwen (2004), has suggested this is attributed to the fact that central and peripheral OT releases are governed by separate mechanisms, where central OT does not readily cross the blood brain barrier (BBB) (Kemeny and Shestyuk, 2008).

It is clear that using peripheral OT as a marker of central OT is under scrutiny, and is further complicated by the current lack of understanding of the biological significance of peripheral OT (McCullough et al., 2013). Given the above issues, the use of measuring peripheral oxytocin as a biomarker of emotional state in animals at this stage is debatable. Future research, specifically investigation into the biological significance of OT in the regulation of psychological and behavioural states, as well as incorporation of species-specific differences, intra-induvial variations in response and well controlled experimental paradigms are, therefore, warranted.

Endorphins

Endorphins can be found in the pituitary gland, as well as the central (CNS) and peripheral (PNS) nervous systems (Jain et al., 2019). They are endogenous opioid neuropeptides produced by the CNS, which preferentially bind to μ-opioid receptors to inhibit the communication of pain signals in PNS by blocking the neurotransmitter substance P (Jain et al., 2019). Within the CNS, the binding of endorphins to μ-opioid receptors inhibits gamma-aminobutyric acid (GABA) release, resulting in increased dopamine synthesis and, in some cases, increased feelings of euphoria and extreme pleasure (Sprouse-Blum et al., 2010). Recently it has been suggested that the release of endorphins is the neurochemical cause for the feeling of pleasure (Mathew and Paulose, 2011). For example, in humans, endorphins are released in response to various activities thought to be pleasurable or those that induce the feeling of pleasure (i.e.,following exercise, , love, listening to music, eating chocolate, laughter, sex and orgasm (Farhud et al., 2014). Furthermore, intravenous administration of μ-opiate and dopamine agonists are associated with positive affective states in humans (Zacny et al., 1994; Drevets et al., 2001).

Endorphins are also produced in response to emotional states such as fear and pain (Dalayeun et al., 1993). The majority of animal welfare research has investigated changes in endorphin levels in response to negative welfare states (i.e., pain and stress). For example, measurements of endorphins have been used as indicators of stress and pain procedures in pigs (Marchant-Forde et al., 2009; Marchant-Forde et al., 2014), compromised welfare in dairy cattle (Zbinden et al., 2017), stress associated with slaughter (Shaw and Tume, 1992), transport stress (Fazio and Ferlazzo, 2003), stereotypies in horses (Briefer Freymond et al., 2019), and pain in lambs following mulesing (Paull et al., 2008). Few studies have investigated correlates between endorphins and positive emotional states in animals. Intravenous administration of μ-opiate and dopamine agonists has been shown to increase specific vocalizations associated with positive emotional experiences in rats (Burgdorf et al., 2001). Additionally, increases in plasma β-endorphins were observed in both humans and dogs during positive dog-human interactions (Odendaal and Meintjes, 2003).

As stated earlier, the majority of the literature on animals' welfare has correlated levels of endorphins with negative emotional states such as pain and stress. Moreover, very few studies have investigated endorphin levels in livestock, even fewer focussing on positive emotional states. There is, however, potential for these compounds to be used in the assessment of positive emotional states but the efficacy and practicality of this technique requires further validation.

Immunological markers

The immune system is crucial in protecting the body from threats of infection and there are direct anatomical and functional links between the CNS and the immune system (Dockray and Steptoe, 2010). Studies relating to stress in both humans and animals have demonstrated links between psychological challenge modifications in the immune response (Padgett and Glaser, 2003; MacQueen and Bienenstock, 2006). It is therefore plausible that there is a contingent of immunological markers that are influenced by both positive and negative emotional states (Yeates and Main, 2008).

In humans, increased cellular immune competence has been linked with positive affect (Lutgendorf et al., 2001), and pro-inflammatory cytokines have been associated with feelings of optimism (Stellar et al., 2015) (Brydon et al., 2009), and happiness (Steptoe et al., 2008). Furthermore, increases in natural killer cell activity (Valdimarsdottir and Bovbjerg, 1997), peripheral white blood cells (Futterman et al., 1992), salivary immunoglobulin-A (IgA), concentrations (Dillon et al., 1986), and interleukin (IL)-2 and IL-3, and decreases in IL-6 and tumour necrosis factors (for review, see (Pressman and Cohen, 2005)) are shown in individuals in positive affective states. Furthermore, in humans, IgA is an antibody and has the potential to be a marker of immune function and increases in response to positive emotional states or pleasant stimuli (Whitham and Wielebnowski, 2013).

In animal studies, the majority of immune based markers are measured in response to negative welfare states, including those pertaining to disease, stress, pain and inflammation. Some of these markers include leucocyte counts, plasma proteins (including the immunoglobulins IgA, IgM and IgG), and pro- and anti-inflammatory cytokines (Colditz, 2002). For example, following castration in calves,

tests indicated higher neutrophil numbers and neutrophil: lymphocyte (N:L) ratios compared with those of control animals (Fisher et al., 1997). The acute phase protein haptoglobin has been shown to be implicated in infection and inflammation in cattle (Eckersall and Bell, 2010). Salivary IgA has been used as a diagnostic measure of immunity in the gastrointestinal nematodes in sheep (Shaw et al., 2012). In dogs, separation stress has shown to increase faecal IgA concentrations (Walker et al., 2014).

Few studies have investigated immunological markers in response to positive welfare states. Calves exposed to positive stimuli (suckling) have shown increased levels of secretatory immunoglobulin A (SIgA), IL-2, IL-3, and lowered tumor necrosis factor (TNFa), compared with calves exposed to negative (frustration feeding) stimuli (Lv et al., 2018). Higher levels of IgG were found in pigs following successful coping in response to rewarding stimuli (food reward) (Ernst et al., 2006). Furthermore, positive and negative psychological experiences have been shown to affect immune system parameters in pigs (Tuchscherer et al., 1998).

Although there is emerging evidence that immune system measures can send signals to the brain and influence emotions (Miller et al., 2005), the exact mechanism by which this happens remains elusive in the current literature. Interactions between immune system functions and HPA activity have been have been extensively investigated (Besedovsky et al., 1991), as well as relationships between the immune system and emotional limbic structures within the brain (Haas and Schauenstein, 1997). Literature about such measurements in humans has started to uncover this dynamic and complex relationship. Affective states in humans are positively correlated with secretory immunoglobulin A, increased levels of certain cytokines (IL-2, IL-3), and decreases in others (i.e., interferon-α and TNFα) (Kemeny and Shestyuk, 2008). Given the anatomical similarity in the neurological structures between human and non-human animals, it is likely that any immunological responses elicited would also be similar in animals and the recent literature suggests this is so (see (Ernst et al., 2006; Gourkow et al., 2014; Lv et al., 2018)).

Conclusions and future directions of current assessments of animal affects

The objective assessment of animal welfare has advanced to include measures of animal emotions that are of particular interest in the assessment of positive emotions. The challenge remains in our ability to develop accurate and reliable markers of the physiological systems associated with emotional states. Although behavioural methods, including measurements of cognitive bias, have been effective at determining the valence of affect in a number of livestock species (see above), limitations in terms of training time, inter- and intra-animal variations in response, and issues relating to study design (i.e., loss of ambiguity), preclude their widespread application in a farming setting. Current physiological indices (discussed above) are commonly applied in livestock; the majority of indices being used to infer negative emotional states in response to stress. Although these measures provide valuable information regarding the emotional arousal being experienced, they often lack the specificity to distinguish between emotional valences (i.e., positive or negative). Furthermore, these measures are frequently taken in isolation, or if they are measured in correlation with other, already established, behavioural or physiological indices, the results are inconsistent across studies. Consequently, there is a need to develop novel biomarkers by which we can measure the emotional states of animals. These measures would ideally be robust, objective, and have the ability to differentiate between valences of affect.

Development of novel biomarkers of animal emotion

One biomarker type that has gained increasing interest in in the field of psychology and animal welfare is microRNAs (miRNAs). These molecules are involved in the regulation of genes, and in humans, have been implicated in number of neurological and affective conditions. MiRNA are ubiquitous throughout the body, including the brain (Sempere et al., 2004), and can be readily measured in blood, saliva and urine. Therefore, circulating miRNA has the potential to be become a biomarker of the processes involved in emotional regulation. Below, I investigate the current literature relating to miRNA, its involvement in the neural regulation of emotional states, and further discuss its potential as a diagnostic marker of emotion in animals.

Brief overview of miRNA

MiRNAs were originally described in 1993, when it was identified that *lin-4 and let-7 controlled the timing of nematode (C. elegans) development through incomplete base pairing to the 3' UTRs of the target mRNAs to repress their translation (Lee et al., 1993; Reinhart et al., 2000; Wahid et al., 2010). Since then,* hundreds of miRNAs have been identified, thanks to recent advancements in technology, including the development of high-throughput sequencing and bioinformatics (Lu et al., 2005; Chen et al., 2019). This has allowed us to identify miRNA target genes and their functions (Lai et al., 2003; Wahid et al., 2010). These small (approximately 19-22 nucleotides), single stranded, non-protein coding RNA molecules have been identified in plants, animals and virusesMiRNA are considered critical regulators of nearly every aspect of mammalian biology (Blahna and Hata, 2012), where It is estimated miRNAs constitute nearly 1% of all predicted genes in mammals (Lim et al., 2003). MiRNAs have been shown to be involved in crucial biological processes, including immunity, fat metabolism, developmental timing, cell proliferation, apoptosis and neural patterning and plasticity (He and Hannon, 2004; Mulligan, 2011; Codocedo and Inestrosa, 2016; Allen and Dwivedi, 2020).

MicroRNAs regulate gene expression post-transcriptionally by binding to complementary sequences at the 3' UTR (untranslated region) of the target messenger RNA (mRNA) (Li et al., 2010; Van Meter et al., 2020). The miRNA target and bind to mRNA and prevent gene expression by either targeting mRNA for degradation, or blocking translation of mRNA into protein. It has been shown that a single miRNA has the ability to control the expression of multiple target mRNAs, and each mRNA can be regulated by multiple miRNAs (Esquela-Kerscher and Slack, 2006; Cai et al., 2009). MiRNA target mRNA with high specificity, and binding is usually restricted to the 'seed' sequence near the 5' terminus (Cai et al., 2009). The seed sequence is highly conserved among species and, as a consequence, variations in the RNA sequence may impact its target capabilities. Alterations in expression and function of miRNAs result in substantial phenotypic changes associated with perturbed development and pathological conditions (Ruepp et al., 2010).

MiRNA biosynthesis

MiRNA are produced from hairpin shaped precursors (Ambros et al., 2003; Van Meter et al., 2020). In animals, the genes for miRNAs are first transcribed in the nucleus by the RNA polymerase III enzyme, Drosha (Borchert et al., 2006), to form long primary miRNAs (pri-miRNA) (Figure 6). The pri-MIRNA is processed within the nucleus to form the precursor miRNA (pre-miRNA). From here, the pre-MiRNA is transported to the cytoplasm via exportin-5 (EXP-5), where it is then further processed by the RNase III type protein Dicer to form the mature miRNA. The mature MIRNA is then encapsulated with an argonaute (ago), protein to produce the RNA-induced silencing complex (RISC) (Wahid et al., 2010). The mature miRNA serves as the target recognition element within the RISC complex, while the ago protein component binds to the target mRNA (Kawamata and Tomari, 2010; Schirle et al., 2014). Once RISC binds to the target mRNA, it results in downregulation of protein expression through repressed translation or mRNA degradation (Van Meter et al., 2020).

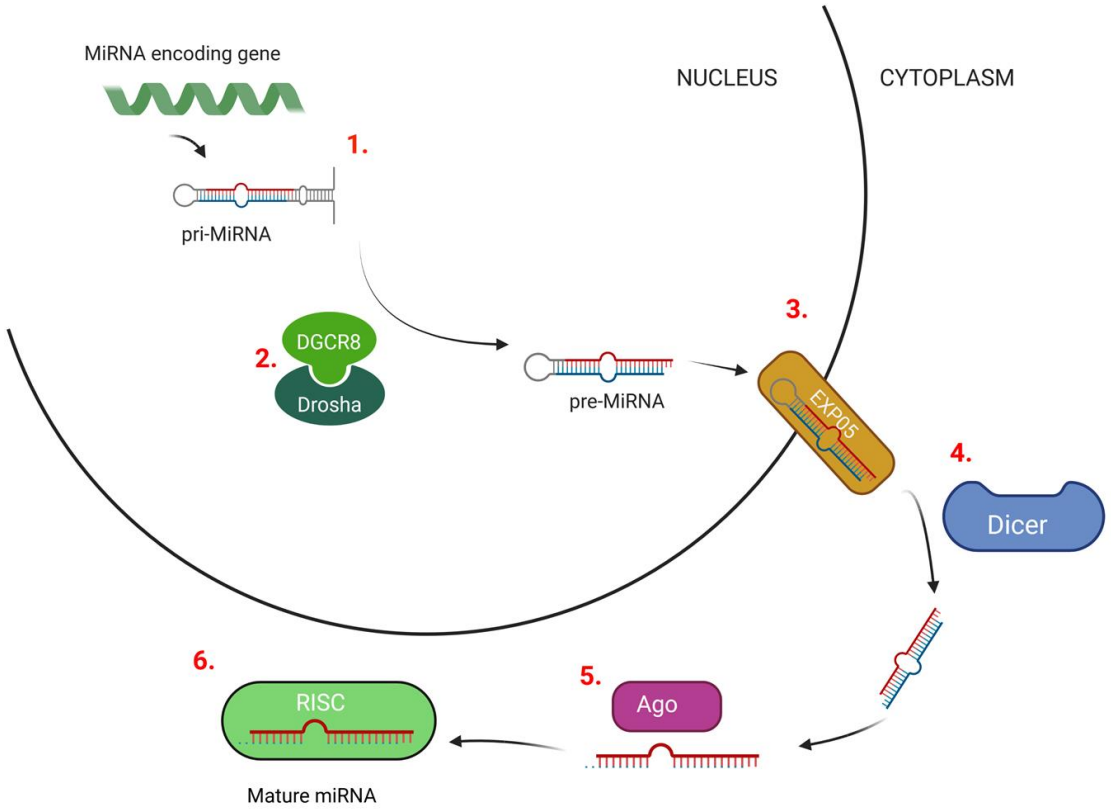

Figure 6: Indicates the pathway of microRNA (miRNA) biosynthesis where 1) the transcription of the miRNA coding gene into primary miRNAs (pri-miRNA); 2) cleavage of pri-miRNA into precursor miRNA

(pre-miRNA) by the RNA polymerase III enzyme, Drosha; 3) the transport of pre-miRNA into the cytoplasm via the transport protein exportin-5 (EXP-5); 4) cleavage of pre-miRNA into mature miRNA via the RNase III type protein, Dicer; 5) The encapsulation of mature miRNA into argonaute (ago) protein; 6) RNA-induced silencing complex (RISC) containing mature miRNA.

miRNA as biomarkers

Since their discovery, over 2500 mature miRNAs have been discovered in humans (Boudreau et al., 2014; Alles et al., 2019; Van Meter et al., 2020). Research pertaining to this discovery has provided knowledge of miRNA biogenesis, modes of action and their function (Van Meter et al., 2020). In humans, research has found that miRNA play a critical role in many biological processes, and dysfunction to miRNA biogenesis and dysregulation of miRNA towards target genes can result in various disease states (Esquela-Kerscher and Slack, 2006). They are involved in cell communication and are actively secreted from cells into the extracellular environment (Miretti et al., 2020). MiRNAs are highly stable in body fluids and have been observed in various biological fluids, such as plasma, serum, saliva, milk, and cerebrospinal fluids (Faruq and Vecchione, 2015) and, as a result, much research to date has investigated miRNAs as diagnostic markers for a number of pathological conditions including, but not limited to, diabetes (Guay et al., 2011), cardiovascular disease (Cheng and Zhang, 2010; Wojciechowska et al., 2017), cancer (Lawrie et al., 2008; Foss et al., 2011; Wang et al., 2019; Moghadasi et al., 2020), and various pain conditions (Sakai and Suzuki, 2015; López-González et al., 2017).

In recent years, the involvement of miRNAs in gene expression within neural networks has warranted interest in their role in human psychological conditions. MiRNAs have been shown to be implicated in numerous mental health conditions, including anxiety (Haramati et al., 2011; O'connor et al., 2012; Andolina et al., 2016; Fonken et al., 2016; Meydan et al., 2016; Narayanan and Schratt, 2020), MDD (Dwivedi, 2011; Mouillet-Richard et al., 2012; Lopez et al., 2014; Serafini et al., 2014; Roy et al., 2017a; Roy et al., 2017b; Dwivedi, 2018; Allen and Dwivedi, 2020), bipolar-mania disorder (Zhou et al., 2009; Kim et al., 2010; Camkurt et al., 2020), PTSD (Balakathiresan et al., 2014; Snijders et al., 2017) schizophrenia (Lai et al., 2011; Cattane et al., 2019; Du et al., 2019b; Santarelli et al., 2019), and OCD (Espinosa-Parrilla et al., 2011; Yue et al., 2020; Lin et al., 2021). MiRNAs and their target genes have

been identified throughout the brain (Boudreau et al., 2014), including the amygdala (Volk et al., 2016; Hoban et al., 2017; Shen et al., 2019; Sun et al., 2019; Roy et al., 2020), prefrontal cortex (Hoss et al., 2016; Hoban et al., 2017; Popa et al., 2020), hippocampus (O'Connor et al., 2013; Sun et al., 2013; Zurawek et al., 2019), and nucleus accumbens (Eipper-Mains et al., 2011; Du et al., 2019a; Guo et al., 2020), among others. In humans and rodents, specific miRNAs measured in the blood can provide information about the cellular processes involved in regulating emotion (Haramati et al., 2011; Issler et al., 2014; Kocerha et al., 2015). For example, a study by Haramati et al. (2011) found that chronic social defeat results in upregulation of miRN-34c in the central amygdala and can lead to anxiety and depression-like behaviours in adult mice. MiR-135a was upregulated in the raphe nuclei of mice following administration of antidepressants and resulted in reduced anxiety-like behaviours (Issler et al., 2014). This study also showed that levels of miR-135 were significantly lower in the brain and blood of depressed human patients and this suggested miRNA is indicative of stress resilience. Furthermore, other studies have identified that miR-9 has been shown to be regulated by chronic unpredictable stress and maternal deprivation, miR-21 is upregulated after multiple types of peripheral pain, and miR-124 was upregulated in key reward centres during rewarding experiences (Balakathiresan et al., 2014; Issler and Chen, 2015; Basak et al., 2016; Codocedo and Inestrosa, 2016).

Once released into circulation, miRNAs can be indicative of cellular processes and states, including those associated with emotional processes. For example, in humans and rodents, circulatory miRNAs have been shown to be reliable and non-invasive diagnostic biomarkers for bipolar disorder, PTSD and schizophrenia (Lai et al., 2011; Rong et al., 2011; Balakathiresan et al., 2014), as well as markers of pain (Qureshi et al., 2016). Furthermore, miR-181a and miR-181b in nucleus accumbens and blood, have been shown to be indicative of positive emotional states in humans (Wingo et al., 2017). Based on these findings, and/or understandings of the neural networks involved in the regulation of emotional processes in humans, there is potential for miRNAs to be biomarkers of emotional processes in animals. Although there is no way of truly knowing if animals experience emotions in the same way as humans, parallels in

neurophysiology, neuroanatomy and behaviour are observed between humans and certain animal species, suggesting they may have emotional experiences similar to humans (Panksepp, 2005; Boissy et al., 2007b; Panksepp, 2011).

MiRNAs as biomarkers of welfare in animals

Although the majority of studies mentioned above have used animals as models to investigate the role of miRNAs in the pathogenesis of human disease and psychological conditions, very limited studies have investigated miRNAs as biomarkers specifically for the assessment of welfare in animals, even fewer on markers of positive welfare states. A recent review by Miretti et al. (2020), identified the current literature investigating miRNAs as biomarkers of health and welfare parameters in livestock, with particular emphasis on those relating to stress. For example, from the literature cited, it was highlighted that differentially expressed miRNAs are observed during stressful events in cattle with mastitis (Li et al., 2014; Luoreng et al., 2018; Srikok et al., 2020)), lameness (Ioannidis et al., 2018), group relocation during lactation (Colitti et al., 2019), and heat stress (Zheng et al., 2014; Li et al., 2018). In sheep miRNA expression is altered with bacterial infections (Qi et al., 2019; Naylor et al., 2020) and viral infection (Du et al., 2017; Bilbao-Arribas et al., 2019; He et al., 2019), as well as in goats following weaning stress (Liao et al., 2019) and feed deprivation (Mobuchon et al., 2015). Altered miRNA expression were also observed in pigs with viral infection (Brogaard et al., 2016; Núñez-Hernández et al., 2017; Brogaard et al., 2018), pain and inflammation (Lecchi et al., 2020), following weaning stress (Tao and Xu, 2013) and heat stress (Hao et al., 2016). Furthermore, ,viral infection (Yang et al., 2017; Kemp et al., 2020)), feed deprivation (Hicks et al., 2019), heat stress (Zhu et al., 2019) and transport (Lecchi et al., 2016) resulted in differential miRNA signatures in poultry.

Whilst the identification of miRNA-based biomarkers is rapidly gaining attention in the field of animal welfare science (see above), most of these studies have investigated miRNA in response to stress. As mentioned earlier in this review, stress is not always a negative experience *per se,* but rather an adaptive response that prepares the organism to deal with challenges. Although stress can influence emotions

through appraisal, the current literature does not allow the distinction between what miRNA might alter in response to emotional processes, and which ones are produced in response to normal biological functioning in the stress response. For example, Lecchi et al. (2020) found differentially expressed miRNA in the saliva of piglets following tail-docking and castration. In this study miR-19b, miR-27b, and miR-365 increased in the saliva of piglets following treatment compared with sham treatments, and that these effects were mitigated by pre-emptive administration of anaesthetic drugs. It is difficult to determine, however, if this change was in response to the affective dimension of pain (emotion appraisal of the stimuli), or perhaps the body's normal response to tissue damage (i.e., an inflammatory response such as focal adhesion, cytokine and macrophage pathways). Furthermore, there seems to be variation in the time it takes for a miRNA response to occur, particularly in the case where miRNAs are measured in various types of body fluids or tissues. For example, miRNA changes were observed as early as 30-45 min in saliva following tail docking in piglets (Lecchi et al., 2020), and in jejunum tissue and serum up to 7 weeks post weaning stress (Tao and Xu, 2013). It may be that the physiological mechanisms by which miRNA are released into circulation (i.e., active secretion or membrane leaking (Miretti et al., 2020)), may differ in response timing to different stimuli as well as differences in tissue type. Additionally, there may be species specific variations in the types of miRNA conserved across species. Although it has been identified that a number of miRNAs are categorized by high sequence conservation across species (e.g., MiR-124) (Quach et al., 2009; Sun et al., 2012), there is evidence of species specific miRNA resulting from specific evolutionary lineages (Mor and Shomron, 2013). Furthermore, variability in miRNA genes between individuals has shown that polymorphisms in miRNA may result in phenotypic differences or disease susceptibility in some species (Jevsinek Skok et al., 2013; Zorc et al., 2015). However, despite increasing research in miRNA expression profiling, the genetic variability within miRNA genes has been much less explored, particularly in livestock (Zorc et al., 2015). Finally, the investigations of miRNAs as biomarkers in livestock have solely focussed on the associations between miRNA and negative welfare

states (i.e., pain and stress). To our knowledge, no study has investigated miRNA as a biomarker of a positive welfare state, in particular positive emotional states.

Conclusions

The inclusion of measures of animal emotion or affective state is now considered a key aspect of animal welfare assessment (Ede et al., 2019). Currently, a range of physiological and behavioural indices are used to infer the emotional states of animals. As highlighted, however, these measures often lack specificity, objectivity or are simply not suitable for a production environment. Physiological measures (including those classically associated with stress systems i.e., HPA and SAM activity), are effective at determining arousal but are often unable to distinguish between valences of affect. Conversely, behavioural parameters (including cognitive bias paradigms), can differentiate valences of affect; however, the time required to train the animals to perform the task, as well as the inconsistency in behavioural outcomes within and between individuals, makes this approach impractical for a production setting. In addition to this, the majority of the research in the field tends to focus on measures of negative affective states, with less focus on identification of measures of positive emotions in animals. Given that affective states in animals are now considered a fundamental aspect in animal welfare thinking, as well as the commitment to the facilitation and promotion of positive welfare states in animals from industry, the ability to assess the emotional states of animals (both positively and negatively valanced) accurately and objectively, remains a crucial dilemma.

As a consequence, there is a need to identify novel biomarkers for affective states in animals. These biomarkers must be robust, objective, easily collectable and have the ability to distinguish valences of affect. Recently miRNAs have emerged as diagnostic markers for a number of neurological conditions, including those associated with emotional processes (Malan-Müller et al., 2013; Wingo et al., 2017). MiRNAs have been implicated in a number of human affective disorders, contributing to the complex functioning of the brain, including neurodevelopment, neurogenesis and neurological disease (De Strooper, 2009; Bredy et al., 2011; Codocedo and Inestrosa, 2016). Once released into circulation,

miRNAs can be indicative of cellular processes and states, including those associated with emotional processes. Therefore, these molecules have the potential to become markers of the activities associated with emotional processes in animals, including livestock species such as the pig.

Chapter 2: Materials and Methods

Materials required:

Table 1: Indicates materials required for the experiments in Chapter 3

Item	Quantity
Animals	
Large White X landrace pigs (3 days old)	48
Drug Treatments	
Pentobarbital sodium	1mL/Kg
Bupivacaine	24mL
Treatment materials	
Bone clipper	1
Blood collection	
BD Vacutainer Heparin Tubes (4mL)	192
21G 1.5 inch hypodermic needle	192
Pipette	1
1000uL pipette tips (RNase free)	50
RNase-zap spray	1 bottle
Test-tube rack (12 X 4)	2
RNeasy protect animal blood tubes	192
Brain collection	
Dry ice	5kg
Plastic chopping board	2
Stainless steel tray	3
Bench coats	4/brain extraction
Paper towel	10 pack
Surgical scissors (sharp)	1
Surgical scissors (blunt)	1
Scalpel	2
Scalpel blades	20
Scales	1
Microtome blade	1 box
150mL specimen tub	120
Ruler	1
Chlorohex	1 litre
Biohazard bag	5
Waste bucket	2
Peristatic pump	1
Saline	24L
Humane killing	
21G 1.5inch needle	1
Disposable syringe (10mL)	1
Laboratory	
RNeasy protect animal blood kit	1
RNeasy universal mini kit	1
RWT buffer	500mL
1000uL pipette tips (RNase free)	1000
200uL pipette tips (RNase free)	1000

200uL pipette tips (RNase free)	1000
20uL pipette tips (RNase free)	1000
2uL pipette tips (RNase free)	1000
RNase-zap spray	1 bottle
Ethanol (100%)	500mL
Ethanol (70%)	500mL
2.0mL microcentrifuge tube sterile	500
High Sensitivity RNA Screen Tape	1box
Screen Tape Sample Buffer	1
Laboratory Vortex	1
Centrifuge	1
Chloroform	1 bottle
Fume hood	1
Tissue rupture/lysis system	1
QIAzol lysis reagent	1 bottle
Scales	1
Brain map	1

Table 2: Indicates materials required for the experiments in Chapter 4

Item	Quantity
Animals	
Large White X landrace gilts (18 weeks old)	8
Drug Treatments	
Fluoxetine hydrochloride	100mg/pig
Saline Sodium Chloride 0.9% (100mL)	1
Amoxycillin (long acting)	(10mg/Kg at 150mg/mL)
Xylocaine 5% Ointment	2 tubes
Heparin in Saline 0.9%	1 litre
Pentobarbital sodium	1mL/Kg
Catheters	
Catheter tubing (1.27 OD x 0.86 ID PVC tubing)	20 meters
Optiva 16G insertion needles	8
Blunt catheter needle (20G)	8
Disposable leur slip syringes (20mL)	8
Disposable leur slip syringes (10mL)	8
Disposable needle (16G)	8
Catheter stoppers	8
Stop Cox	8
Tensoplast (7.5cm x 2.4m elastic adhesive)	1.5roll/pig
Paper towel	5
Chlorhexidine Gluconate sponges	8
Chlorhexidine Gluconate	1litre
Betadine solution	1 litre
Ethyl Alcohol (80%) spray	1
Superglue tube	1
Scissors	1
Saline Sodium Chloride 0.9% (100mL)	1

Rope snare	1
Catheter pouch	8
Metal tray	1
Blood collection	
Saline Sodium Chloride 0.9% (1 litre)	5
Heparin in Saline 0.9% (1 litre)	1
BD Vacutainer Heparin Tubes (4mL)	500
Disposable syringe (5mL)	1000
Humane killing	
Disposable syringe (20mL)	8
Sharp knife	1
Laboratory	
Eppendorf microcentrifuge tube (2mL)	1000
Disposable transfer pipettes	1000
Cortisol Coated Tube RIA Kit	1000
20mL falcon tube	4
Pipette	1
50 µL pipette tips	1000
1000 µL pipette tips	1000
Test tube rack	3 X (12 X4) minimum
Laboratory vortex	1
Water bath (37 ± 1°C)	1
Aspirator with trap	1
Gamma counter	1
Centrifuge (4'C)	1

Table 3: Indicates materials required for the experiments in Chapter 5

Item	Quantity
Animals	
Large White X landrace pigs (3 weeks-20 weeks old)	24
Drug Treatments	
Pentobarbital sodium	1mL/Kg
Housing treatments	
Dog rope toys	12
Tennis balls	12
Basket balls	12
Hula Hoops	4
Metal chains	4
PVC piping	4
MnMs	5kg
Group stalls	4
Barron stalls	12
Heat lamps	4
Rubber matting	4
Judgment bias training/testing	
Arena	1

Chalk	1 box
Go-pro camera	2
Camera stand	2
Dog bowl (red, black, blue)	1 of each
Clicker	1
Stopwatch	2
Paper to record	1
Blood collection	
BD Vacutainer Heparin Tubes (4mL)	50
18G 1.5inch hypodermic needle	50
Pipette	1
1000uL pipette tips (RNase free)	50
RNase-zap spray	1 bottle
Test-tube rack (12 X 4)	2
RNeasy protect animal blood tubes	50
Rope snare	1
Brain collection	
Hydraulic bench	1
Liquid nitrogen	5 litres
Dewar	1
Dry ice	5 kg
Plastic chopping board	2
Stainless steel tray	3
Bench coats	4/brain extraction
Paper towel	10 pack
Surgical scissors (sharp)	1
Surgical scissors (blunt)	1
Scalpel	2
Scalpel blades	20
Bone saw	1
Scales	1
Microtome blade	1 box
Hammer	1
Chisel	1
150mL specimen tub	120
50mL specimen tub	12
Ruler	1
Chlorhexidine	1 litre
Biohazard bag	5
Waste bucket	2
Humane killing	
Butterfly needle system (18G)	4
Disposable syringe (20mL)	8
Sharp knife	1
Xylocaine topical anaesthetic (2%)	1 tube
Surgical tape	1
Laboratory	
RNeasy protect animal blood kit	1

RNeasy universal mini kit	1
RWT buffer	500mL
1000uL pipette tips (RNase free)	1000
200uL pipette tips (RNase free)	1000
200uL pipette tips (RNase free)	1000
20uL pipette tips (RNase free)	1000
2uL pipette tips (RNase free)	1000
RNase-zap spray	1 bottle
Ethanol (100%)	500mL
Ethanol (70%)	500mL
2.0mL microcentrifuge tube sterile	500
High Sensitivity RNA Screen Tape	1box
Screen Tape Sample Buffer	1
Laboratory Vortex	1
Centrifuge	1
Chloroform	1 bottle
Fume hood	1
Incubator (55-65'C)	1
Tissue rupture/lysis system	1
QIAzol lysis reagent	1 bottle
Heated magnetic stirrer	1
Magnetic stirring rod	1
Beaker (2 litre)	1
Conical flask (200mL)	1
2mm disposable biopsy punch	12
1mm disposable biopsy punch	12
Ammonium sulphate	9kg
Sodium citrate	1kg
0.5M EDTA	500mL
Milli-Q water	12 litters
Scales	1
Brain map	1

Experimental procedures:

Animal Procedures

Brain extraction (gilt)

1. Ensure brain in removed from the animal within 15 minutes of death in order to preserve the integrity of the RNA.
2. Directly following humane killing, place the pig on top of a hydraulic bench in a supine position.
3. Using a sharp knife, remove the head of the animal by cutting through the neck in a dorsal direction. (*Note: cut around the spine and then turn the head in a clockwise direction to separate the neck vertebrae*).

4. Place entire pig head on metal tray.
5. Using a scalpel and scalpel blade, remove the skin around the cranium.
6. Once the skull is exposed, use a bone saw to cut through the skill in a window formation (Figure 7). (*Note: Ensure to cut through the skull carefully so as not to damage the brain tissue with the saw)*
7. Expose the brain by breaking away pieces of the skull using a hammer and chisel (Figure 8).
8. Remove the brain gently. (*Note: in some cases, turning the head upside down will encourage the brain to come away from the skull).*

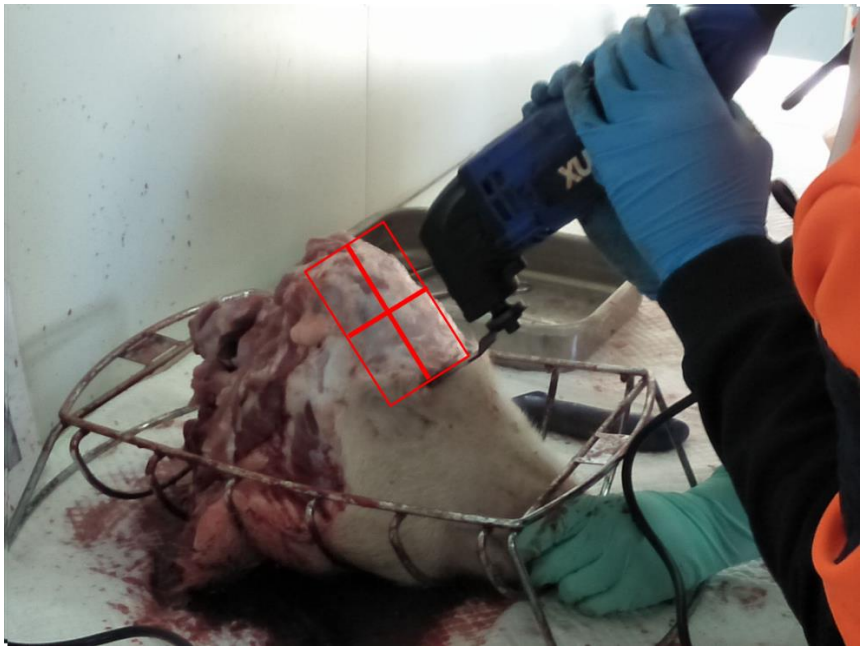

Figure 7: Indicates cutting of the skull in a window formation using a bone saw

.

Figure 8: Indicates exposure of the pig brain using hammer and chisel

Brain extraction (piglet)

1. Ensure the brain in removed from the animal within 15 minutes of death in order to preserve the integrity of the RNA.
2. Directly following humane killing, place the pig on top of the work bench in a supine position.
3. Perfuse the brain with ice cold saline by inserting a needle (blunt 18Gx 1.5inch), attached to a hose, into the right aorta and then the carotid artery.
4. Turn on perfusion machine.
5. Make an incision in the left ventricle to ensure the saline is circulating through the brain before returning to the heart.
6. Keep perfusion machine running until liquid from the left ventricle runs clear (approximately 1 minute).

7. Using a sharp knife, remove the head of the animal by cutting through the neck in a dorsal direction. (*Note: cut around the spine and then turn the head in a clockwise direction to separate the neck vertebrae*).
8. Using a scalpel and scalpel blade, remove the skin around the cranium (Figure 9).
9. Once the skull is exposed, use surgical bone clippers to cut through the skull in a circular manner.
10. Expose the brain by breaking away pieces of the skull using surgical forceps
11. Remove the brain gently. (*Note: in some cases, turning the head upside down will encourage the brain to come away from the skull).*

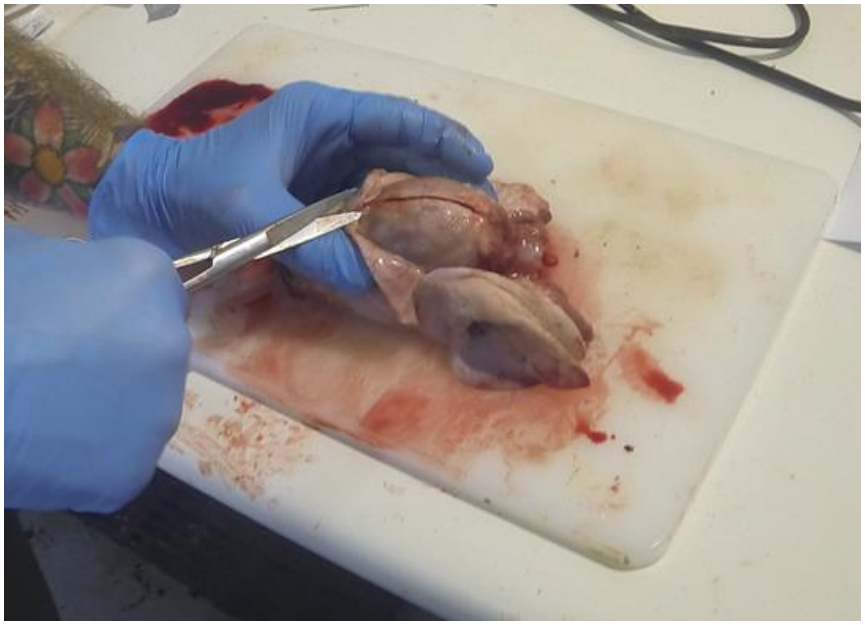

Figure 9 Indicates exposure of the skull by removing the skin on top of the cranium

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Tissue preparation for RNA analysis

1. Once the brain is removed, immediately submerge the entire brain into a tub with ice cold saline.
2. Place the whole brain on a chopping board (Note: place the chopping board on a bed of ice to ensure the working surface is cold prior to brain extraction.
3. Using a microtome blade, first cut the entire brain into two sagittal sections (Figure 10). (*Note: Do not use a saw action to slice the brain tissue, instead use long clean slices).*

4. Immediately place the right cerebral hemisphere into a 50mL specimen tub, submerge in liquid nitrogen and then store at - 80˚C for further analysis.
5. Using a microtome blade and a ruler, make 5mm coronal sections (rostral to caudal) along the left cerebral hemisphere.
6. Place each section into a labelled 150 mL specimen tub containing 100 mL RNA stabilising solution. Store at - 20˚C for further analysis.

Figure 10: A) entire pig brain; B) brain cut into right and left cerebral hemispheres, and C) left hemisphere prior to coronal sectioning

Laboratory Procedures:

Drug preparation

Fluoxetine hydrochloride

1. Prepare 4 × 20 mL syringes by adding 100 mg of fluoxetine hydrochloride powder to each syringe.
2. Immediately prior to drug administration add 10 mL saline to each tube.
3. Slowly invert tubes to ensure all fluoxetine is dissolved.
4. Using a 20 mL syringe, draw up 15 mL fluoxetine solution.
5. Immediately but slowly (5 mL / min) administer fluoxetine solution through ear vein catheter line.
6. Flush line with 2 mL saline.
7. Dispose of syringe once used.

RNA stabilizing solution preparation

This procedure makes 1 litre of RNA stabilising solution:

1. Weigh 700 g of ammonium sulphate and place into a 2-litre beaker.
2. Add 935 mL of milli-Q water.

3. Place beaker on magnetic heat stirrer and spin with magnetic flea (approximately 10 minutes until solution is dissolved).
4. Meanwhile, in a 200 mL conical flask, mix 1 M sodium citrate by adding 58.8 g of sodium citrate to 100 mL of milli-Q water.
5. Once ammonium sulphate has dissolved, add 25 mL of the sodium citrate solution and 40 mL of 0.5 M EDTA.
6. Mix well and distribute 100 mL of the RNA stabilising solution into 150 mL specimen tubs.
7. Store at room temperature until use.

Radio-immunosorbent Assay (RIA)

1. For detection of cortisol in plasma, ensure collection of whole blood into 4 mL BD Vacutainer Heparin tubes.
2. Following blood collection, centrifuge BD Vacutainer tubes (10 min at 1000 × g).
3. Using a disposable pipette, transfer the top plasma layer (clear) into labelled 2 mL microcentrifuge tubes and store at - 20'C until RIA analysis *(Note: a minimum of 25 µL of plasma is required to perform cortisol RIA analysis)*.
4. Prior to RIA analysis, label coated tubes for standards, controls and samples.
5. Bring all standards, samples, controls, coated tubes, and CORTISOL-125I (tracer), to room temperature prior to use.
6. Turn on the water bath and set to 37'C.
7. Turn on the gamma counter.
8. Prepare standards to working concentrations of 0, 1.0, 3.0, 10, 30 and 100 µg / dL.
9. Pipette 25 µL of each standard, control and patient sample into its respective coated tube.
10. Add 1.0 mL of the tracer to all tubes and vortex for 15 sec.
11. Incubate each tube for 45 min at 37 ± 1°C.
12. Working behind a fume hood, aspirate or decant the contents of each tube. (*Note: If decanting, blot the rim of the tubes on absorbent paper before turning upright.)*

13. Immediately count the tubes in a gamma counter calibrated for tracer 125I.
14. Discard all leftover reagents in the biohazard bin.

Extraction of brain RNA

1. Remove specimen tubs containing 5 mm thick coronal brain sections from the freezer and thaw to room temperature.
2. Using the stereotaxic atlas of the pig brain developed by Félix et al. (1999), identify the amygdala (Figure 11).
3. Prior to extraction, bring all reagents from RNeasy plus the universal mini kit to room temperature and prepare stock solutions according to the manufacturer's instructions.
4. Turn on centrifuge and set to 4°C.
5. Clean all equipment with RNase-zap spray prior to use.
6. If working alone, it is recommended to extract total RNA from no more than six samples at a time.
7. Label 6 × 3 mL round-bottom test tubes and add 2 mL QIAzol to each.
8. Using a 2 mm biopsy punch, remove tissue sample from withing the amygdala from the coronal section, weigh and place in a test tube (Figure 12).
9. Immediately disrupt the tissue and homogenise the lysate using the tissue lysis system, a process usually taking 30-40 sec. (*Note: clean the rupture probe between samples using RO water)*.
10. Place tubes on ice on the bench for 5 min to promote dissociation of nucleoprotein complexes.
11. For each sample, pipette 1 mL of the sample into a 2 mL microcentrifuge tube and store at -80°C.
12. Pipette the remaining 1 mL into a labelled 2 mL Eppendorf tube, add 100 µL gDNA eliminator solution, secure cap and vortex for 15 sec.
13. Working behind a ventilation hood, add 180 µL of chloroform, vortex for 15 sec and place on the bench at room temperature for 3 min.

14. Centrifuge samples at 4°C (20000 × g) for 15 min. *Note: this step should cause samples to separate into 3 phases, an upper aqueous phase, a middle white interphase and a lower red phase. The aqueous phase should be approximately 600* µL.
15. Set centrifuge to room temperature.
16. Transfer the upper aqueous phase into a new, labelled 2 mL microcentrifuge tube, add 1 volume (usually 600 µL) of 70% ethanol, pipette up and down and vortex for 15 sec.
17. Transfer 700 µL of the sample into a RNeasy mini spin column, then place in a 2 mL collection tube.
18. Centrifuge at room temperature (8000 × g) for 15 sec.
19. Carefully remove the column and discard the flow through.
20. Repeat steps 18-19 with any remaining samples.
21. Add 700 µL of RWT buffer to the RNeasy spin column and centrifuge at room temperature (8000 × g) for 15 sec.
22. Carefully remove the Column and discard the flow through.
23. Add 500 µL of RPE buffer to the spin column and centrifuge at room temperature (8000 × g) for 15 sec.
24. Carefully remove the Column and discard the flow through.
25. Add 500 µL of RPE buffer to the spin column and centrifuge at room temperature (8000 × g) for two min.
26. Carefully remove the Column and place back into a new 1.5 mL collection tube.
27. Add 30 µL - 50 µL of RNase-free water directly into the spin column.
28. Close the lid and centrifuge at room temperature (8000 × g) for one min to elute the RNA.
29. Immediately test the integrity of the RNA using RNA screen tape assay.
30. Store total RNA at -80°C.

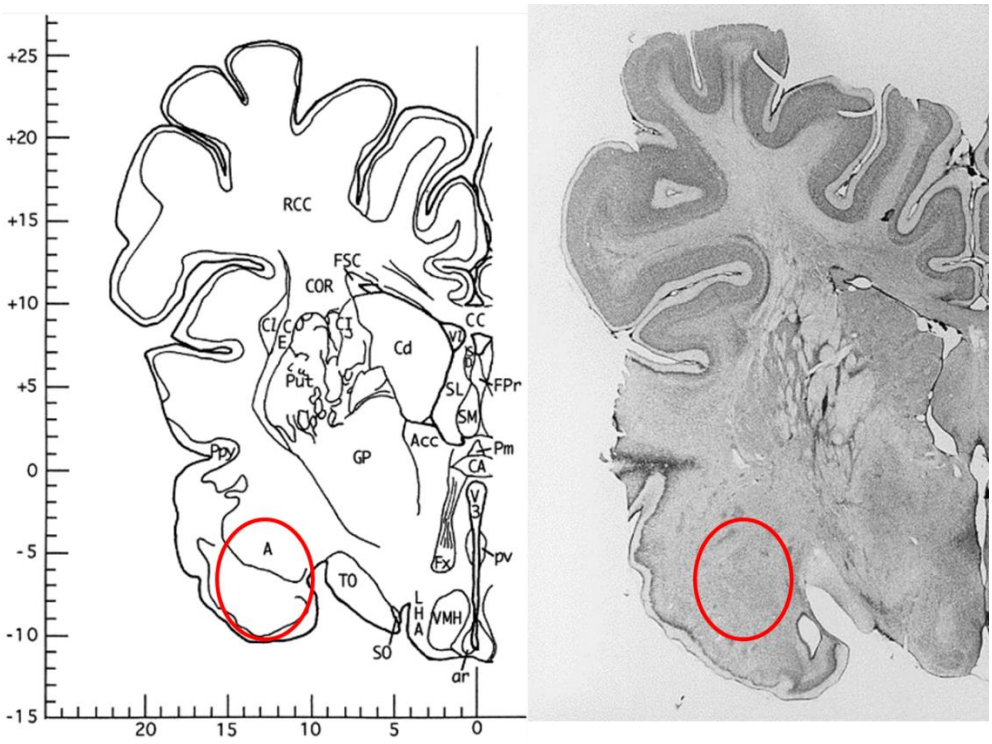

Figure 11: Indicates identification of the amygdala (red circle), in the coronal section of a pig brain. Region identified and figure adapted from Félix et al. (1999).

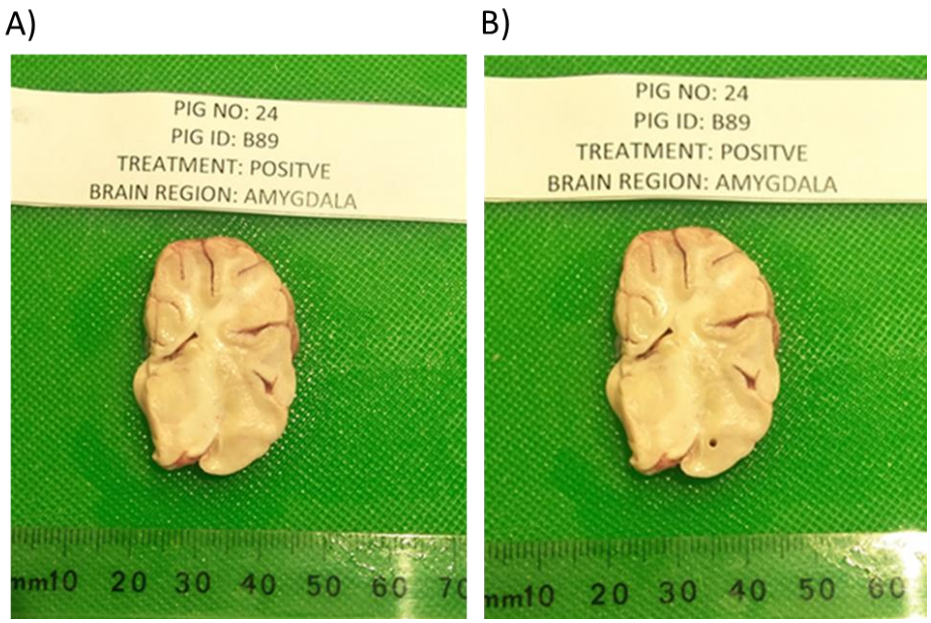

Figure 12: Indicates A) the left hemisphere coronal section of a pig brain with the amygdala intact, and B) Indicates the left hemisphere coronal section of a pig brain with the amygdala dissected.

Extraction of whole blood RNA

1. Remove RNA, protect animal blood tubes from freezer, and thaw at room temperature for 2 h.

2. Set incubator to 55°C
3. Prior to extraction, bring all reagents from the RNeasy protect animal blood kit to room temperature and prepare stock solutions according to the manufacturer's instructions.
4. Centrifuge animal blood tubes at room temperature (5000 × g) for 3 min.
5. Decant the supernatant and add 450 µL of RNase-free water to the pellet and vortex until dissolved.
6. Centrifuge animal blood tubes at room temperature (5000 × g) for 3 min.
7. Decant the supernatant and add 240 µL RSB buffer and vortex until dissolved.
8. Transfer sample to a new 2 mL microcentrifuge tube and separately add 200 µL RBT buffer and 20 µL of proteinase K, vortex for 15 sec and incubate at 55°C for 10 min.
9. Remove samples and set incubator to 65°C.
10. Pipette sample into QIAshredder column.
11. Centrifuge at full speed for 3 min.
12. Transfer the flow-through to a new 1.5 mL tube. *Note: do not disturb the pellet.*
13. Add 690 µL of 100% ethanol and vortex for 15 sec.
14. Pipette 700 µL of the sample into a RNeasy mini-elute column.
15. Centrifuge at room temperature for 1 min (8000 × g) and discard flow-through.
16. Repeat steps 14-15 until all of the sample is used.
17. Add 350 µL of RWT buffer, centrifuge at room temperature for 15 sec (8000 × g), and discard the flow-through.
18. For each sample, mix 10 µL of DNase 1 stock solution with 70 µL of RDD buffer (80 µL total). *Note: do not vortex.*
19. Pipette 80 µL of DNase 1 incubation mix (from step18) directly into the spin column, incubate at room temperature for 15 min.

20. Add 350 µL of RWT buffer to the column, centrifuge at room temperature for 15 sec (8000 × g), discard the flow-through.
21. Add 500 µL of RPE buffer to the column, centrifuge at room temperature for 15 sec (8000 × g), discard the flow-through.
22. Add 500 µL of 80% ethanol to the column, centrifuge at room temperature for 2 min (8000 × g), discard the flow-through
23. Carefully remove the column and place into a new 2 mL microcentrifuge tube (lid cut off).
24. Centrifuge at room temperature for 5 min (10000 × g) and discard the flow-through.
25. Carefully remove the column and place into a new 1.5 mL microcentrifuge tube and add 30 µL of REB buffer directly into the column, centrifuge at room temperature for 1 min (8000 ×g).
26. Incubate the eluted RNA at 65°C for five min.
27. Immediately test the integrity of RNA using RNA screen tape assay.
28. Store total RNA at -80°C.

Total RNA concentration and integrity testing

1. Allow reagents to equilibrate at room temperature for 30 min.
2. Thaw total RNA samples of ice or perform on ice immediately following extraction.
3. Prepare sample by mixing 5 µL of RNA sample buffer and 1 µL of RNA sample.
4. Vortex and spin down at 200 ×RPM for one min.
5. Load samples into tape station instrument.
6. Place RNA screen tape into instrument and specify file name.
7. Click start.

Chapter 3: MicroRNA as a biomarker of negative emotional state in pigs

Contextual Statement

It is generally agreed that the assessment of animal welfare should consider incorporation of emotional states. Negative emotions such as pain can have detrimental effects on livestock productivity, as well as implications for animal welfare and public perception. Consequently, there are economic and social incentives in being able to identify and mitigate animal pain on farm.

To date, measures to assess the physiological aspects of pain and their effects on emotional processing in animals are currently limited. Current measures used in the assessment of pain in animals tend to rely on a number of behavioural and physiological indices. These methods can be seen as subjective or lacking in sensitivity, can be variable in outcome, and are unable to distinguish between the type of pain or degree of pain experienced by the animal. Furthermore, these measures may more closely reflect arousal responses rather that emotional responses to pain given their change in activity during both positive and negative experiences.

In light of these issues with current pain assessment methods, new methods of accurately assessing pain are needed. Recently, miRNAs have emerged as potential biomarkers of the neural activity associated with pain, including the affective-dimension of pain processing. These molecules regulate gene expression and are implicated in neuronal network plasticity within the nervous system. They are easily measured in blood, saliva, urine and faeces, and therefore have the potential to be biomarkers of pain processes in animals. In **Chapter 3,** I investigated if miRNAs measured in the blood of piglets could be used as markers of the neural activity in the brain associated with pain processing in response to tail docking. I further investigated if changes in brain miRNAs were similar to those in blood, thus indicating the efficacy of circulating miRNA as a proxy marker of brain miRNA activity associated with pain processes. This chapter is formatted for publication only and not yet submitted for publication.

Body of work

MicroRNAs as biomarkers of pain processing in piglets following tail-docking

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Abstract

Background: The accurate determination of the welfare state of animals requires an assessment of their emotional or affective state. Negative emotional states such as pain are of particular concern in livestock industries due to their damaging effects on productivity, welfare and public perception. Behavioural and physiological indices are often used to infer emotional states in animals but can be challenging to interpret. Therefore, there is a need to develop novel ways to accurately and objectively measure emotional states of animals. Here we aimed to develop miRNA-based biomarkers of pain in pigs. We hypothesized that pigs exposed to tail-docking would exhibit altered microRNA expression in the brain and blood, and that this response would be mitigated by local anesthetic with analgesic properties. **Methods**: Forty-eight Large White X Landrace piglets (24 male, 24 female) were randomly assigned to four treatments (n=6/treatment). Treatments were 1) no tail-docking (Control); 2) tail-docking (TD); 3) tail-docking plus anesthetic (TDA) and 4) anesthetic only (A). Brain samples (Amygdala, AMY; Periaqueductal grey, PAG) +6h post treatment) and blood (-1h pre- and +1h, +2h and +6h post treatment) were collected and miRNA expression profiles obtained using Affymetrix gene chip technology. Validation of differentially expressed miRNA identified by gene chip (miR-7a, miR-99, miR-124, miR-129, miR-137, miR-381, let-7 and mir-412) was performed using TaqMan probes. **Results**: miR-412-3p and miR-7a-2-3p indicated differential expression within the PAG (H (2) = 6.538, P=0.038 and H (2) = 6.229,

P=0.044 respectively). Following TD and TDA, decreased expression of miRNA-412-3p was observed compared to Control animals (Z = -1.992, *P* = 0.046 and Z = -1.992, *P* = 0.046 respectively). The expression of miR-7a-2-3p in TD animal was decreased compared to TDA animals (Z = -1.992, *P* = 0.046).

No significant treatment effect was observed for any candidate miRNA in AMY or blood (P>0.05). **Conclusions:** Although MiRNA expression was altered in the PAG of pigs following tail-docking, this change was not reflected in miRNA of blood. Thus, the utility of miRNA as a diagnostic tool to assess pain requires further study. However, our data provide a basis for further investigation into the use of miRNA as diagnostic tools of animal emotional state.

Keywords: MicroRNA, Pain, Brain, Blood, Welfare, Tail-dock, Affect, Emotion.

1 Introduction

The ability to ascertain the welfare of animals relies on accurate assessment of their emotional or affective states. Emotions are complex, and can be defined as intense but short lived physiological and psychological responses which are associated with specific bodily changes (Boissy et al., 2007). Negative emotional states such as pain are of particular concern in livestock industries due to the damaging effects on productivity, welfare and public perception. Therefore, there are economic and social license-based incentives in being able to identify and mitigate animal pain on farm.

The relationship between the physiological aspects of pain and its effect on emotional processing is complex. Pain has been defined as "An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (Lumley et al., 2011; Raja et al., 2020). Thus, pain is partially an emotional experience that gives rise to rapid physiological and behavioural changes (Rhudy and Meagher, 2001; Lumley et al., 2011). Human clinical practice considers three dimensions of the overall pain experience; 1) the sensory-discriminative dimension associated with noxious stimuli identification, i.e. location, timing and physical characteristic, 2) the affective-motivational dimension associated with the unpleasantness towards the noxious stimulus and activation of defense behaviours, and 3) the cognitive evaluative dimension associated with appraisal or meaning of the consequences of the pain (Melzack and Casey, 1968; Lumley et al., 2011). During noxious stimulation to the body, pain information is transmitted from peripheral nociceptors along the lateral- and medial-spinothalamic tract, associated with the sensory and affective components of pain, respectively. Projections to lateral nuclei of the thalamus and somatosensory cortices are responsible for the sensory quality of pain, while the emotional experience of pain occurs following medial projections within the spinothalamic tract to several limbic structures including the limbic cortex, periaqueductal grey (PAG), amygdala and anterior cingulate cortex (Lumley et al., 2011).

In mammals, the components involved in the transduction, transmission and perception of noxious stimuli are similar and although non-human animals may not be able to perceive or communicate pain in the same way, it is likely they experience pain just as humans do (Ahrens et al., 2007). Despite this similarity in pain experience, the assessment of pain in animals is difficult, perhaps arising from the high inter- and intra-individual variation in response to

77 painful stimuli (Anil et al., 2005). Currently the assessment of pain in animals relies on
78 behavioural and physiological indices. Specifically in pigs, pain related behaviours such as
79 body posture/movement (Noonan et al., 1994; Edwards et al., 2009), vocalizations (Taylor and
80 Weary, 2000; Briefer and Le Comber, 2012), and grimace scoring (Di Giminiani et al., 2016;
81 Akintola et al., 2017; Reid et al., 2018), are often used as indicators as they are relatively easy
82 to apply. However, these methods could be viewed as subjective or lacking in sensitivity,
83 variable in outcome (Wilson and Mogil, 2001; Stasiak et al., 2003) and unable to distinguish
84 between the type of pain or degree of pain experienced by the animal. A range of physiological
85 parameters traditionally associated with stress, e.g., glucocorticoid and catecholamine
86 production (Prunier et al., 2005) or ANS function, e.g., heart rate and HRV, as well as markers
87 of inflammation such as pro-inflammatory cytokines (Zhang and An, 2007) have also been
88 used to determine pain by inference (Hernandez-Avalos et al., 2019). However, these systems
89 may become activated during both positive and negative stimuli and may not always be related
90 directly to pain or its severity. Therefore, such measures may not always provide clear guidance
91 for pain mitigation, e.g., through analgesic intervention. The assessment of pain would
92 therefore benefit from new markers and indicators of pain. Such measures need to be
93 applicable in a farm setting, relatively non-invasive, and provide an objective measure of the
94 affective experience in the same way people do (Ahrens et al., 2007).

95 Recently, microRNA (miRNAs), have emerged as potential biomarkers of neural activity
96 associated with pain due to their role in the regulation of gene expression and neuronal network
97 plasticity within the nervous system and distinct expression signatures (López-González et al.,
98 2017; Popa et al., 2020). These short non-coding 22-24 nucleotides long RNA molecules have
99 the ability to modulate or inhibit the expression of multiple genes post-transcriptionally. The
100 dysregulation of individual or entire families of miRNA have been associated with a number
101 of human conditions including chronic pain disorders (Descalzi et al., 2015), inflammatory and
102 neuropathic pain (Bai et al., 2007; Andersen et al., 2014), complex regional pain syndrome
103 (Orlova et al., 2011), osteoarthritis (Beyer et al., 2015) and migraine (Andersen et al., 2016).
104 More recently these molecules have been used as markers of negative welfare states in animals
105 including pain in horses with laminitis (Lecchi et al., 2018), inflammation in pigs following
106 tail-docking and castration (Lecchi et al., 2020), mastitis detection in cattle (Srikok et al.,
107 2020), and dermal wound healing in mice (Jin et al., 2013). Pain models in rodent have shown
108 altered miRNA expression to brain areas associated with the emotional components of pain
109 perception (Bai et al., 2007; Aldrich et al., 2009; Imai et al., 2011; Poh et al., 2011). A potential
110 advantage of miRNA markers for pain is that they are produced across tissues and organs,
111 including the brain, are secreted or excreted into extracellular space (Pigati et al., 2010;
112 Weiland et al., 2012; Cho et al., 2019), and can be measured relatively non-invasively in blood,
113 urine and saliva (Sheinerman and Umansky, 2013; Burgos et al., 2014; Di Ieva et al., 2014;
114 Wang et al., 2016).

115 Tail docking is a routine animal husbandry practice in pigs and aimed at reducing the
116 occurrence of tail biting. The procedure is performed in piglets, often without the use of
117 anesthetic or analgesic and therefore pain, stress and discomfort associated with the procedure
118 is considered a significant welfare concern (Backus and McGlone, 2018). Here we investigated
119 if miRNA measured in the brain and blood of piglets could be used as a marker of the neural
120 activity in the brain associated with pain processing in response to tail docking. To validate

that differential expression was modulated by pain, we applied an analgesic to test for dampening of the response. We hypothesized that 1) the pain in response to tail docking would result in differentially expressed miRNA in both the brain and blood of piglets, and that this expression would be modulated by local anesthetic, 2) that the expression of miRNAs in brain and blood follow the same pattern, thus indicating that circulating miRNA could be used as a proxy markers of brain miRNA expression associated with pain processes.

2 Materials and Methods

2.1. Animals and housing

This study was conducted at the Roseworthy piggery, South Australia between July 1st and July 4th, 2017. Forty-eight Large White X Landrace piglets (24 male/ 24 female) were selected from 12 multiparous sows (parity 2-4), at day 3 of life. Piglets were pseudo-randomized into one of four treatments so that each treatment (n=12) contained 6 males and 6 females. Treatments were 1) no tail docking (Control); 2) tail docking (TD); 3) tail docking plus analgesic (TDA) and 4) analgesic (A). Tail docking was performed under manual restraint using bone pliers where the tail was cut leaving 3.8cm (1.5inch) of tail remaining. Animals in Control treatment were subjected to manual restraint only for 30 seconds to mimic time taken to tail-dock. Piglets in TDA treatment received local anesthetic 15 minutes prior to tail docking, and again at +3h following tail docking to ensure the drug was effective throughout the experiment. Piglets in treatment A received analgesic once at the time of treatment and again +3h following treatment. Based on veterinary advice and following manufacturer's recommendations, we used 0.5mL bupivacaine hydrochloride (BH) at 2.5mg/mL injected subcutaneously twice (dorsal and ventral) at the base of the piglet's tail. All animal procedures were approved by the University of Adelaide Animal Ethics Committee (S- 2017-051) and conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013), and the Animal Welfare Act, 1985 (SA).

2.2. Treatment protocol

For one hour prior to and +1h, +2h and +6 h following treatment, animals were manually restrained for approximately 1 min in dorsal recumbency and 2.5mL of blood was taken from the anterior vena cava of each piglet using vacutainer needle (21G x 1 inch) attached to 5ml EDTA tube (Zebravet, Australia). Five-hundred µL of each blood sample was immediately aliquoted into a 2mL RNeasy protect animal blood tube (QIAGEN, Chadstone, Australia; cat. no. 76554), stored at 4°C for 24 hours and then frozen at -80°C as per manufacturer's instructions until RNA extraction. Six hours following treatment, 24 animals were randomly selected and returned to the herd, while the remaining 24 animals (6/treatment, 3 male and 3 female) were euthanized with 1 mL/10 kg of pentobarbital sodium iv (Virbac Pty Limited, Milperra, NSW, Australia), followed by exsanguination. The brain was perfused with ice cold saline by inserting a needle (blunt 18G x 1.5inch), attached to a hose, into the right aorta and then the carotid artery. A small incision was made in the left ventricle to ensure the saline was circulating through the brain before returning to the heart. The perfusion machine remained on until liquid from the left ventricle was running clear (approximately 1 minute). The whole brain was then removed and 5mm coronal sections sliced rostral to caudal were immediately placed in a 200mL specimen tub containing 100mL of RNA-stabilizing solution and stored at -20°C.

2.3. Tissue sampling and extraction of total RNA

A stereotaxic atlas of the pig brain (Félix et al., 1999), was used to identify regions of the brain including the Amygdala (AMY) and Periaqueductal Grey (PAG). Using a biopsy punch (Ted Pella, Redding, Canada; cat # 15110-20), a 2mm tissue sample was taken from each region in the left cerebral hemisphere (Figure 1). Total RNA was extracted from blood and tissue samples using RNeasy protect animal blood kit (QIAGEN, Australia; cat. # 73224), and RNeasy plus Universal kit (QIAGEN, Chadstone Australia; cat. # 73404) respectively, according to manufacturer's guidelines and kit protocol. miRNA quantity and quality was assessed using RNA tape station (Agilent technologies, Mulgrave, Australia; cat. # 5067-5579), where RIN values greater than 7.8 were used in analysis.

2.4. Expression profiling of miRNA

Differential expression of miRNA was initially investigated from brain (AMY and PAG, n=6/treatment (3M:3F)) and blood (-1 and +6 following treatment, n=6/treatment (3M:3F)) from 24 animals using Affymetrix gene chip technology (GeneChip™ miRNA 4.0 Array, Thermofisher Scientific, Australia) in accordance with manufacturer's instructions. Poly(A)Tailed, biotin-labelled miRNA was prepared from 500ng total RNA samples, using the FlashTag Biotin HSR RNA Labeling Kit for GeneChip miRNA Arrays (Thermofisher Scientific, Australia). Labelled RNA samples were hybridised to GeneChip miRNA v4.0 arrays. Arrays were incubated in a GeneChip Hybridization Oven 645 for 16 hours at 48°C. Array washing and staining was performed on the GeneChip Fluidics Station 450, and scanned using GeneChip Scanner 3000 7G. CEL files were generated using Affymetrix GeneChip Command Console Software.

2.5. Statistical analysis of Affymetrix data and miRNA candidate selection

Affymetrix data was imported as CEL files into genomic software package TAC (Transcriptome analysis console 4.0, Applied biosystems, Thermofisher Scientific). A custom comparative analysis was conducted for brain (AMY and PAG, n=6/treatment), and blood (-1- and +6-hours following treatment, n= 6/treatment), parameters using an ANOVA with eBayes analysis to correct for variance due to low animal numbers. Pairwise and interaction comparisons were conducted between groups and included fixed effects of time, sex and treatment. Differences were considered significant when a gene-level fold change of < -2 or > 2 occurred with an FDR adjusted P-value less than 0.05 (FDR $P<0.05$). Seven candidate miRNAs were selected for q-PCR validation firstly by significance level, and then prioritized based on their involvement in pain processing previously published using data from processes in PubMed and MiRbase databases. Additionally, two novel miRNAs were selected based on level of significance derived from the array data, but had not been previously shown to be involved in pain processing, giving a total of 9 miRNA candidates selected for validation.

2.6. MiRNA candidate validation with RT-qPCR

Validation of selected candidate miRNA was performed on brain (PAG, n=6/treatment), and blood (-1, +1, +2, +6 hours following treatment, n= 6/treatment), for animals exposed to Control, TD and TDA treatments only. The parameters chosen to be included for validation of candidate miRNA (treatment, bleed number and tissue type), were based on findings from the

TAC analysis software as per results presented below. Treatment groups (A) and tissue type (AMY) were removed from analysis as no significant effects on miRNA expression were observed in the TAC analysis (FDR P>0.05). MiRNA validation was performed using custom TaqMan™ MicroRNA Reverse Transcription Kit (Thermofisher Scientific, Australia; cat # 4366597) according to the manufacturer's instructions. cDNA was normalized to 5ng/µL and twelve sets of RT reactions were prepared using 2µL of total RNA (total of 10ng). The TaqMan® Fast Advanced Master Mix (cat # 4444964) was used to perform the Real Time PCR on the QuantStudio 12K Flex instrument, with the following modifications to the recommended protocol: All samples, plus 3 no template controls, were arrayed in triplicate into a 384-well optical plate, therefore the reaction volume was set to 10µL. Each reaction plate profiled a single miRNA candidate, eliminating the need for inter-plate calibrators. The default cycling conditions were used; however, the cycle number was set to 50 due to the low abundance of some miRNAs. All reverse transcription reactions were performed in triplicate. Probes included three endogenous control miRNA (ssc-miR-17, ssc-miR-103 and ssc-miR-10) previously reported in pig , and 9 candidate miRNA probes; mml-miR-99b-3p (Thermofisher Scientific, Australia; cat # 4440886), mmu-miR-137-5p (Thermofisher Scientific, Australia; cat # 4440886), mmu-miR-7a-2p* (Thermofisher Scientific, Australia; cat # 4440886), hsa-miR-99b* (Thermofisher Scientific, Australia; cat # 4427975), hsa-let-7e* (cat # 4427975), hsa-miR-129 (cat # 4427975), hsa-miR-124* (Thermofisher Scientific, Australia; cat # 4427975), hsa-miR-412 (Thermofisher Scientific, Australia; cat # 4427975), and mmu-miR-381* (Thermofisher Scientific, Australia; cat # 4440886).

2.7. Statistical analysis of TaqMan data

Raw data from RT-qPCR was checked for basic quality metrics on the 12K Flex software, and the relative quantification score (RQ) was calculated using the Vandesompele relative gene expression method (Vandesompele et al., 2002; Hellemans et al., 2007) in Microsoft Excel. Relative gene expression (RGE) was then calculated against the geometric mean of the house genes. Samples with a Ct score lower than 40 were not included in the analysis. Data were tested for normality and non-parametric analysis was applied where appropriate in statistical software package IBS SPSS. Both Kruskal-Wallis and Wilcoxon signed-rank tests were used to determine differences between treatments for each miRNA candidate individually for brain (PAG, n=6/treatment) and at each bleed (-1, +1, +2, +6 hours following treatment, n= 6/treatment). Data visualization was performed in Graphpad Prism 7.

3 Results

3.1 Affymetrix chip miRNA expression profiling and candidate MiRNA selection

Within the PAG, a significant difference in miRNA expression was observed between Control and TD animals where 127 miRNA species were differentially expressed at the FDR adjusted significance level (FDR P<0.05, Figure 2A). However, after correcting for species-dependent redundancy of miRNA on the array a total of 51 differentially expressed miRNA was observed (Table 1) and 9 candidate miRNAs were selected for validation following the selection criteria mentioned in section 2.5 (Figure 3). Animals in TDA and A treatments did not differ in miRNA expression in the PAG compared to Control animals (Figure 2B, C). Treatment had no

significant effect on miRNA expression in the AMY (Figure 2D-F) or in blood (-1- and +6-hours following treatment) (FDR P>0.05).

3.2 TaqMan Analysis

Results from the TaqMan analysis indicated that two out of the 9 selected miRNA candidates (miR-412-3p and miR-7a-2-3p) showed differential expression within the PAG against the housekeeping genes (H (2) = 6.538, P=0.038 and H (2) = 6.229, P=0.044 respectively, Figure 4A). Following TD and TDA, there was a significant decrease in expression for candidate miRNA-412-3p compared to Control animals (Z = -1.992, P = 0.046 and Z = -1.992, P = 0.046 respectively, Figure 4B). No significant difference was observed in miRNA-412-3p expression within the PAG between TD and TDA animals (Z = -0.314, P = 0.753). Conversely, there was no difference in the expression of miR-7a-2-3p between TD and Control animals (Z = -0.943, P = 0.345) or TDA and Control animals (Z = -1.782, P = 0.075), however there was decreased expression of miR-7a-2-3p in TD animal compared to TDA animals (Z = -1.992, P = 0.046, Figure 4C). In blood no significant treatment effect was observed for any candidate miRNA at any bleed point (P>0.05, Figure 5).

4 Discussion

In the present study we observed differential expression in 59 miRNA species within the PAG between Control and TD animals. Validation of selected candidate miRNAs miR-7a, miR-99, miR-124, miR-129, miR-137, miR-381, let-7 and Mir-412 revealed that two miRNAs (miR-412 and miR-7a) were differentially expressed in the PAG. Significant downregulation of miR-412 was observed in pigs exposed to TD and TDA compared to control animals but no difference was observed between TD and TDA. Conversely, miR-7a indicated downregulated expression between TD and TDA animals but no difference in the expression levels was observed between TD or TDA and Control animals. Furthermore, there was no significant effect of treatment on miRNA patterns within the AMY. No effect of treatment was observed on miRNA expression in blood at any of the sampling timepoints.

The results of this study were partly consistent with our hypothesis that pain experienced in the animal would elicit an effect measurable as differences in miRNA expression in regions of the brain associated with pain processing and that these effects would be mitigated by the administration of analgesic in TDA animals. Although no differences in miRNA expression were observed in AMY tissue, differences in expression of miR-412 and miR-7a were observed in the PAG. Following treatment, mir-412 was significantly downregulated in comparisons between TD and Control and TDA and Control, suggesting involvement of this particular miRNA in pain processes in the brain. Interestingly however, we observed no difference in miR-412 expression between TD vs. TDA groups. We would have expected the expression levels between TD vs. TDA to be different, providing evidence that the administration of BH was mitigating the pain experienced of the animal. In human, BH is commonly used as a local analgesic, where it acts by blocking sodium influx into nerve cells, consequently inhibiting the conduction of pain signals to the brain (Chahar and Cummings, 2012). The onset of action for BH is rapid; however, the duration of its effects is relatively short, i.e., less than 8 h with a half-life of 2.7 h in human adults (Hu et al., 2013). Levels of bupivacaine in human plasma were shown to peak between 30-45 min after administration, followed by a decline over 3-6 h (Hu

et al., 2013). Similar anatomy and physiology, including skin structure and wound healing capacity, between human and pig (Sullivan et al., 2001; Zel et al., 2019), as well as similarity in pain pathways across mammalian species (Grubb and Lobprise, 2020), suggest the efficacy of anaesthetic treatments are likely to be comparable. In our study BH was first administered to pigs 15 min prior to treatment and thus its onset of action would have been in effect at the time of treatment, and, presumably, for the first 3 h following treatment. However, effects of the second dose of BH at + 2.75 h post tail docking may have worn off by the time brain samples were collected at 6 h post tail docking. Therefore, any changes in miRNA expression in the TDA treatment may have in fact been similar to responses elicited in the TD treatment group. A relatively short duration of drug efficacy has been observed in pigs where BH was used as a sensory nerve block following invasive dental surgery (Holman et al., 2014). The study found that injections of BH lasted only 1-3 h before the animals responded to the sensory stimulation with a reflexive movement. Furthermore, although BH is a well-established local anaesthetic used in veterinary medicine, a relatively short duration of action of < 6 h has also been observed in sheep (Lucky et al., 2007), rabbits (Dollo et al., 2004), cattle (Nakthong and Homat, 2016), and horses (Le et al., 2020). Subsequently, in order to ensure that the pain signals from tail-docking were sufficiently blocked at the time of tissue collection in TDA treated animals, it would have been beneficial to provide piglets with either an additional dose of BH closer to the time of brain tissue collection, or to perhaps administer an extended-release form of bupivacaine, i.e., bupivacaine liposome injectable suspension, which has been reported to provide longer lasting analgesic effects (Lascelles et al., 2016; Alter et al., 2017).

It should also be noted that there were also differences in both the type and method of administration of pain relief given. Administration of local anesthetics can be applied topically, intradermally or subcutaneously to anesthetize local tissues (Latham and Martin, 2014). Although direct infiltration on local anaesthetic into the subcutaneous layer is considered effective in blocking pain signals from free nerve endings within these layers, pharmacokinetic differences between subcutaneous and intradermal administration of the bioavailability of certain drugs has been observed, including differences in anaesthetic potency, time of onset, and metabolism and absorption (Becker and Reed, 2012; Milewski et al., 2015). In the study by Lecchi et al. (2020), pigs were administered with both local anesthetic (procaine plus adrenaline) and anti-inflammatory (meloxicam) drugs which were given intradermally and intramuscular respectively. Here we administered BH subcutaneously and perhaps the combination of drugs used by lecchi et al. (2020), as well as the route of administered, was more effective at mitigating the pain caused by the tail-docking treatments. Therefore, pharmacokinetic differences between drug type and administration method should be considered in future studies.

To our knowledge changes in the expression of miR-412 in response to pain processes has not been reported previously. Until now the majority of research citing changes in expression levels of miR-412 have been focused on cancer biology, where it has been used as a predictor of cancer progression (Lenherr et al., 2017) with high expression in extracellular vesicles from patients with oral squamous cell carcinoma (Gai et al., 2018). The selection of this novel miRNA was based solely on its significance level in the array data analyses. Although in this study mir-412 is a promising biomarker for processes related to pain following tail-docking in

pigs, further research is required to understand what potential target genes are involved and which signaling molecules and pain pathways may be affected.

Within the PAG miR-7a expression was downregulated between TD vs. TDA animals but no difference in expression levels was observed between TD or TDA vs. Control animals. This is an interesting and unexpected result. Previously miR-7a has been identified as an important miRNA in the regulation of neuropathic pain (Sakai et al., 2013). Following spinal nerve ligation in rats, miR-7a was downregulated in the dorsal root ganglion (DRG) during the late phase of neuropathic pain (Sakai et al. (2013). Mir-7a targets protein expression of the β2 subunit of voltage-gated sodium channels, leading to both neuronal and behavioural disturbances. Tissue and nerve injuries resulting from mechanical noxious stimuli are associated with the formation of traumatic neuromas, a tangle of truncated neural fibers and connective tissue that can result in neuropathic pain or pain resulting from inflammation (Di Giminiani et al., 2017; Sandercock et al., 2019). Traumatic neuromas are common in cases of amputation or surgical removal of body parts (Flor, 2002), and may correspond with residual stump pain or phantom limb pain (Flor, 2002; Rajput et al., 2012; O'Reilly et al., 2016). In pigs, the process of tail-docking has been implicated in traumatic neuroma development and proliferation, resulting in acute and long-term changes in tail stump sensitivity, thus suggesting a resemblance to neuropathic pain conditions (Sandercock et al., 2016; Di Giminiani et al., 2017). Furthermore, tail amputation in piglets has shown differentially expressed genes in caudal DRG compared to control animals (Sandercock et al., 2016). The authors identified functional gene families linked to inflammation, macrophage, neurohormone and opioid peptide activity, concluding the majority of the observed differentially expressed genes were implicated in promoting neuronal survival (Sandercock et al., 2019). It may be that the observed difference in miR-7a expression in the current study, was potentially implicated in processes relating to the inflammatory responses. Following injury, the activation of different types of immune cells aid in the control of the immune response, including the modulation of inflammatory processes (Wu et al., 2019). However, given that miR-7a indicated downregulation between TD vs. TDA animals without expression differences between TD or TDA vs. Control, it could also be that the anaesthetic was having a direct effect on miR-7a expression. It is well documented that local anesthetic contain anti-inflammatory properties and can directly impact immune cell functioning, including their adherence and migration into the site of injury (Cruz et al., 2021). Furthermore, many miRNAs have shown to regulate molecular processes involved in mediating or attenuating drug effects (Li et al., 2016; Rupaimoole and Slack, 2017), including analgesic treatments in human pain models (Douglas et al., 2015). It would have been useful to incorporate a measure of inflammation such as pro- and anti-inflammatory cytokines, or an additional anti-inflammatory treatment to investigate if in fact, miR-7a expression is influenced by inflammatory processes. .

In this study we further hypothesized that tail-docking effects on expression of miRNA in the brain of pigs could be correlated with differential miRNA expression patterns in blood. Here we observed no change in miRNA signatures in the blood of piglets in any treatment at any timepoint. To our knowledge, only one other study has investigated circulating miRNA in response to tail-docking in the pig. In the study by Lecchi et al. (2020), miRNA in saliva was measured as a marker of inflammation in response to tail-docking plus castration in male piglets either with or without analgesic. Their study found differentially expressed miRNA in

374 the saliva 30-45 minutes following treatment compared to control animals, and the change in
375 expression was mitigated by analgesic. However, the treated animals received tail-docking as
376 well as castration, whereas our study used tail-docking only. One explanation for differences
377 in results is that additional tissue trauma through castration enabled a faster miRNA response.
378 If this were the case, the time of sampling in the current study, starting at + 1 h following
379 treatment, may not have been sufficient to pick up any acute change in miRNA expression.
380 Additional blood samples taken closer to the time of treatment could have provided a more
381 comprehensive account of any potential miRNA differences and should be considered in future
382 studies. Additionally, the process of blood collection, e.g., manual restraint and potential needle
383 pain, could be a source of acute stress in pigs (Martínez-Miró et al., 2016). Finally, the manual
384 restraint and repeated blood sampling in this study may have elicited the bodies stress response
385 and potentially abrogated or masked any changes in miRNA expression specific to the pain in
386 response to treatment. Such effects could be mitigated in future studies where multiple samples
387 are required over short time periods to capture acute miRNA responses, if less invasive
388 biofluids such as saliva were used. Furthermore, the study by Lecchi et al. (2020), only used
389 male pigs, where as our study incorporated both male and female animals. There is research in
390 human and rodent to suggest apparent sex differences in pain response to noxious stimuli
391 (Mogil, 2020), including differences in neurotransmitter signaling within PAG between males
392 and females (Yu et al., 2021). Furthermore, a recent study has identified sex-specific miRNA
393 profiles within the PAG of pig (Pawlina-Tyszko et al., 2020). Although sex was included as a
394 factor in the statistical model, where no effect was observed, there may not have been sufficient
395 animal's numbers to rule this possibility.

396 miRNA have shown to play critical roles in the development and pathophysiology of various
397 pain states (Sakai and Suzuki, 2014). Following a noxious stimulus, nociceptive signals are
398 transmitted to brain areas including the somatosensory cortices and limbic structures (Sakai
399 and Suzuki, 2015), associated with the sensory aspect of pain (i.e., intensity and location) and
400 the affective aspects of pain (i.e., perception and attention), respectively (Apkarian et al., 2011).
401 Accordingly, in order to determine that the observe differential expression in miRNA was in
402 response to the affective-dimension of pain, we would expect to see this difference reflected in
403 specific brain regions, particularly those incorporated in limbic structures. Here we only
404 observed differential expression of miRNA within the PAG where no differences in miRNA
405 patterns were observed within the AMY. This was unexpected as the AMY serves an important
406 brain center for the affective-dimension of pain and is perhaps most well-known for its role in
407 fear conditioning and regulation, memory formation and modulation of pain related behaviour
408 (Simons et al., 2014; Thompson and Neugebauer, 2017). In humans, neuroimaging studies have
409 shown extensive connectivity (both afferent and efferent) between AMY and various brain
410 networks, including the PAG (Simons et al., 2014; Li and Sheets, 2018). Following a
411 nociceptive stimulus, the lateral nuclei of the AMY receive afferent nociceptive information
412 from the spinal cord, subcortical and cortical areas where this information is then transmitted
413 to the central nucleus of the AMY (Neugebauer et al., 2009). The central AMY then projects
414 to the PAG which is the primary center for descending pain modulation, and responsible for
415 outputs to autonomic and behavioural functioning (Mokhtar and Singh, 2020). Pain related
416 plasticity in both AMY and PAG have been demonstrated in human and rodent pain conditions
417 (Jensen et al., 2018), and the effects of pain states on gene expression within these regions have
418 been investigated (Neugebauer, 2015; Descalzi et al., 2017) Based on this, we would therefore

expect to observe differential expression of miRNA in both AMY and PAG brain regions, thus providing evidence that the affective- dimensions of pain had been activated. It may be that we have only observed differential expression of miRNA in within the PAG as a result of efferent projections from other brain regions that are not primarily associated with affective-dimension of pain signals. Another possibility is that the region of AMY tissue that was sampled (ie., 2mm biopsy punch from within the AMY structure) was not specific enough to capture any potential differences in miRNA expression resulting from more specific areas within the AMY. For example, the AMY is a structure of the limbic system that comprises several major subregions and include the basolateral AMY (BLA), the centro-medial AMY (CMA), the frontoparietal or central executive network (CEN), the dorsal anterior cingulate-anterior insula or salience network (SN), and the medial prefrontal-medial parietal or default mode network (DMN) (Jiang, 2016). In human studies, functional magnetic resonance imaging (fMRI)has identified that each of these structures play important but specific roles in pain processing (Roy, 2009). It is therefore possible the specific area of the AMY responsible for AMY-PAG interactions/connectivity may have been missed in this study. Unfortunately, without the use of FMRIor micro-dissection of AMY tissue, the biopsy of specific subregions of AMY tissue was not possible in this study. It would have been useful to incorporate behavioural indices into the experimental design, specifically operant behavioural tests or conditioned place avoidance paradigms which may provide information on an animals learned responses related to the emotive attributes of a noxious stimuli (Gracely and Harte, 2009). Facial grimace scoring is a validated behavioural measure of pain and has been previously applied in pigs in response to tail-docking (Di Giminiani et al., 2016). This may then provide evidence of AMY activation which could be validated with behavioural outputs. Additionally, measures of autonomic functioning such as heart rate, respiration rate, body temperature and inflammatory cytokines may have also been beneficial to assist in teasing apart the miRNA response observed in the PAG.

5 Conclusion

To date methods to assess pain in animals has relied heavily on a number of behavioural and physiological parameters. However, these measures, especially when used in isolation, often cannot provide an accurate or objective account of the affective dimension of pain or the severity of pain an animal is experiencing. Given that the ability to assess the welfare of animals is critically dependent on our ability to incorporate the assessment of emotional or affective state, the development of novel measures that can be used to accurately measure these emotional states of animals are required. Here we sought to investigate the role of miRNA as marker of pain in piglets following tail-docking with and without analgesic. We found miR-412 and miR-7a were differentially expressed within the PAG, but the use of analgesic did not mitigate this response. No change in miRNA expression was observed within the AMY or blood of pigs following treatment compared to control animals and therefore this study provides a basis for further investigation into the use of miRNA as diagnostic tools of animal emotional state.

6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

Conceptualization, C.R.R. and S.H.; methodology, C.R.R., S.H. and L.E.M.; validation, C.R.R., S.H. and L.E.M.; formal analysis, L.E.M., G.N. and J.R.; investigation, L.E.M.; resources, C.R.R.; data curation, L.E.M. and J.R.; data interpretation, L.E.M., S.H., A.L.W., P.V.; writing—Original draft preparation, L.E.M.; writing—Review and editing, S.H., A.L.W., P.V.; visualization, L.E.M.; supervision, S.H, A.L.W., C.R.R., and P.V.; project administration, C.R.R., P.V.; funding acquisition, C.R.R. All authors have read and agreed to the published version of the manuscript. Funding agencies had no role in study design and data interpretation.

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10 Data Availability Statement

Data are available upon request from corresponding author.

The datasets [GENERATED/ANALYZED] for this study can be found in the [NAME OF REPOSITORY] [LINK]. Please see the Data Availability section of the Author guidelines for more details.

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Figures and tables

1 Figures as they appear in the text

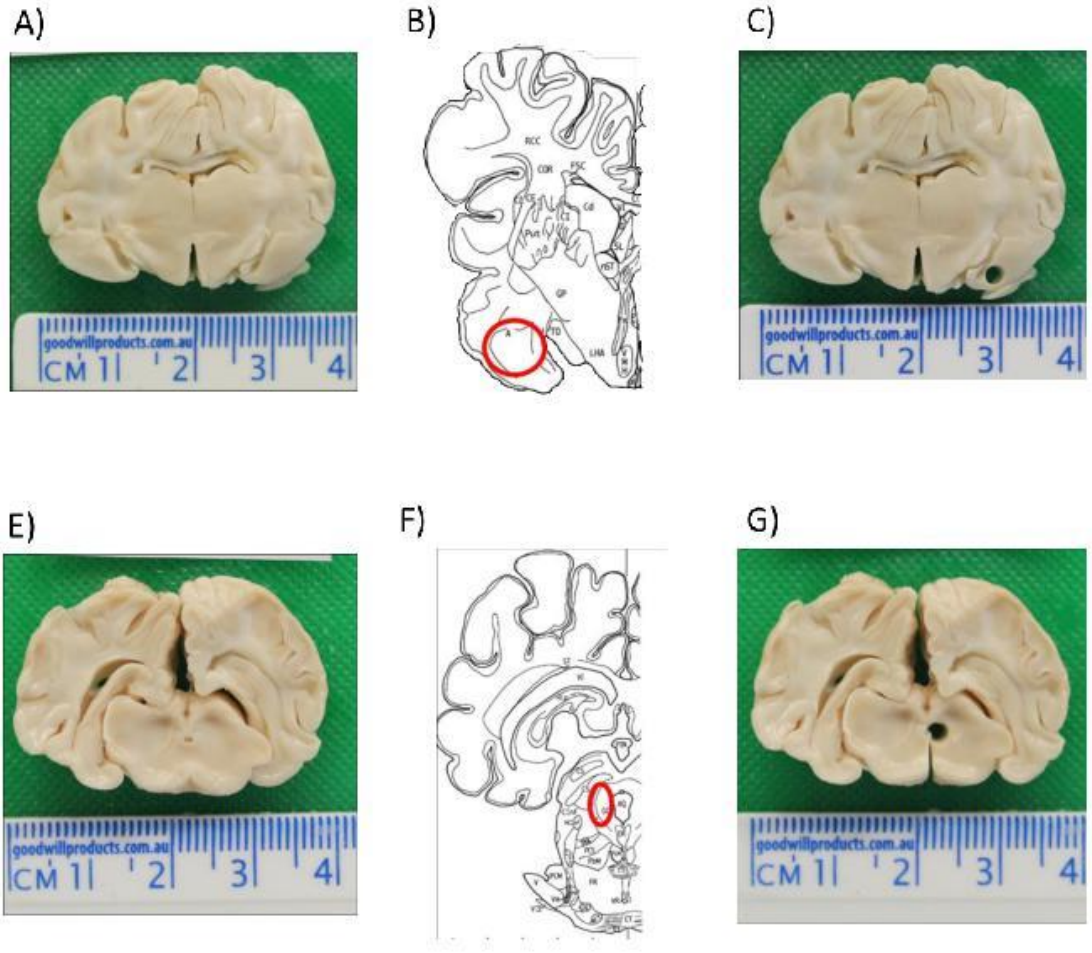

Figure 1. Indicates coronal sections of the piglet brain where **A, E**) indicate brain sections intact prior to tissue dissection, **B, F**) Identification of Amygdala and Peri-aqueductal Grey, respectively, using stereotaxic atlas (Félix et al., 1999), **C, G**) Dissection of the Amygdala and Peri-aqueductal Grey using 2mm biopsy punch.

Figures and Tables

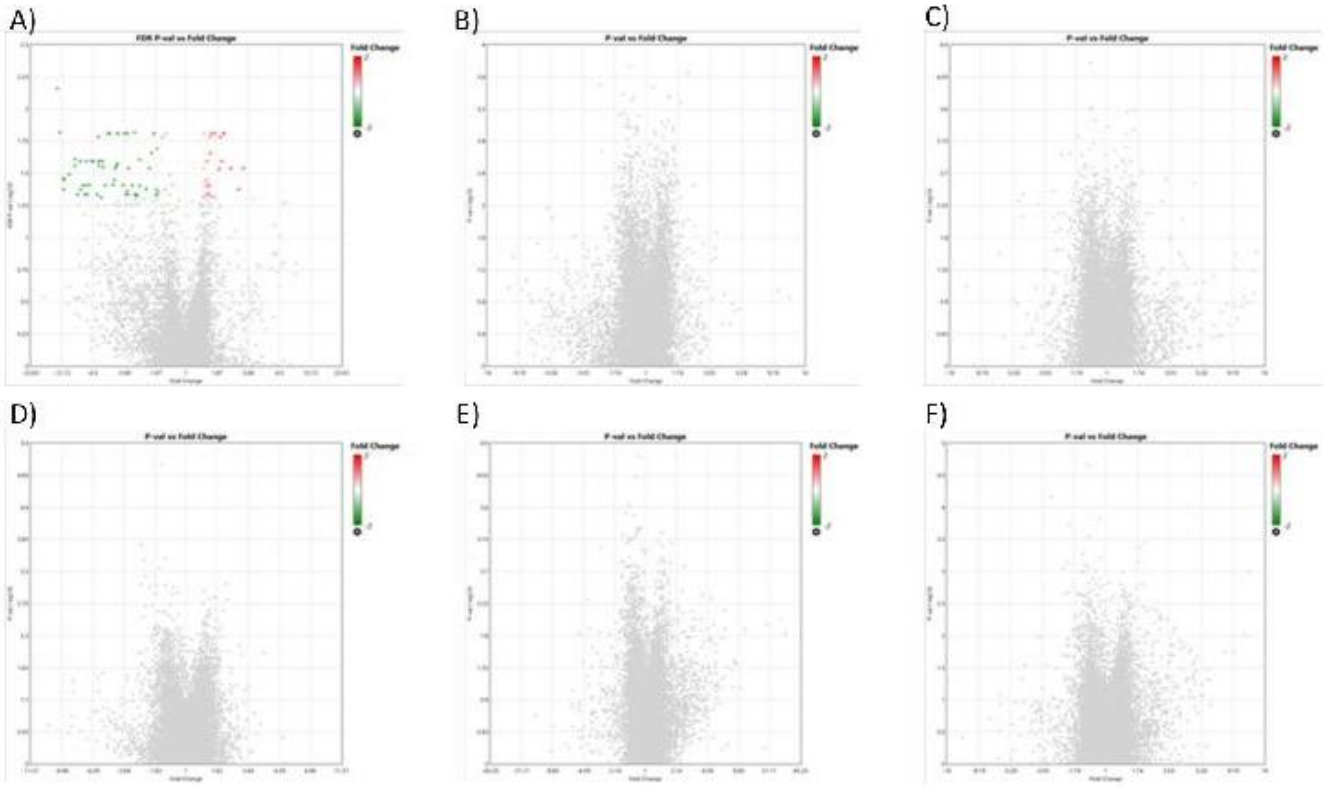

Figure 2. Indicates differentially expressed MiRNA within the Peri-aqueductal Grey (PAG) of pigs exposed to treatment groups **A)** Control vs. Tail-docked (TD); **B)** Control vs. Tail-docked plus Analgesic (TDA); **C)** Control vs. Analgesic (A). Differentially expressed MiRNA within Amygdala (AMY), of pigs exposed to treatment groups **D)** Control vs. TD; **E)** Control vs TDA; **F)** Control vs. A. Data represents 2-fold change in expression (green= down regulation, red= up regulation) of miRNA at significance level FDR adjusted P value <0.05.

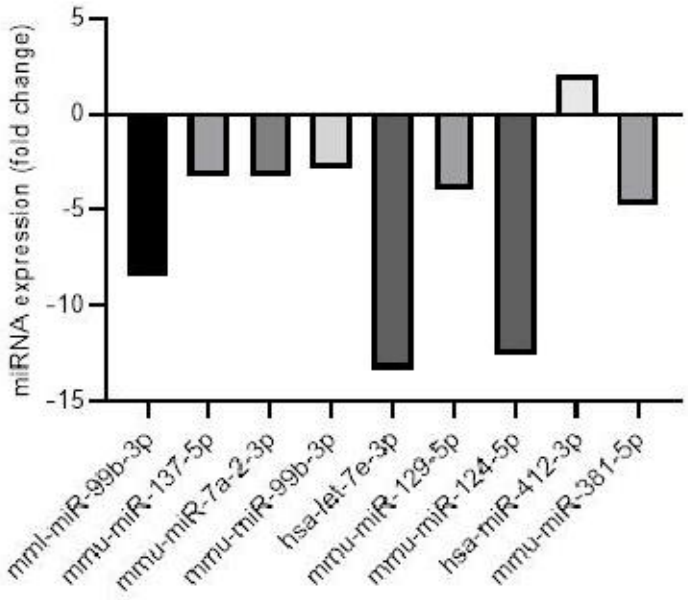

Figure 3. Indicates fold change (FRD P=<0.05), of selected miRNA candidates derived from PAG tissues between Control (Control) and Tail-Docked (TD) treated animals (n=6/treatment).

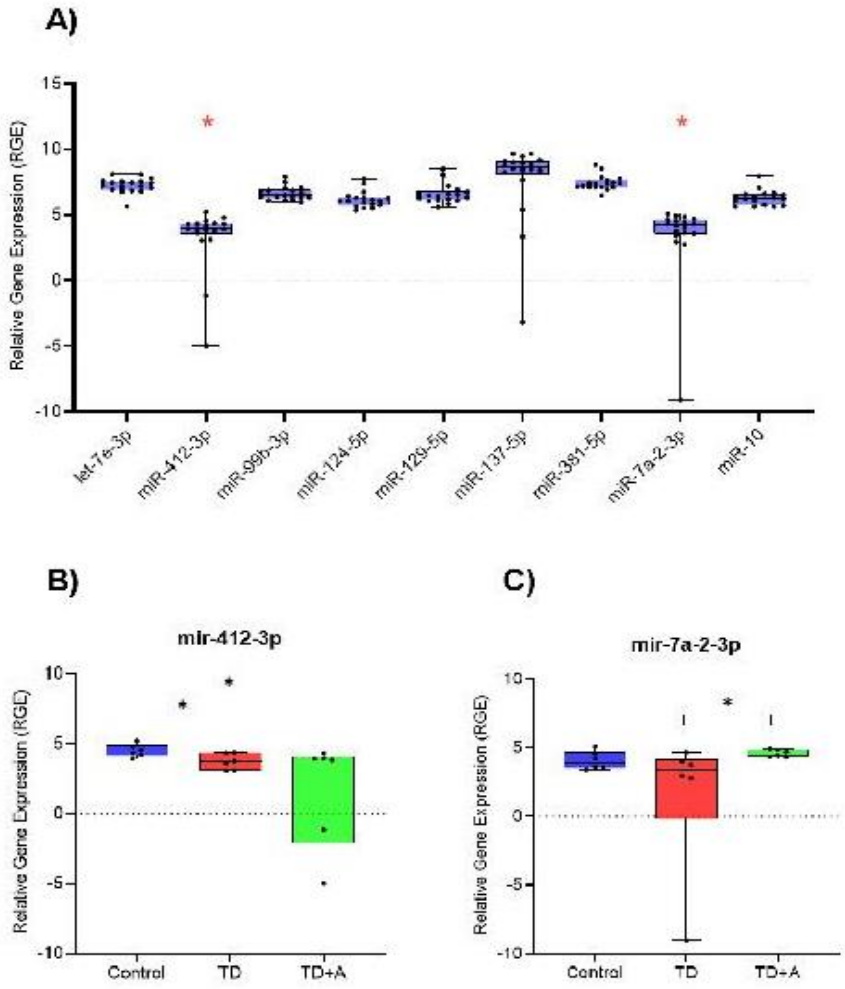

Figure 4. A) Indicates relative gene expression (RGE) of selected miRNA candidates within the periaqueductal grey (PAG); **B)** Relative gene expression (RGE) of selected miRNA candidate miR-412-3p within the PAG in pigs exposed to either tail docking (TD), tail-docking plus analgesic (TDA) or sham tail-docking (Control); **C)** Relative gene expression (RGE) of selected miRNA candidate miR-7a-2-3p within the PAG in pigs exposed to either tail docking (TD), tail-docking plus analgesic (TDA) or no tail-docking (Control). Data are log2 transformed and significance indicated by presence of asterix at P=<0.05.

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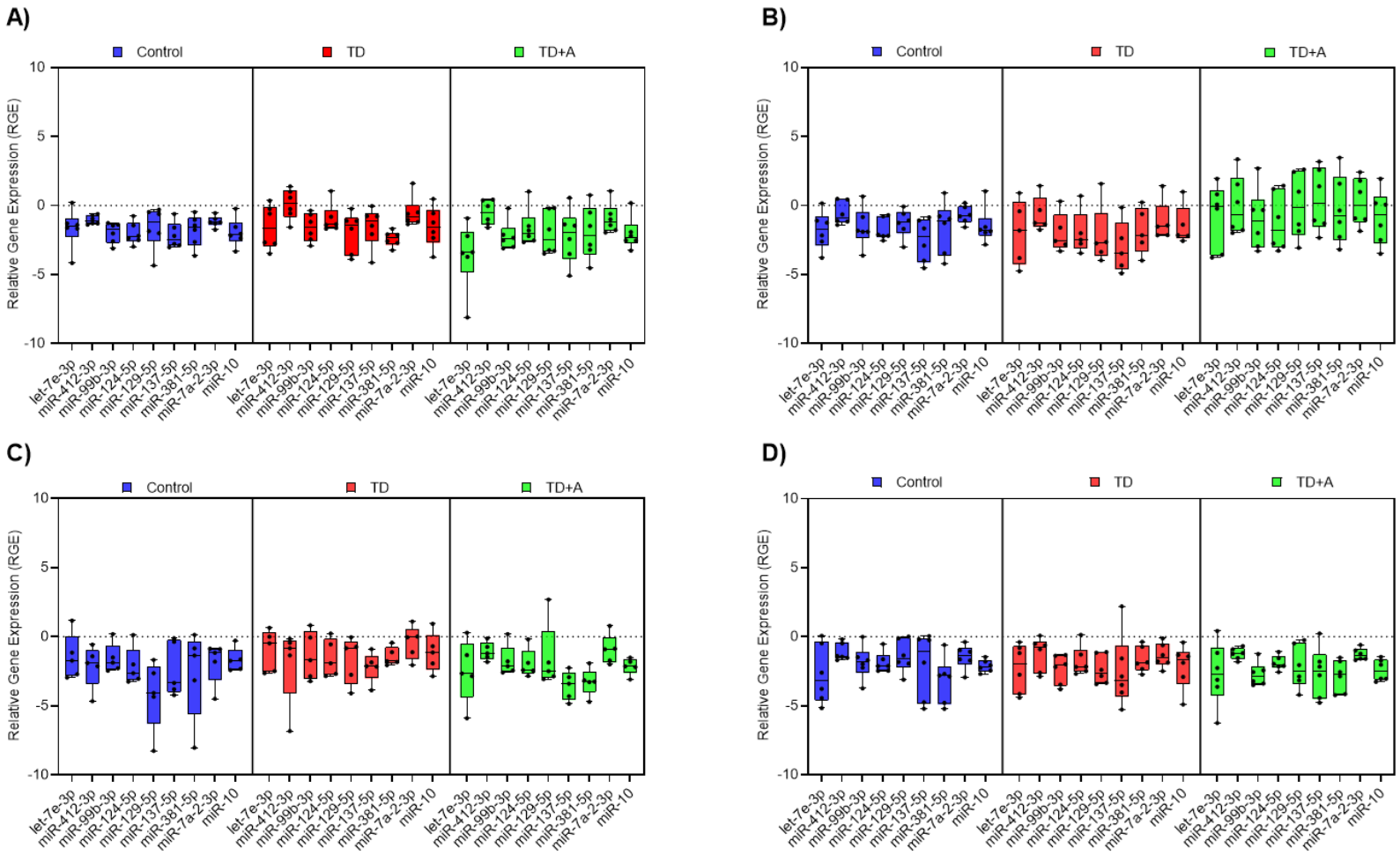

Figure 5. Indicates relative gene expression of selected miRNA candidates following treatment in blood at -1 (**A**), +1 (**B**), +2 (**C**) and +6 (**D**) hours following treatment. Data are Log2 transformed where significant difference is indicated by presence of asterix at P=<0.05.

Table 1. Indicates the 51 differentially expressed miRNA within the Periaqueductal-Grey (PAG), between animals exposed to tail-docking (TD) and control (Control). MiRNA candidates highlighted in blue represent those selected based firstly on order of significance and then previously found to be involved in pain processes. MiRNA candidates highlighted in grey represent novel miRNA candidates selected based on order of significance.

ID	miRbase ID	Fold Change	P-val	FDR P-val	Sequence	References
20500122	hsa-let-7e-3p	-13.26	1.92E-07	0.007	CUAUACGGCCUCCUAGCUUUCC	(Orlova et al., 2011; He and Wang, 2012)
20500256	mmu-miR-99b-3p	-2.83	1.03E-06	0.0153	CAAGCUCGUGUCUGUGGGUCCG	(Bai et al., 2007; Cui et al., 2018)
20500259	mmu-miR-124-5p	-12.55	1.26E-06	0.0153	CGUGUUCACAGCGGACCUUGAU	(Bai et al., 2007; Willemen et al., 2012; Elramah et al., 2017)
20500285	mmu-miR-137-5p	-3.25	1.17E-05	0.0155	ACGGGUAUUCUUGGGUGGAUAAU	(Liu et al., 2009)
20501266	mmu-miR-381-5p	-4.75	4.77E-06	0.0155	AGCGAGGUUGCCCUUUGUAUAUU	
20501150	mmu-miR-7a-2-3p	-3.25	4.00E-06	0.0155	CAACAAGUCCCAGUCUGCCACA	(Sakai et al., 2013)
20501795	mmu-miR-412-3p	-5.81	1.92E-05	0.0155	UCACCUGGUCCACUAGCCG	
20500269	mmu-miR-128-1-5p	-3.95	1.31E-05	0.0155	CGGGGCCGUAGCACUGUCUGA	
20500361	mmu-miR-129-5p	-3.95	1.31E-05	0.0155	CUUUUUGCGGUCUGGGCUUGC	(Yu et al., 2019; Tian et al., 2020)
20500612	dme-miR-285-3p	-3.25	1.17E-05	0.0155	UAGCACCAUUCGAAAUCAGUGC	
20501957	gga-miR-29b-2-5p	-5.81	1.92E-05	0.0165	AGCUGGUUUCACAUGGUGGCUUAGA	
20501439	rno-miR-127-5p	-5.81	1.92E-05	0.0165	CUGAAGCUCAGAGGGCUCUGAUU	

20501792	mmu-miR-411-5p	-5.81	1.92E-05	0.0165	UAGUAGACCGUAUAGCGUACG	
20501793	mmu-miR-411-3p	-5.81	1.92E-05	0.0165	UAUGUAACACGGUCCACUAACC	
20502243	mmu-miR-431-5p	-2.54	3.42E-05	0.0248	UGUCUUGCAGGCCGUCAUGCA	
20504209	rno-miR-543-3p	-7.39	5.13E-05	0.0255	AAACAUUCGCGGUGCACUUCU	
20508386	mml-miR-99b-3p	-8.37	3.84E-05	0.0255	AAGCUCGUGUCUGUGGGUCCG	(Bai et al., 2007; Bjersing et al., 2013)
20503095	hsa-miR-412-3p	2.05	5.43E-05	0.0255	ACUUCACCUGGUCCACUAGCCGU	
20508249	gga-miR-1767	-5.36	4.35E-05	0.0255	AGGCGAGGAGAACAGCAGCU	
20508161	gga-miR-1700	-6.68	4.96E-05	0.0255	CAUCAGAGGGAUAGGAUGGAC	
20504153	rno-miR-412-3p	-7.39	5.13E-05	0.0255	CUUCACCUGGUCCACUAGCCGU	
20502708	dre-miR-129-5p	-6.47	5.77E-05	0.0255	CUUUUUGCGGUCUGGGCUUGCU	
20505092	bta-miR-345-5p	2.05	5.43E-05	0.0255	GCUGACUCCUAGUCCAGUGCU	
20506711	hsa-miR-1180-5p	-5.79	5.46E-05	0.0255	GGACCCACCCGGCCGGGAAUA	
20504217	hsa-miR-487b-5p	2.05	5.43E-05	0.0255	GUGGUUAUCCCUGUCCUGUUCG	
20506632	cre-miR1155	2.05	5.43E-05	0.0255	UAGUCCUGCACGAGGAAGGAGC	
20502759	dre-miR-218a	-6.47	5.77E-05	0.0255	UUGUGCUUGAUCUAACCAUGUG	

20513904	eca-miR-703	-11.56	0.0001	0.0356	AAAACCUUCAGAAGGAAAGGA	
20511457	osa-miR2096-3p	-4.22	0.0001	0.0356	CCUGAGGGGAAAUCGGCGGGA	
20519489	hsa-miR-4685-5p	-2.95	0.0002	0.039	CCCAGGGCUUGGAGUGGGGCAAGGUU	
20518817	hsa-miR-4442	-7.44	0.0002	0.039	GCCGGACAAGAGGGAGG	
20515560	hsa-miR-3147	-7.44	0.0002	0.039	GGUUGGGCAGUGAGGAGGGUGUGA	
20521895	mmu-miR-299b-3p	-7.94	0.0002	0.039	UAUGUGGGACGGUAAACC	
20518958	oar-miR-379-5p	-7.44	0.0002	0.039	UGGUAGACUAUGGAACGUAGGC	
20515196	tgu-miR-29b-2-5p	-7.94	0.0002	0.039	UGGUUUCACAUGGUGGCUUAGA	
20514056	eca-miR-412	-7.44	0.0002	0.039	UUCACCUGGUCCACUAGCCG	
20525220	gga-miR-6587-3p	-2.23	0.0002	0.0416	UGCUGGAAGUGGUGCUGGAGGCU	
20526754	sbi-miR6224a-3p	-11.7	0.0002	0.0423	CUUAUAUACUAGGACGGAGGG	
20525306	gga-miR-6666-3p	-8.26	0.0002	0.0423	UCGGGUGAUGAGGACAGGAGGA	
20526031	mmu-miR-7033-5p	-11.7	0.0002	0.0423	UCUCCAGGAGUCUGAGGGGCAGG	
20525383	hsa-miR-6716-5p	-11.7	0.0002	0.0423	UGGGAAUGGGGGUAAGGGCC	
20525993	mmu-miR-7014-5p	-11.7	0.0002	0.0423	UUGGGUGCUGUGGAAGGGACAG	

20526885	hsa-miR-7162-3p	-7.6	0.0002	0.046	UCUGAGGUGGAACAGCAGC	
20526865	hsa-miR-7152-3p	-7.6	0.0002	0.046	UCUGGUCCUGGACAGGAGGC	
20528998	bdi-miR7766-3p	-7.28	0.0003	0.0462	CGAGGCUGACUGGGACUAAGCGGC	
20529781	hsa-miR-8071	-3.22	0.0003	0.0462	CGGUGGACUGGAGUGGGUGG	
20530037	ppe-miR395a-3p	-7.28	0.0003	0.0462	CUGAAGUGUUUGGGGGGACCC	
20528074	lja-miR7539	-7.28	0.0003	0.0462	UCGAGAGAGAGAGCGACGAGG	
20527773	gga-miR-7466-3p	-5.97	0.0003	0.0462	UUUUCCUGUAGAGGAAGCAGGAG	
20530334	hbv-miR-B14RC-3p	-2.68	0.0003	0.0465	AGGAGGGGUCUGGGAGAGAAGGG	

Chapter 4: Pronounced inter-individual variation in plasma cortisol response to fluoxetine hydrochloride in the pig

Contextual Statement

In order to validate novel biomarkers for the assessment of positive emotions in animals, it is first necessary that we have the ability to identify when regions of the brain associated with positive emotional processes become activated. Accurate identification of this activity can then provide a basis from which to investigate novel biomarkers, including miRNA. Activation of specific neural networks, including the serotonergic-reward pathway, are possible through pharmacologically-induced means (i.e., through administration of selective serotonin reuptake inhibitors, or SSRIs). Traditionally, SSRI compounds have been used to modify affective states in human patients suffering from various psychological disorders, where the majority of these exhibit their effects by restoring neurotransmission along various neural circuits. A chain reaction to this is that SSRI administration often results in alterations to other, down-stream biological systems. One such alteration includes increased activity of the hypothalomo-pituitary-adrenal (HPA) axis, resulting from increased neural firing to the hypothalamus. A corollary to this is of increased synthesis in neuropeptides that ultimately increase circulating levels of glucocorticoids (i.e., cortisol). Thus, circulating glucocorticoids, including cortisol, have the potential to be markers of brain activity in response to SSRI treatment.

Therefore, the purpose of **Chapter 4** was to investigate the efficacy of measuring circulating cortisol as an indicator for the effective delivery of a single intravenous dose of the SSRI, fluoxetine hydrochloride, to a pig brain.

Statement of Authorship

Title of Paper	Pronounced inter-individual variation in plasma cortisol response to fluoxetine hydrochloride in the pig
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Principal Author

Name of Principal Author (Candidate)	Laura Marsh		
Contribution to the Paper	Conducted experiment, performed laboratory analysis of samples, performed statistical analysis of data, interpreted data, wrote manuscript.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	22/06/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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animals

MDPI

Brief Report

Pronounced Inter-Individual Variation in Plasma Cortisol Response to Fluoxetine Hydrochloride in the Pig

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Simple Summary: Holistic animal welfare assessment requires measures for emotional (affective) state, in particular positive states. Pharmacological agents such as antidepressants that create a predictable positive affective state can be valuable tools to assess novel welfare biomarkers. However, efficacy of pharmacological action in the brain needs to be demonstrated before such an approach is applicable. Counterintuitively, in humans and sheep, effective delivery of antidepressant agent, i.e., selective serotonin reuptake inhibitors, has been demonstrated by an increase in downstream cortisol levels. Here, we tested the efficacy of measuring circulating cortisol as an indicator for effective delivery of a single intravenous dose of the selective serotonin reuptake inhibitor fluoxetine hydrochloride to the pig brain. Antidepressant treatment resulted in increased plasma cortisol levels 15–165 min after treatment as compared with saline controls, suggesting that, similar to the other species, plasma cortisol is an indicator of fluoxetine hydrochloride efficacy. However, individual cortisol profiles of pigs treated with the antidepressant were highly variable with either the expected—an unorthodox, or no response. We conclude that significant inter-individual variation in cortisol response currently precludes the use of cortisol as a reporter for fluoxetine hydrochloride efficacy in the pig. These data need to be verified in a larger study.

Abstract: Animal welfare assessment requires measures for positive affective state. Pharmacological agents that manipulate affective state can be used to evaluate novel biomarkers for affective state assessment. However, to validate that an agent has modified brain function, a reliable indicator is required. Circulating cortisol has been used as a reporter for effective delivery of the antidepressant selective serotonin reuptake inhibitor (SSRI) fluoxetine hydrochloride to the brain in humans and sheep. Here, we tested cortisol as a reporter for effective delivery of fluoxetine hydrochloride to the pig brain. We hypothesized that following administration of SSRI, innervation of the serotonergic reward pathway would result in activation of the hypothalamic-pituitary-adrenal (HPA) axis, leading to increased circulating cortisol levels. Large White-Landrace gilts received either a single intravenous dose of 100 mg fluoxetine hydrochloride suspended in 10 mL saline (n = 4), or 10 mL saline alone (n = 4). Blood samples were collected every 15 min for one hour prior to, and six hours post-treatment. The interaction of treatment x time on mean plasma cortisol levels between 15–165 min post-treatment was significant ($p = 0.048$) with highest cortisol concentrations of SSRI treated pigs versus controls (+98%) at 135 min post-treatment. However, individual cortisol profiles after SSRI treatment revealed

high inter-individual variation in response. We conclude that, while combined data imply that plasma cortisol may be a readout for SSRI efficacy, inter-individual variation in SSRI response may preclude application of this approach in the pig. Given the current limited sample size, further research to confirm these findings is needed.

Keywords: cortisol; antidepressant; fluoxetine hydrochloride; pig; affective state; welfare

1. Introduction

The assessment of animal affective state can be challenging, in particular the evaluation of positive states. At present, behavioral measures and affective bias tests are the predominant assessment methods for positive states [1]. However, these methods are less suited to a production environment, because they are time-consuming and arguably subjective [2,3]. There is, therefore, an urgent need to identify and validate novel physiological and molecular markers of positive affect, such as miRNA [4,5], to complement or even replace behavioral [6] measures.

Validation of novel biomarkers for affective state requires robust means to manipulate affective state in a consistent manner. Pharmacological agents, including antidepressant selective serotonin reuptake inhibitors (SSRI) are candidates for this approach [7,8]. However, in order to use SSRI in validation experiments of novel biomarkers, a measure that demonstrates the effective delivery of the SSRI to the brain is required. In humans and sheep, effective SSRI delivery to the brain has been associated with an increase in adrenocorticotropic hormone (ACTH), leading to a downstream increase in cortisol level [9–12]. Rodent studies have reported similar findings using serotonergic inhibitors and/or 5HT1a receptor antagonists [13–15]. To our knowledge, no such data are available for the pig.

Here we tested whether plasma cortisol is a reliable indicator of effective delivery of SSRI to the pig brain. We hypothesized that pharmacological stimulation of the serotonergic system with an intravenous dose of the SSRI fluoxetine hydrochloride, would activate the HPA axis resulting in increased plasma cortisol levels. This peripheral cortisol response would thus provide evidence for successful activation of the serotonergic system in the brain of the pig.

2. Materials and Methods

2.1. Animals and Housing

Animal procedures were approved by the University of Adelaide Animal Ethics Committee (S-2018-053) and conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013), and the Animal Welfare Act, 1985 (SA). Eight Large White-Landrace females at 18 weeks of age (mean weight 85 kg, range of 72–92 kg) were sourced and housed at the Roseworthy Piggery, South Australia. Animals were kept in individual stalls (240 cm × 60 cm) and thus restricted in their movement, and within sight of other individuals, throughout the experiment. Water was available *ad libitum* and 4 kg standardized grower feed (Barastoc MP Pig 1300, Ridley's, Adelaide, South Australia) was provided every morning. The study was conducted in December, the southern hemisphere summer.

2.2. Treatment Protocol

Pigs were habituated to individual stalls for seven days prior to study commencement. To aid in adjustment to human presence, pigs had human contact daily. On day 1 of the study, topical local anesthetic (Xylocaine, Provet, Adelaide, Australia) was applied to the ear vein and catheterization performed under manual restraint with a rope snare. Catheter tubing was secured to the neck of the animal using adhesive tape (Elastoplast, Zebravet, Adelaide, South Australia). Computer-generated randomization (Microsoft Excel 2016, Microsoft Corporation) was used to assign pigs into two groups

of 4 animals each. On day 2 all animals received either intravenous (i.v.) 100 mg SSRI fluoxetine hydrochloride (Complimentary Compounds, Ballina, NSW, Australia) dissolved in 10 mL 0.9% saline (Zebravet, Adelaide, South Australia) or i.v. 10 mL 0.9% saline at 8:00 am. The dose was chosen based on previous studies, where an initial 10 mL bolus injection containing 70 mg of fluoxetine hydrochloride, followed by a continuous infusion of 98.5 µg/kg/d for eight days was effective at increasing ACTH and cortisol in pregnant sheep [10,11]. The higher dose was chosen because we aimed to test cortisol response after a single intravenous injection. Considering previously published data and standardization applied to mitigate factors known to affect cortisol response such as age and breed [16] sex [17], feed intake [18] and level of exercise [19], the use of 4 animals per treatment group was deemed sufficient for this study to minimize animal usage; a retrospective power calculation with the acquired data revealed a power of 71%.

2.3. *Sampling and Cortisol RIA*

Blood sampling started at 7:00 am, one hour before treatment at 8:00 am, with sampling performed every 15 min until six hours post-treatment. Each sample of 3 mL blood was collected into 5 mL Lithium-Heparin coated tubes (Vacuette, Greiner Labortechnik, Kremsmünster, Austria). Samples were immediately centrifuged at 1000× *g* for 10 min and plasma stored at −20 °C until further analysis. Animals that had received SSRI treatment could not re-enter the commercial herd and were euthanized with 1 mL/10 kg of pentobarbital sodium (Virbac Pty Limited, Milperra, NSW, Australia). Saline treated animals reentered the commercial herd. Plasma samples were assayed for cortisol in duplicate by RIA following the manufacturer's instructions (ImmuChem CT cortisol kit, MP Biomedicals, Orangeburg, NY, USA). Sensitivity of the kit was 0.17 pg/dL and intra and inter-assay coefficients of variation <15% and <10%.

2.4. *Statistical Analysis*

Statistical analyses were conducted with SPSS, Version 25 (IBM, Armonk, NY, USA). A linear mixed model analysis with time as the repeated measure was used to analyze the data. Normality and homogeneity of the dataset were tested by examining the correlation between the residuals and predicted values. As the data were not normally distributed, cortisol values were log10-transformed for the final analysis. The effect of animal weight on cortisol levels was tested in the model and removed due to lack of significance ($p > 0.10$). Statistical significance level was $p < 0.05$.

3. Results

A significant treatment by time interaction was observed between 15 and 165 min after treatment ($p = 0.048$). The greatest increase in mean plasma cortisol concentration of SSRI treated pigs as compared with saline controls (+ 98%) was measured 135 min post-treatment (Figure 1A). However, individual cortisol response profiles of SSRI treated animals varied considerably (Figure 1B). While the elevated cortisol profiles of SSRI treated Animals 5 and 7 were consistent with an SSRI induced cortisol response, two other SSRI treated animals displayed unorthodox cortisol response profiles (Figure 1B). Animal 6 did not respond to SSRI treatment, and Animal 8 revealed an initial spike in cortisol at 120 min post-treatment that quickly returned to baseline levels (Figure 1B). Saline treatment of control animals had no effect on circulating cortisol levels (Figure 1B).

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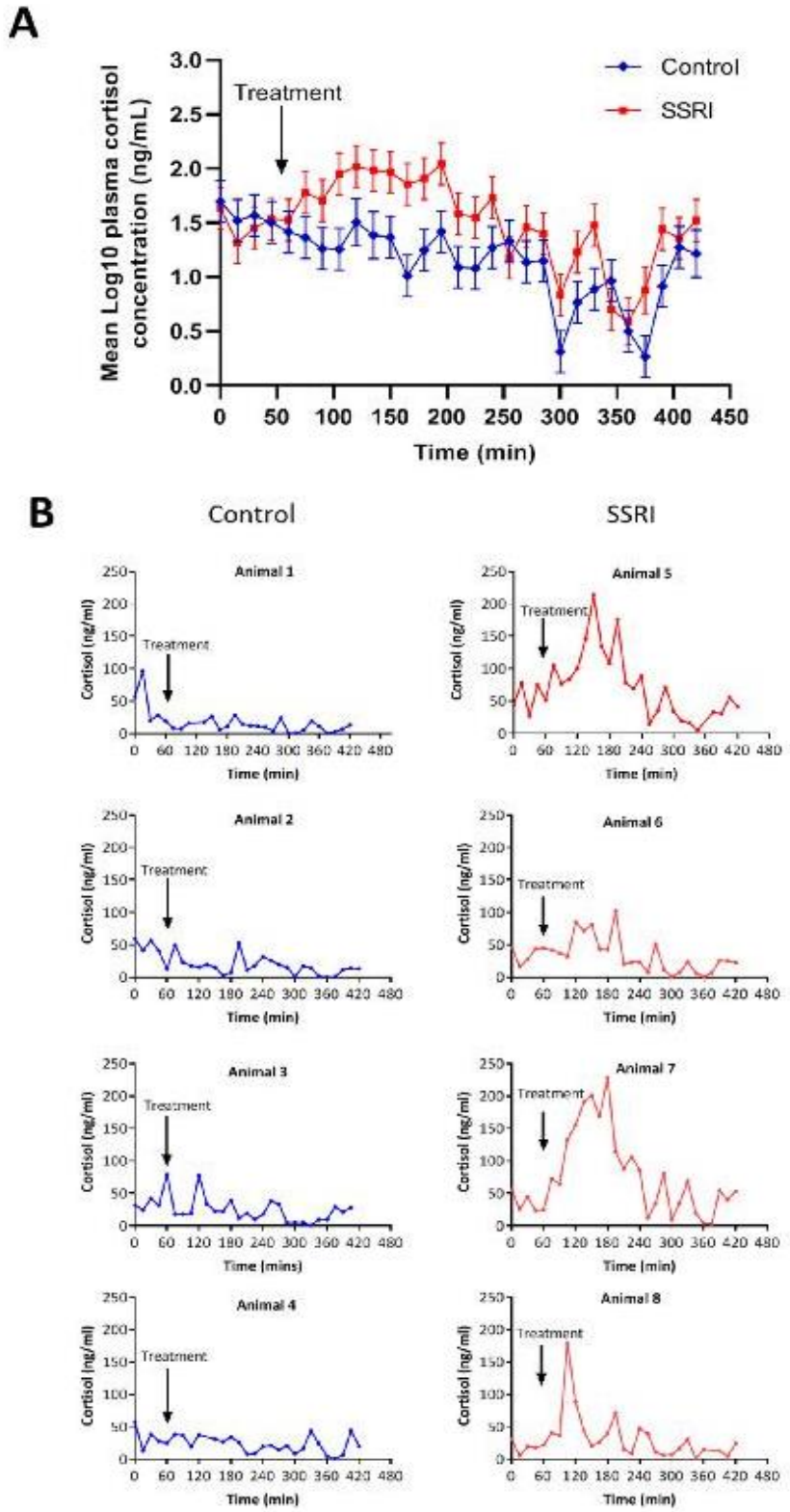

Figure 1. Plasma cortisol concentrations of pigs treated with either a single intravenous dose of the selective serotonin reuptake inhibitor (SSRI) fluoxetine hydrochloride (100 mg suspended in 10 mL saline) or saline control (10 mL). (**A**) Mean Log10 plasma cortisol concentration (ng/mL) ± SEM of animals with SSRI treatment in comparison to control animals that received saline. (**B**) Individual cortisol response profiles of control (animals 1–4) and SSRI treated (animals 5–8) individuals.

4. Discussion

Here we tested whether measurement of circulating cortisol levels could be used as a reliable indicator of effective delivery of SSRI to the pig brain.

We observed a substantial increase in mean plasma cortisol levels after SSRI treatment 15–165 min post-treatment as compared with saline controls. This is consistent with activation of brain regions involved in reward processing and in agreement with observations in human and sheep [9,12]. However, examination of individual cortisol response profiles after SSRI treatment revealed an unexpected degree of inter-individual variation in cortisol response. While two SSRI treated pigs displayed the expected cortisol response curve with an initial peak followed by a gradual decline over time, two other pigs had a very different and unorthodox cortisol response. One of the cortisol profiles indicated a lack of response while the other one indicated a short spike in cortisol followed by a sudden return to baseline. Considering the standardization of factors known to affect cortisol response (i.e., sex, time of day, time of feeding, level of exercise [16–20], and finding no significant effect for the co-variate body weight, this degree of inter-individual variation was unexpected and has, to our knowledge, not been described previously. It is noteworthy that, in humans, psychiatric research has revealed individual variability in patient response to SSRI treatment, where genetic variation, environmental exposure and gene-environment interactions likely influence treatment outcomes [21,22]. We therefore propose the following explanations for the observed differences in individual cortisol response profiles after SSRI treatment in the pig: (1) inherent differences in the pharmacological pathway of the drug, including differences in receptor number, structure or function, or (2) differences in HPA axis responsiveness to SSRI, or (3) a combination of these. Regardless of the causes for the observed variation in response, in order to understand the dynamic relationship between the neurobiology of the serotonergic system and its effect on HPA activity, and thus cortisol, it appears essential that inter-individual differences are taken into account.

Furthermore, our data caution against over-reliance on statistically significant results obtained from group means without due regard for the individual data that constitutes the finding. Further research is needed regarding plasma cortisol as a biomarker of SSRI delivery in the pig.

Author Contributions: Conceptualization, C.R.R. and S.H.; methodology, C.R.R., S.H. and L.E.M.; validation, C.R.R., S.H. and L.E.M.; formal analysis, L.E.M., R.T.; investigation, L.E.M.; resources, C.R.R.; data curation, L.E.M., R.T.; data interpretation, L.E.M., S.H., A.L.W., R.T, C.R.R; writing—Original draft preparation, L.E.M.; writing—Review and editing, S.H., A.L.W., R.T; visualization, L.E.M., R.T.; supervision, S.H, A.L.W., R.T. and C.R.R.; project administration, C.R.R., R.T., S.H.; funding acquisition, C.R.R. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 5: Evaluation of miRNA as biomarkers of emotional valence in pigs

Contextual Statement

Due to recent trends in animal science, the assessment of animal welfare has now advanced to incorporate measures of emotional or affective states, with particular emphasis placed on tools that can assess positive emotions in animals. In line with this change in research direction, the development of novel biomarkers that can accurately and objectively assess positive emotional states in animals are necessary. These biomarkers must be robust, have the ability to differentiate between valences of affect, and require validation against traditional behavioural and physiological indices.

One such biomarker that has recently gained attention is miRNA. In human research, circulating miRNAs have been used as diagnostic markers for a number of conditions and disease states, including those associated with emotional processes. Given their similarities to humans, including likeness in neuroanatomy and neurophysiology, these molecules therefore have the potential to be biomarkers of emotional processes in animals.

Chapter 5 investigates miRNA expression in the brain and blood of pigs. I explore the efficacy of miRNAs as biomarkers of positive emotional states following exposure to husbandry conditions assumed to elicit alterations in emotional processes. Judgment bias and brain neurotransmitter concentrations were used as corroborating markers of positive valence in pigs.

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Laura Marsh		
Contribution to the Paper	Conceptualisation, conducted experiment, performed laboratory analysis of samples, performed statistical analysis of data, interpreted data, wrote manuscript.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Evaluation of miRNA as Biomarkers of Emotional Valence in Pigs

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Simple Summary: It is widely recognized that the assessment of animal welfare should include measures of positive emotional (affective) state. Existing behavioral and physiological indicators of a positive affective state frequently lack sensitivity, objectivity or are unsuitable in a production environment. Therefore, there is a need to develop new approaches to accurately and objectively measure a positive emotional state in animals, including novel molecular markers such a miRNA. These biomarkers must be measurable in the peripheral circulation and provide an accurate account of the physiological and molecular activity in regions of the brain associated with emotional processing. Further, such markers require validation against established behavioral and physiological indices. Here we investigated the efficacy of circulating miRNA as biomarkers of emotional state in the pig.

Abstract: The ability to assess the welfare of animals is dependent on our ability to accurately determine their emotional (affective) state, with particular emphasis being placed on the identification of positive emotions. The challenge remains that current physiological and behavioral indices are either unable to distinguish between positive and negative emotional states, or they are simply not suitable for a production environment. Therefore, the development of novel measures of animal emotion is a necessity. Here we investigated the efficacy of microRNA (miRNA) in the brain and blood as biomarkers of emotional state in the pig. Female Large White × Landrace pigs (n = 24) were selected at weaning and trained to perform a judgment bias test (JBT), before being exposed for 5 weeks to either enriched (n = 12) or barren housing (n = 12) conditions. Pigs were tested on the JBT once prior to treatment, and immediately following treatment. MiRNA and neurotransmitters were analyzed in blood and brain tissue after euthanasia. Treatment had no effect on the outcomes of the JBT. There was also no effect of treatment on miRNA expression in blood or the brain (FDR $p > 0.05$). However, pigs exposed to enriched housing had elevated dopamine within the striatum compared to pigs in barren housing ($p = 0.02$). The results imply that either (a) miRNAs are not likely to be valid biomarkers of a positive affective state, at least under the type of conditions employed in this study, or (b) that the study design used to modify affective state was not able to create differential affective states, and therefore establish the validity of miRNA as biomarkers.

Keywords: welfare; biomarkers; positive affective state; miRNA; pigs

1. Introduction

The assessment of emotional or affective state in animals can be challenging, particularly the assessment of positive emotion since there are fewer identified behaviors or biomarkers specific to these states. Emotions have been operationally defined as "specific, intense and short-lived responses to stimuli" whilst mood refers to "longer, more ambiguous, and nonattributable affective feelings of lower intensity" [1,2], both of which can vary along two main axes, including arousal, or strength of response, and valence (direction of response, being positive or negative) [3]. Emotions are recognized as complex, multifaceted phenomena, that give rise to rapid physiological and behavioral changes which likely evolved to achieve goals related to survival, such as attainment of valuable resources/rewards and avoidance of harm/punishment [4]. Animal welfare encompasses a long-lasting state comprising the summed-up experiences of the individual [5] and can be defined in terms of affective states and their relative weighting over time [6]. Therefore, the assessment of animal welfare should include measures of animal emotion [7]. However, in order to study animal emotional state, it is first imperative to identify methods that accurately and objectively measures the emotional state of animals.

A number of physiological and behavioral indices are currently used to infer the emotional state of animals. For example, physiological indices including hypothalamo–pituitary–adrenal axis (HPA) activity, sympathetic and autonomic functioning, endocrine function, as well as behavioral parameters have been used as makers of emotional state in animals. However, although these measures can indicate emotional arousal, they are often unable to distinguish between the valence or direction of the emotion being elicited. Furthermore, these measures tend to relate to negative affect, with less focus on, and development of, indicators of positive emotional state [8]. One assessment tool recently shown to have value in this respect is the judgment bias test (JBT), which use an animal's behavioral response as an indicator of its underlying affective state in response to an unknown stimulus. [9]. Animals first learn to discriminate between a positive stimulus, such as a high value reward, and an aversive or nonrewarding stimulus, such as no reward or punishment [10]. Once animals have learnt to discriminate between positive and aversive stimuli, they are then tested on an ambiguous stimulus, intermediate between the two learned stimuli. These tests are based on the assumption that if, under ambiguity, the animal behaves in a manner normally associated with a positive reward, that animal has an enhanced expectation of a positive outcome that, thus, implies a positive emotional state [11]. Conversely, if the animal displays behaviors consistent with an aversive outcome, that animal has reduced anticipation of a positive outcome, which implies the animal is in a negative affective state [11]. The JBP has been used successfully in a variety of species including rats [12], sheep [13], dogs [14], chickens [15], and pigs [16–18], but while JBPs are considered to have good validity [19], they are less suited to production environments due to the time it takes to train animals to perform the test [20]. There is therefore an urgent need to identify and validate objective physiological or molecular markers of positive affect [21,22], in order to complement or even replace existing behavioral and physiological measures [23,24]. Following validation, new technologies may be able to be developed to analyze these biomarkers rapidly on farm using relatively noninvasive sampling, thus making them applicable for production environments (i.e., sensor-based technologies in blood or saliva).

MiRNA are small, noncoding RNA molecules involved in the regulation of genes post-transcriptionally. These molecules are ubiquitous throughout the body, including the brain, and are involved in the regulation of genes, including those associated with emotional processing [22]. For example, dysregulations of specific miRNAs have been used as diagnostic tools for a number of psychological conditions including anxiety [25,26], major depressive disorder (MDD) [27], post-traumatic stress disorder (PTSD) [28], bipolar disorder [29], and schizophrenia [30]. These molecules are involved in the regulation of emotional processes, and are released into the circulation, enabling measurement in the blood, urine or saliva [31,32]. As a result, they have the potential to be biomarkers

of the activity associated with emotional processing, including those neuronal systems involved in the regulation of positive emotions such as the serotonergic and dopaminergic reward pathways [22,33,34]. For example, miRNA-16 has recently been implicated in the modulation of serotonergic transmission in the mouse brain [35]. In another mouse study, specific miRNAs, including miRNA-212, were shown to regulate the motivational properties of drug addiction within the prefrontal cortex (PFC) and striatum following the self-administration of addictive drugs [36]. Nevertheless, most miRNA research conducted in humans and rodents has focused on negative physiological or disease related conditions [37], including neuropathic pain or psychological conditions that can impact emotional state. Few studies have investigated miRNA with the specific intention to identify miRNA as correlates of positive emotional state, and to our knowledge no such studies have been conducted in pigs.

To identify and validate novel measures of positive emotion in the pig, including molecular markers such as miRNA, requires an accurate assessment of different affective states in the animal as well as the implementation of a robust means to manipulate affective state in a controlled experimental setting. Husbandry practices are known to influence production outcomes and impact welfare parameters. For example, increased floor space was shown to produce healthier pigs with high immunity and increased comfort and play behavior [38]. Pigs that are socially isolated from pen mates have shown increased behaviors indicative of stress and a decrease in behaviors indicative of positive welfare such as play [39]. The provision of enrichment to animals in farmed systems is suggested to improve biological functioning, as well as increase overall wellbeing, as it allows the animal to perform rewarding and motivated species-specific behaviors [40,41]. Furthermore, the provision of enrichment to pigs has been shown to induce a positive judgment bias compared to animals housed in barren systems, suggesting pigs provided enrichment were in a more positive emotional state [16].

In this study, we investigated the efficacy of miRNA in the brain and blood as biomarkers of positive emotional state in the pig. We anticipated that husbandry practices known to result in positive welfare outcomes would lead to a more positive emotional state in the animals compared to practices known to compromise welfare outcomes. The level of brain neurotransmitters, as well as judgment bias testing, were used as corroborating measures to infer the emotional status in pigs. We hypothesized that (i) exposing pigs to enriched housing conditions would result in a more positive judgment bias, increased neurotransmitter concentration, and differential miRNA patterns in the brain and blood compared to pigs exposed to barren environments, (ii) that changes in expression of miRNA in the brain could be corroborated with changes of miRNA expression in blood, allowing peripheral miRNA response to be used as a proxy marker for positive emotional state in the pig.

2. Materials and Methods

2.1. Animals and Housing

All animal procedures were approved by the PIRSA Animal Ethics Committee (No. 01/19), and conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013), and the Animal Welfare Act 1985 (SA). A total of 24 female Large White × Landrace pigs with an average weight of 6.4 kg (range 5.0–8.2 kg), were selected at weaning from 12 multiparous sows and housed for two weeks in groups containing 12 animals per pen (2.0 m (W) × 4.0 m (L) × 0.8 m (H)) at the Roseworthy piggery, South Australia. During this period, pigs were exposed twice daily to 15 min of positive human interaction (patting, rubbing and scratching), and given sweet treats (M&M's®, Mars Wrigley, Ballarat, Vic, Australia).

At five weeks of age all pigs were moved into group pens comprising 6 animals/pen (Figure 1). The pens were (2.26 m (W) × 4.46 m (L) × 0.86 m (H)) with flooring that consisted of half concrete and half slatted floor. Each pen contained one feed hopper, 6 nipple drinkers and an overhanging heat lamp that was turned on daily between 18:00–06:00. Pigs had

access to water and ad libitum grower feed (Barastoc MP Pig 1300, Ridley's, Adelaide, SA, Australia).

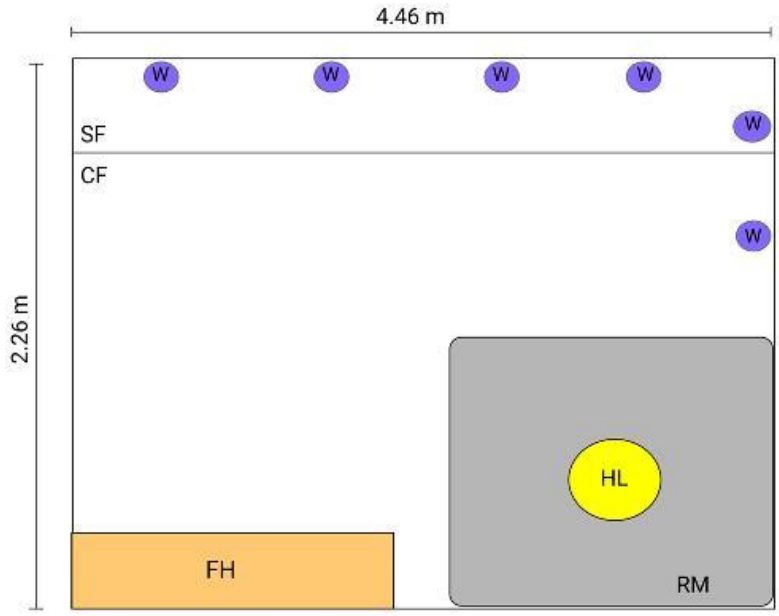

Figure 1. Group housing at Roseworthy piggery with six animals per pen. The pens were 2.26 m (W) × 4.46 m (L) × 0.86 m (H). The flooring consisted of half concrete (CF) and half slatted floor (SF). Each pen contained one feed hopper (FH), runner matting (RM), 6 nipple drinkers (W), and overhanging heat lamp (HL).

2.2. *Spatial Judgment Bias Task*

From 5 weeks of age pigs were first trained to perform in a judgment bias test that consisted of a spatial, go/no go task. During the training phase pigs discriminate between positive and aversive stimuli within a test arena (Figure 2A). Each stimulus was associated with two cues, (1) bowl color (blue = positive and red = aversive) and (2) bowl location (right or left). Each cue was reinforced with either a food reward (M&M's, positive) or no food reward plus a scare from human (see below, aversive). To ensure pigs could not discriminate between the positive and aversive reinforcer, the red bowl (aversive cue) contained chocolate treats that were unattainable to pigs due to a plastic covering (Figure 2B). The location and color of bowl were randomized for each pig using computer generated randomization in Excel (Microsoft Excel 2016, Microsoft Corporation, Redmond, WA, USA). Ordering was consistent for each pig across time. Pigs that did not learn to discriminate between the positive and aversive stimulus during training were excluded from the analysis. Exclusion criteria were based on previous literature [42], where pigs were excluded if their individual mean latency to approach the aversive cue was equal to, or lower than their individual mean latency to approach the positive cue on their last day of training. The timeline of training is provided in Figure 3 and details are provided below.

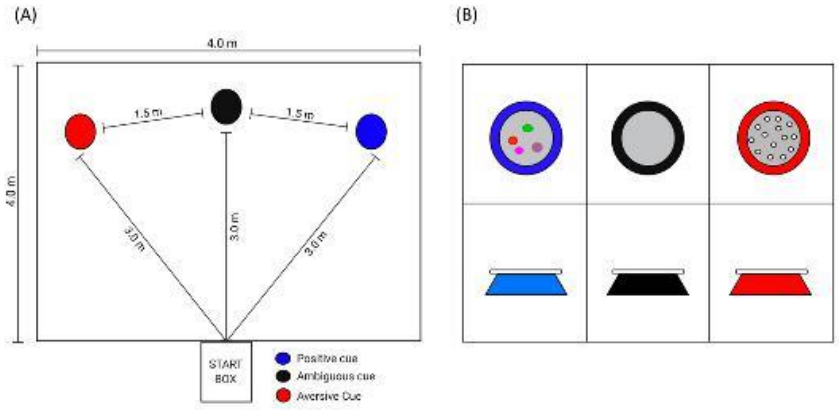

Figure 2. Illustrates (**A**) training arena for spatial go/no go task in pigs with positive, aversive and ambiguous cue locations depicted, (**B**) showing positive (blue bowl with food reinforcer), ambiguous (black bowl, no food reinforcer), and aversive (red bowl, no food reward plus a scare from human) stimulus.

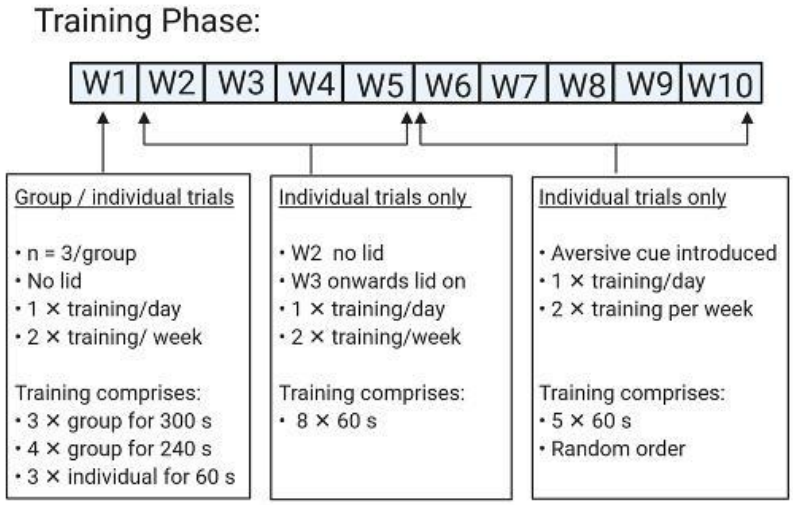

Figure 3. Indicates simple timeline of training protocol for spatial go/no go task where pigs were trained for a ten-week period in both group and individual trials.

2.2.1. Training Protocol

Week 1 Training

During week 1, pigs were habituated to the test arena once a day for two days. On each training day, pigs were exposed to ten consecutive trials (3× group for 300 s, 4 × group for 240 s and 3 × individual for 60 s). Each group trial consisted of the 3 animals housed in the same pen. Pigs entered the arena and were allowed to familiarize themselves with the arena and the positive stimulus. For each positive stimulus the positive cue was placed (no lid), at either left or right side of the testing arena and was filled with chocolate treats (M&M's). If pigs had not approached the bowl by the end of the trial, they were given additional time to approach the positive stimulus and eat the sweet treats. If pigs in individual trials displayed distress, i.e., high pitch screams, escape attempts, erratic movements or loud grunting, the individual was removed from the test arena and an additional group run was performed thereafter. Following an additional group run the pig was then trialed individually until all trials were completed.

Week 2–5 Training

Animals were trained twice a week on alternate days. On each training day pigs were exposed to eight individual consecutive trials of 60 s each. During week two training, the lid remained off the bowl in the positive stimulus. The positive cue contained five sweet treats and pigs that approached the bowl were allowed to eat the treat before being removed from the arena. From week 3 of training the lid was placed on top of the bowl and remained on for the remainder of the training and testing sessions. If the pig approached the bowl and flipped the lid, it was considered a pass and the pig was allowed to eat the treat before being removed from the test arena. If the pig failed to flip the lid it was considered a fail. Training continued until all pigs passed and were able to flip the lid within 60 s upon entering the arena.

Week 6–10 Training

Pigs were trained individually twice a week on consecutive days where the aversive stimulus was introduced. Each day pigs performed 5 trials (individual for 60 s that comprised 3× positive and 2× aversive cues). The order of trials was pseudorandomized so that no more than two positive or aversive cues were conducted in secession, but the final trial was always positive and was adapted from similar training protocols conducted previously in pigs [43] and sheep [44]. Six trials were deemed sufficient per training session based on learning ability during training weeks 1–5. If pigs approached the aversive stimulus and flipped the bowl lid, an investigator holding a toy clapper would move the clapper vigorously close to the pig's face until the pig retreated. The pig was then removed from the arena. Pigs who approached the positive stimulus were allowed to eat the reward before being removed from the arena.

Refresher Training

Refresher training occurred once a week between test 1 and test 2 (JBT1 and JBT2, respectively). This was performed to reinforce the associations between positive and aversive cues between the first and second tests. The refresher training followed the same training protocol as week 6–10 training (see above).

Once trained, pigs then underwent two judgment bias tests where the ambiguous stimulus was introduced and included a black bowl placed between the positive and negative stimulus and was unrewarded with treats. JBT1 occurred following week ten training and was prior to treatment allocation, and JBT2 occurred four weeks later following treatment allocation.

2.2.2. Testing Protocol

The test protocol was the same for both JBT1 and JBT2. Each test consisted of eight consecutive trials of 60 s each, and the trial order remained the same for each pig being tested (P, N, P, A, N, P, N, A). The sequence of trials was planned to ensure that, for all animals, the number of times each ambiguous location followed immediately after a rewarded location, and immediately after an unrewarded one, was the same [17]. The test period began when pigs moved from the start box and both front legs had entered the test arena and ended after 60 s. Latency to approach bowl was recorded for each trial using a stopwatch and times were confirmed with video data derived from one video camera (HERO5, GoPro Inc., San Mateo, CA, USA) mounted on either side of the test arena. The stopwatch was started when the pigs two front legs entered the testing arena from the start box. Following the last trial each pig was moved away from the testing area and a blood sample was taken before the pig was returned to its home pen.

2.3. *Treatments*

Treatment allocation was randomized from JBT1 data so that each treatment group comprised the same number of pigs classified as having either positive bias, negative bias or unknown bias. Individual pigs who took longer to approach the ambiguous stimulus

in JBT1 relative to the mean latency of all pigs to approach the ambiguous stimulus in JBT1 were considered to have negative bias. Conversely, individuals who took less time to approach the ambiguous stimulus relative to the mean latency of all pigs were considered to have positive bias. Individuals on the mean were considered unknown bias and randomly allocated between treatments. Pigs were then allocated between two treatments with $n = 12$ each: barren housing or enriched housing (Figure 4). Barren housing entailed animals being individually housed in barren stalls (0.6 m (W) × 2.24 m (L) × 1.7 m (H)), where pigs had sight of neighboring pigs but were unable to physically interact. Each stall contained a feed hopper and nipple drinker. Pigs had access to water and were fed 4 kg standardized grower feed (Barastoc MP Pig 1300, Ridley's, Adelaide, SA, Australia) daily. No human contact was present except for the person feeding and cleaning in the morning. In enriched housing pigs were in groups of 3 per pen (2.0 m (W) × 4.0 m (L) × 0.8 m (H)) and exposed to positive human contact (patting, rubs and scratches) for 15 min daily. Toys were also provided for enrichment and included tennis balls, basket balls, chains, ropes and PVC piping, and rubber matting. Each day the toys were placed back into the appropriate pen so that each pig had access to one of each type of toy continuously. The choice of enrichment was based on previous studies investigating the effects of providing various enrichments on welfare parameters in pigs [41,45–47].

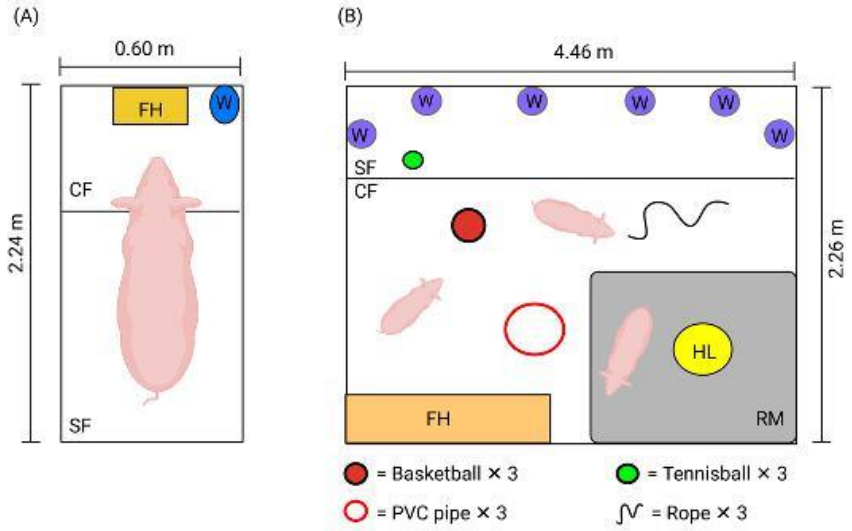

Figure 4. Indicates (**A**) barren housing (individually stalled, no human contact and no enrichment) and (**B**) enriched housing (group housed in pens, positive human interaction and enrichment provided). Barren housing conditions contained a feed hopper (FH), nipple drinker (W), concrete (CF), and slatted flooring (SF). Enriched housing contained a feed hopper (FH), nipple drinkers (W), concrete (CF), and slatted flooring (SF), a heat lamp (HL), rubber matting (RM) and enrichment materials (see legend in figure).

2.4. Blood MicroRNA Collection

Immediately following JBT1 and JBT2, pigs were restrained using a rope snare and a 3 mL blood sample collected from the jugular vein of each pig into a 4 mL-Lithium-Heparin coated tube (Vacuette, Greiner Labortechnik, Kremsmünster, Austria). Following this, 500 µL of whole blood was aliquoted into 1mL animal blood tube (Qiagen, Hilden, Germany). The blood tubes were then stored at 4 °C for 24 h and then frozen at −80 °C, following manufacturer guidelines, until further analysis.

2.5. Brain MiRNA and Neurotransmitter Collection

One day following JBT2, 6 randomly selected animals of each treatment were humanely killed with 1 mL/10kg i.v. of pentobarbital sodium (Virbac Pty Limited, Milperra, Australia) and the brain removed immediately following protocol developed by Bjarkam et al. [48]. The remaining twelve animals were returned to the commercial herd. Once removed from the skull, the brain was then submerged in ice cold saline and then sectioned into right and left cerebral hemispheres. The right cerebral hemisphere was placed directly into liquid nitrogen and frozen at −80 °C for subsequent HPLC analyses. The left cerebral hemisphere was sectioned into 5 mm coronal sections (rostral to caudal, Figure 5), and each section placed in a 150 mL specimen tub containing 100 mL of RNA stabilizing solution and then stored at −20 °C.

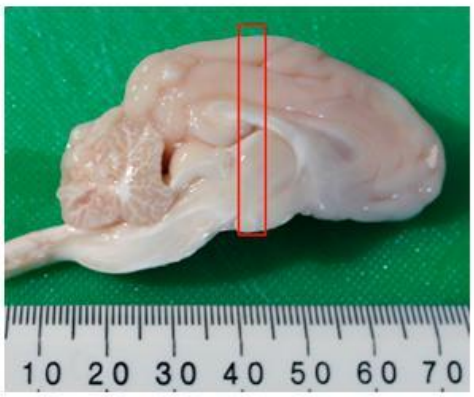

Figure 5. Example of the left cerebral hemisphere of the pig brains recovered in the experiments. The brain was further sliced into 5 mm coronal sections and placed into RNA stabilizing solution. The red box shows the approximate location of the 5 mm section where tissue from the amygdala was obtained.

2.6. Extraction of miRNA

A stereotaxic atlas of the pig brain [49], was used to identify the amygdala. Using a 1 mm biopsy punch (Ted Pella, Redding, CA, USA), a sample was taken from the amygdala (see Figure 6), weighed and immediately underwent extraction of total RNA. Isolation of total RNA was performed from the blood and tissue samples using RNeasy protect animal blood kit (Qiagen, Hilden, Germany), and RNeasy plus Universal kit (Qiagen, Hilden, Germany), respectively, according to the manufacturer's instructions. Integrity of RNA was determined using 2200 Tape-Station Analysis software (Agilent, Mulgrave, Australia), and samples with RIN values greater than 7.5 were used in the analysis.

2.7. Expression Profiling of miRNA

Differentially expressed miRNA in blood and amygdala RNA were detected using Affymetrix gene chip technology (GeneChip™ miRNA 4.0 Array, Thermofisher Scientific, Thebarton, SA, Australia), and performed by ACRF Cancer Genomics Facility (Centre for Cancer Biology, SA Pathology, Adelaide, SA, Australia), in accordance with manufacturer's instructions. Briefly, poly(A)Tailed, biotin labelled miRNA was prepared from 500 ng of total RNA sample using the FlashTag Biotin HSR RNA Labelling Kit for GeneChip miRNA Arrays (Thermo Fisher Scientific, Thebarton, SA, Australia, cat. no. 901910). Labelled RNA samples were hybridised to GeneChip miRNA v4.0 arrays with arrays incubated in a GeneChip Hybridization Oven 645 for 16 h at 48 °C. Array washing and staining were performed on the GeneChip Fluidics Station 450, and scanned using GeneChip Scanner 3000 7G. CEL files were generated using Affymetrix GeneChip Command Console Software v4.0 (Thermo Fisher Scientific, Thebarton, SA, Australia).

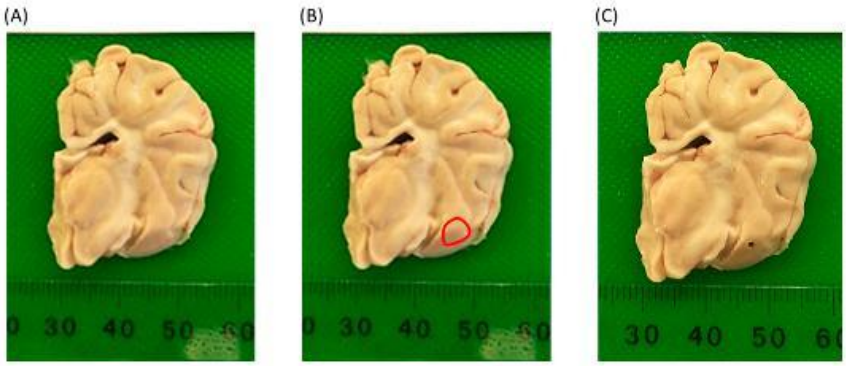

Figure 6. Sampling location for amygdala tissue, (**A**) Left hemisphere coronal section of pig brain with entire amygdala; (**B**) identification of amygdala (red circle), from pig atlas derived from Felix et al. [49]; (**C**) left hemisphere coronal section of pig brain with amygdala sample removed by punch biopsy.

2.8. HPLC Analysis

Regions of the brain including the striatum, amygdala and prefrontal cortex were dissected working on ice from the right cerebral hemisphere using the stereotaxic atlas of the pig brain derived from Félix et al. [49]. High performance liquid chromatography (HPLC), analysis was conducted to detect dopamine (DA), serotonin (5-HT), and their respective metabolites (DOPAC and 5-HIAA), using previously published methodology [50].

2.9. Statistical Analysis

2.9.1. Behavior

Behavior data were analyzed in statistical software package IBS SPSS to investigate differences in judgment bias between treatment groups. All behavior data were tested for normality and homogeneity and nonparametric analysis was conducted where appropriate. Training data were analyzed using a Friedman test to determine differences in latency to approach positive and aversive cues over time (training week 1–10 for positive and training weeks 6–10 for aversive, $n = 24$). A Wilcoxon signed-rank test was then conducted to determine differences between individual weeks. A Kruskal–Wallis test was then performed to determine difference in latency between positive and aversive cues at week ten of training.

JBT1 data were analyzed using Mann–Whitney–Wilcoxon test to determine differences in latency towards cue location, and was performed on 23 pigs ($n = 12$; enriched, $n = 11$; barren), as one pig had to be euthanized on humane grounds. Kruskal–Wallis and Mann–Whitney–Wilcoxon tests were then performed to look at treatment effects on latency towards the ambiguous cue at JBT1 and JBT2 and between JBT1 and JBT2. To control for possible intrinsic differences between pigs (i.e., walking speed, food motivation and body size), an adjusted judgment bias index (JBI), was calculated for each pig at JBT1 and JBT2 following a formula described by Horback et al. [43]. The JBI normalizes the animal's response toward the ambiguous stimulus based on its previous responses to the positive and negative stimulus. The index ranges from 0–1 where animals with a JBI < 0.2 are considered negatively biased, a score of > 0.8 are positively biased and a score between 0.3–0.7 are unknown bias. A Fisher's Exact Test analysis was performed to determine the change in proportions in JBI between pigs exposed to positive or negative housing at JBT1 and JBT2. Latency data are presented as medians with upper and lower range and JBI data are presented as proportions. Data were considered significant when $p \leq 0.05$ unless stated otherwise.

2.9.2. Blood and Brain MiRNA

Analysis of differentially expressed genes in blood and brain were conducted following a similar statistical protocol performed previously [51]. Affymetrix data were imported into genomic software package TAC (Transcriptome analysis console 4.0, Applied biosystems, Thermofisher Scientific, Thebarton, SA, Australia). Independent *t*-tests to determine between and within treatment effects at bleed 1, bleed 2 and in Amygdala were performed. Differences were considered significant when a gene level fold change of <2 or >2 occurred with an FDR adjusted *p*-value of less than 0.05 (FDR $p < 0.05$).

2.9.3. Dopamine, Serotonin and Metabolites

Brain dopamine (DA), serotonin (5HT), and their respective metabolites DOPAC and 5H1AA were analyzed in statistical software package IBS SPSS to investigate differences in expression between treatments. Data were tested for normality and homogeneity using the Kolmogorov and Levene's test, respectively. A Mann–Whitney–Wilcoxon test was then performed to investigate treatments differences in Amygdala, Striatum and Prefrontal cortex. Data are presented as medians ± range with a significance level of $p < 0.05$.

3. Results

3.1. Behaviour Data

3.1.1. Identification of Positive and Aversive Cue

During the learning phase (weeks 1–10) pigs were able to successfully identify the positive cue as shown by the decreased mean latency to approach the positive cue over time (χ^2 (9) = 117.7, $p = 0.000$, Figure 7A). During the learning phase from weeks 6–10 there was a significant difference in the latency towards the aversive cue over time (χ^2 (4) = 12.99, $p = 0.012$, Figure 7B). During week ten of training the latency to approach the positive cue was significantly lower compared to the aversive cue ($Z = -5.8$, $p = 0.000$, Figure 8).

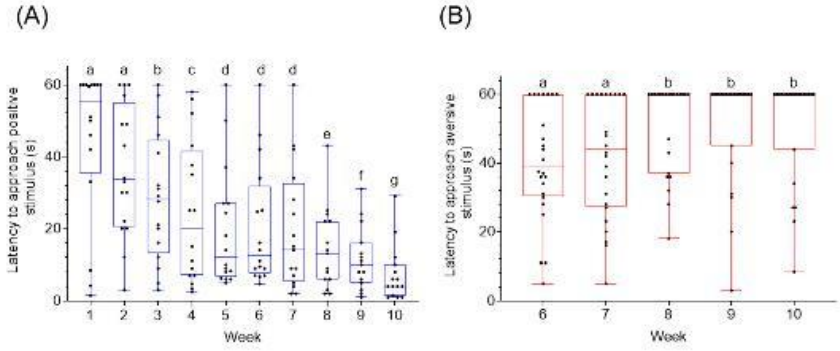

Figure 7. (**A**) Latency to approach (s) the positive stimulus during training weeks (1–10) in pigs ($n = 24$), (**B**) indicates latency to approach aversive stimulus during training weeks (6–10) in pigs (n24). Data are medians with range. Significant difference is indicated with differences in subscripts ($p < 0.05$).

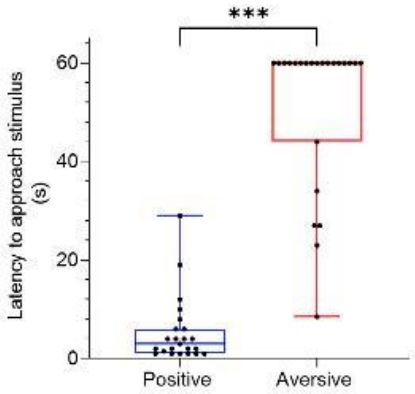

Figure 8. Latency to approach (s) positive and aversive stimulus at week ten of training in pigs (n = 23). Data are medians with range. Significant difference is indicated with presence of asterisk ($p < 0.05$).

3.1.2. Cue Location and Latency to Approach

An overall effect of cue location on latency to approach was observed in all pigs in both JBT1 and JBT2 ($\chi^2(2) = 21.7$, $p = 0.000$; Figure 9). During JBT1, an increased latency to approach was observed towards the aversive location compared to both the ambiguous ($Z = -404.0$, $p = 0.000$) and positive ($Z = -3.88$, $p = 0.000$) locations. Pigs further had increased latency towards the ambiguous location compared to the positive location ($Z = -3.6$, $p = 0.020$; Figure 9A). During JBT2, an increased latency to approach was observed towards the aversive location compared to both the ambiguous ($Z = -3.99$, $p = 0.000$) and positive ($Z = -3.\ 7$, $p = 0.001$) locations, but no increased latency towards the ambiguous location compared to the positive location was observed ($Z = -1.4$, $p = 0.16$; Figure 9B). Between JBT1 and JBT2, there was no difference in latency to approach the ambiguous location in pigs exposed to either enriched or barren housing treatments ($Z = -1.2$, $p = 0.250$ and $Z = -1.22$, $p = 0.360$ $p = 0.36$; Figure 9C). There was no significant effect of treatment on latency towards the ambiguous cue during JBT2 ($Z = 2.11$, $p = 0.48$; Figure 9D).

3.1.3. Treatment Effects on Judgment Bias

No effect of treatment on JBI between JBT1 and JBT2 was observed (χ^2 (20) = 2.0, $p = 0.5$).

3.1.4. Blood and Brain MiRNA

At bleed 1 there were 51 differentially expressed miRNA between pigs exposed to enriched and barren housing (14 up regulated and 37 down regulated) but none were significant (FDR $p > 0.05$). Similarly, following bleed 2 there were 71 differentially expressed miRNA between pigs exposed to enriched and barren housing (43 up regulated and 28 down regulated) but none were significant at the FDR threshold (FDR $p > 0.05$). Within the amygdala, a total of 185 miRNA were differentially expressed (122 up regulated and 63 down regulated), but no significant effect of treatment was observed (FDR $p > 0.05$). The top 10 genes that were closest to achieving statistical significance, for each comparison, are listed in Tables S1–S3 (Supplementary Materials).

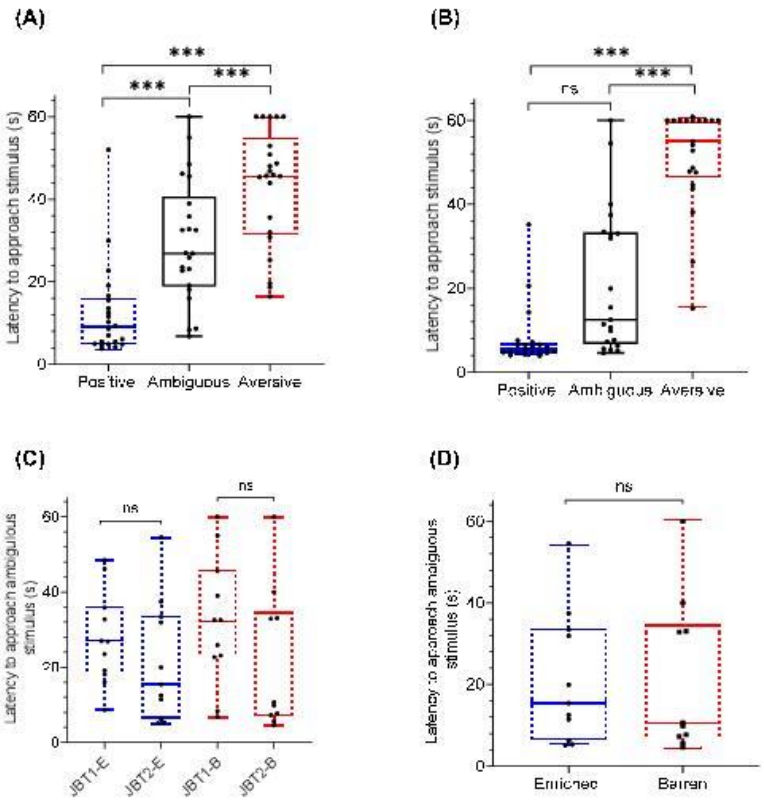

Figure 9. (**A**) Latency for pigs ($n = 23$) to approach positive, aversive and ambiguous stimulus at judgment bias test 1 (JBT1), (**B**) latency to approach positive, aversive and ambiguous stimulus in judgment bias test 2 (JBT2) in pigs exposed to enriched housing ($n = 12$), or barren housing ($n = 11$), (**C**) indicates latency to approach ambiguous stimulus between JBT1 and JBT2 in pigs exposed to enriched ($n = 12$), or barren housing ($n = 11$), and (**D**) indicates latency to approach ambiguous stimulus during JBT2 in pigs exposed to enriched ($n = 12$) or barren housing ($n = 11$). Data are medians with range. Significant difference is indicated with presence of asterisk ($p < 0.05$).

3.1.5. Dopamine, Serotonin and Metabolites

Pigs exposed to enriched housing had an increased concentration of dopamine (DA) (2838.8 ng/g vs. 1002.3 ng/g, $Z = -2.26$, $p = 0.02$) and its metabolite DOPAC (620.1 ng/g vs. 266.6 ng/g, $Z = -2.26$, $p = 0.02$) within the striatum, compared to pigs housed in barren conditions (Figure 10). No significant effect on DA or DOPAC was observed in the amygdala ($Z = -0.94$, $p = 0.37$ and $Z = -0.53$, $p = 0.68$) or prefrontal cortex ($Z = -1.60$, $p = 0.37$ and $Z = -1.60$, $p = 0.37$). Furthermore, treatment had no significant effect on serotonin (5HT) or its metabolite 5-HIAA in the striatum ($Z = -0.8$, $p = 0.12$), amygdala ($Z = -1.60$, $p = 0.13$), or prefrontal cortex ($Z = -1.2$, $p = 0.68$).

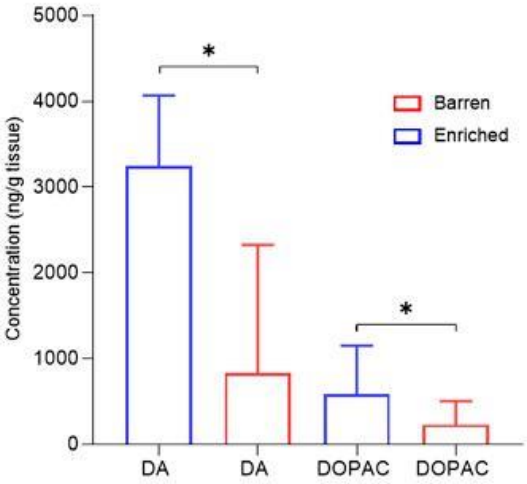

Figure 10. Concentration (ng/g tissue) of dopamine (DA), and its metabolite (DOPAC), in the striatum of pigs exposed to either enriched ($n = 6$) or barren ($n = 6$) housing treatments. Data are median ± range. Significant differences are indicated by presence of asterisks ($p < 0.05$).

4. Discussion

In this study we investigated the suitability of circulating miRNA as biomarkers to distinguish valence of emotional state in the pig. We proposed that miRNA would be differentially expressed in the brain and blood during positive emotional states, and that a change in miRNA could be corroborated with already existing behavioural and physiological indices of emotional valence. We hypothesized that (i) exposing pigs to enriched housing conditions would result in a more positive judgment bias, increased neurotransmitter concentration, and differential miRNA patterns in the brain and blood compared to pigs exposed to barren environments, (ii) that changes in the expression of miRNA in the brain could be corroborated with changes of miRNA expression in blood, allowing peripheral miRNA response to be used as a proxy marker for emotional state in the pig. We found that treatment had no effect on behaviour during the JBT, nor did we observe differences in miRNA profiles in the brain or blood of pigs. There was an increase in concentrations of DA and its metabolite DOPAC in the striatum, but this increase was not observed in amygdala or prefrontal cortex. No difference in the neurotransmitter serotonin (5-hydroxytryptophan or 5-HT), nor its metabolite 5-HIAA, was found in any brain region between treatment groups. The results of this study imply that either (a) miRNAs are not likely to be valid biomarkers of positive affective state, at least under the type of conditions employed in this study, or (b) that the study design employed with enriched housing versus barren housing as a modifier of affective state was not sufficient to create differential affective states, and therefore establish the validity of miRNA as biomarkers.

With regard to the first possible interpretation—that miRNAs are not likely to be valid biomarkers of affective state—there is some limited evidence from the porcine literature on the validity of miRNAs, at least as biomarkers of negative states. Weaning stress [50], and heat stress [52], altered miRNA expression in intestinal and muscle tissue respectively. Lecchi et al. 2020 [53], also demonstrated that certain miRNA expression changes in saliva were present following castration and tail docking without analgesia. Our null finding, in contrast to these studies, might be explained by the assumed relatively low impact on physiological processes created in our study. The effects of heat and pain variously create cell damage, tissue degradation and inflammatory pathway activation which may not occur as a result of environmental change. MiRNA may therefore only be useful biomarkers where a relatively invasive change occurs that has a notable effect on physiology.

A significant increase in the tonal concentration of DA, and its metabolite DOPAC, in the striatum of animals exposed to enriched housing conditions was observed. This finding is consistent with our hypothesis and suggests that the provision of enrichment resulted in a chronic shift in affective state, leading to a more positive emotional state in the animals. It is difficult to know if the relationship between the treatment and increased DA was a causative effect, or perhaps a response elicited by another biological process. Given that DA is implicated in behavioural control and is essential for reward related processes including reward learning [54,55], we anticipated this same difference to be reflected in the judgment bias data. For example, here we observed a treatment effect on tonal DA (i.e., a sustained level of DA neuron firing) where enriched housing increased tonal DA compared to animals housed in barren conditions. Subsequently, we would anticipate that the tonal increase in DA would influence behaviour, where pigs would, under ambiguity, have an enhanced expectation of a positive outcome and behave in a manner normally associated with a positive reward. Here, we did not detect a treatment effect on behavioural parameters; however, potential issues with the design of the behaviour paradigm may account for this and are discussed below. Furthermore, it is interesting that we did not see an increase in DA in the amygdala or the prefrontal cortex. Following rewarding experiences, dopaminergic neurons project widely throughout the brain. The ventral striatum is the region of the brain most closely associated with reward processing such as reward-based learning [56], and is directly innovated by the orbital prefrontal cortex and amygdala [57]. The amygdala plays a critical role in the coordination of the conscious experience of emotion and, along with the prefrontal cortex, forms reciprocal connections that allow learning and experience of the cognitive aspects of emotion [58]. It is unusual, then, given the interconnections between these regions, that no increase in DA was apparent in the amygdala or prefrontal cortex. However, there is some evidence from human studies that an increased reactivity in the ventral striatum occurs during adolescence, leading to stronger striatal activation in response to primary, secondary and social rewards [56]. We speculate that the age of the pigs used in the present study may have resulted in similar effects, where enhanced activity within the striatum may have occurred but was obscured in other brain regions (i.e., amygdala and prefrontal cortex) due to potential developmental differences in the brain. Further research is necessary to clarify and confirm this.

Serotonin is a key neurotransmitter abundant throughout the body and involved in a number of biological systems. Central 5-HT, however, is implicated in behavioural and neuropsychological processes including, but not limited to, mood regulation, appetite, sexuality and attention. In humans, chronic dysregulation of serotonergic activity, including alterations in serotonergic tone, is considered a key component underlying a number of affective disorders including anxiety and depression [59,60]. Serotonergic neurons originating from the raphe nucleus project to multiple brain structures involved in emotional regulation and behaviour response; this includes the amygdala [61], striatum [62], and prefrontal cortex [63]. Previously, administration of the 5-HT antagonist pCPA resulted in pessimistic judgment bias in sheep [44] and pigs [64], and depleted 5-HT concentration in brain regions including the rostral anterior cingulate cortex, prefrontal cortex, striatum, amygdala, hippocampus, hypothalamus and brain stem [65]. Furthermore, pharmacologically induced increases in 5-HT led to a positive judgment bias in rats with a dose dependent response [65]. Unexpectedly, we observed no difference in tonal 5-HT concentrations in the brain of pigs housed in enriched conditions. An explanation for this may be that the duration animals were exposed to the enriched treatment (four weeks), or the enrichment itself, was not sufficient to alter tonal 5-HT concentrations. Another factor may be that alterations in 5-HT levels are more closely associated with the body's stress systems, including HPA activity in response to negative stimuli [66]. For example, following acute handling stress, 5-HT has been shown to be reduced from baseline levels in hippocampus and amygdala in fearful pigs, with the same reduction not occurring following non-stressful handling [66]. Another study has shown hippocampal 5-HT is positively correlated with standing alert time (freezing) during a novel object test, indicating a higher

level of anxiety or fear in pigs [67]. It is plausible that the effect of enrichment was not sufficient to stimulate the bodies HPA axis, and thus no chronic changes in 5-HT levels were observed.

We expected that animals housed in enriched conditions would experience a more positive emotional state leading to the judgment of ambiguous stimuli with an enhanced expectation of a positive outcome, and, therefore, result in reduced time to approach the ambiguous cue provided. However, in this study no change in judgment bias was observed in response to enriched housing. There are two likely reasons for this: (i) there was no change in affective state in response to the treatments and/or (ii) the possibility that factors related to the training and test design may have compromised the JBT results.

Whilst increased space allowance, as provided in the enriched housing, has been shown to have beneficial effects on welfare in several studies [68], enrichment may be a determining factor in effects observed. Although the provision of enrichment has been previously shown to improve welfare outcomes and induce a positive bias in pigs [16,41,69], the type of enrichment given in this trial may not have been considered a rewarding stimulus by the pigs, and thus not been integrated at a cellular level. For example, for enrichment to be effective it should stimulate an animal's visual, somatosensory, and olfactory systems whilst maintaining its novelty [70], where natural substrates, such as straw, green fodder, root vegetables and pressed or chopped miscanthus, are considered optimal for animal welfare. Unfortunately, the use of natural substrates for enrichment was not feasible in this trial due to the negative impact this may have had on the effluent system on this particular farm. Consequently, the substrate used may not have been sufficient to provide a rewarding stimulus. Furthermore, the provision of enrichment may have, in fact, affected the pigs in a negative manner, perhaps leading to aggression due to competition for the limited resource. Furthermore, the social structure of pigs is based on a dominance hierarchy, which is vigorously established through fighting when unacquainted pigs are brought together [71]. Although pen mates in the enriched housing group remained the same throughout this experiment, there may have been some incidences of aggression following training or testing, as individual animals were frequently removed from and then reintroduced to the group. Competition for resources could also have been a factor of disturbance for the pigs housed in groups. If the objects provided were insufficient then the social competition from pen mates may not allow all animals to use the enrichment at the same time, leading to adverse events such as aggression and tail biting [72]. It would have been beneficial to make additional behavioural observations of individuals in the enriched housing treatment to gain a better understanding of the level of activity and types of behaviour shown toward enrichment objects, as well as an account of behaviours considered to reflect positive emotions such as play behaviours [73,74].

Similar issues may have arisen in pigs housed in barren conditions. We would expect that that the effect of isolation in a barren environment would have a negative impact on the pigs and result in a more negative judgment bias. It may be that the animals exposed to barren environments did not find the environment extreme enough to alter behavioural outcomes in the judgment bias test. This has been observed in piglets where repeated social isolation had no effect on behaviour parameters toward ambiguous stimuli [75]. It may also be the case that the pigs housed in the negative environment were displaying rebound behaviour during the test. Rebound behaviour can be described as an increased tendency to perform a specific behaviour, i.e., an activity rebound, after a period of prevention [76]. If pigs were unable to perform locomotive behaviour due to the isolated and restricted housing, they may have developed or built up the urge to display increased locomotive behaviours once released into the test arena. If the pigs that were confined showed increased locomotive behaviour due to rebound effects, some may have touched the ambiguous probe (through choice or accidentally) quicker than if they were not confined, and thus confounded the latency to approach results. The test design itself may also have not been sensitive enough to successfully identify differences in affective state in the pigs in response to the housing treatment. During testing, a number of factors may have

arisen which could have affected latency outcomes. It is common for judgment bias trials, including the present study, to leave the ambiguous cue unrewarded [9]. However, such an approach has, in some cases, led to loss of ambiguity towards the ambiguous cue and pigs learn to associate the ambiguous stimulus with an unrewarded outcome [9]. If pigs in this trial learned that the ambiguous stimulus was unrewarded during JBT1, and then remembered this during JBT2, their responses may have led to false measures of judgment bias, as seen previously in sheep [77] and pigs [78]. It has been suggested that rewarding ambiguous cues may maintain optimistic choices throughout testing [78], although similar issues may still arise through associative learning in relation to ambiguous trials that are rewarded. Furthermore, it has been suggested that the measurement of latency alone may lead to the false detection of pessimism in cases where animals are exposed to repeated ambiguous trials [79]. This was observed in rats, where exposure to repeated ambiguous trials was associated with increased latency. However, this increase in latency was also associated with optimistic responses in an active choice test [79]. As the authors in this study conclude, modification to the experimental designs that include both active-choice and latency measures would have been beneficial to minimize ambiguity of interpretation of latency data.

5. Conclusions

No changes in miRNA profiles in the brain or blood of pigs were observed in pigs exposed to either enriched or barren housing conditions. Although increased concentrations of dopamine and its metabolite DOPAC were observed in the striatum, this was not the case in the amygdala or prefrontal cortex. There was no difference is the neurotransmitter serotonin nor its metabolite 5-HIAA in any brain region between treatment groups. No difference was observed in judgment bias in any treatment group. There are two likely reasons for this: (i) there was no change in affective state in response to the treatments and/or (ii) the possibility that factors related to the training and test design may have compromised study outcomes. Therefore, in the absence of an adjunct measure indicative of valence of response (i.e., behavioural and physiological indices), we are unable to confirm the validity of miRNA as biomarkers of emotional state. However, given their promise as suggested in the literature, we recommend that further investigation of their utility as biomarkers for positive affective state should be undertaken.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani11072054/s1, Table S1: Indicates the top 10 miRNA genes with highest, albeit non-significant, *p*-values at bleed 1 in pigs exposed to either enriched or barren housing. Column 1 indicates gene ID; column 2 indicates fold change; column 3 indicates *p*-value ($p < 0.05$); and column 4 indicates *p*-values with an adjusted false detection rate (FDR $p < 0.05$). Table S2: Indicates the top 10 miRNA genes with highest, albeit non-significant, *p*-values bleed 2 in pigs exposed to either enriched or barren housing. Column 1 indicates gene ID; column 2 indicates fold change; column 3 indicates *p*-value ($p < 0.05$); and column 4 indicates *p*-values with an adjusted false detection rate (FDR $p < 0.05$). Table S3: Indicates the top 10 miRNA genes with highest, albeit non-significant, *p*-values within the amygdala of pigs exposed to either enriched or barren housing. Column 1 indicates gene ID; column 2 indicates fold change; column 3 indicates *p*-value ($p < 0.05$); and column 4 indicates *p*-values with an adjusted false detection rate (FDR $p < 0.05$).

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Institutional Review Board Statement: This study was conducted in accordance with the Australian code for the care and use of animals for scientific purposes (NHMRC 2013) with approval from the University of Adelaide Animal Ethics Committee (Animal Ethics Number 2018-053).

Informed Consent Statement: Not applicable.

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General discussion

Research Summary

Over the last few decades, the field of animal welfare science has rapidly gained momentum and emphasis has been placed on the identification of methods that can provide accurate welfare assessment. In wake of this, the assessment of welfare in animals used in agriculture has now advanced to include measures of their emotional/affective state. The affective experience can vary in terms of valence, arousal and motivation, and comprises physiological, behavioural and cognitive components. To date, the most meaningful assessments of animal emotion incorporate the biological functioning/activity arising when animals attempt to cope with their environment, and involve physiological systems classically associated with stress, as well as a range of neurological, immunological, endocrinological and behavioural responses. Although these markers (or biomarkers) can provide information about the activation of these systems to infer the emotional experience in animals, they can often lack objectivity and specificity, are often unable to differentiate the valence of affect or are simply not suitable for a production environment. Furthermore, these measures are often used to evaluate negative affect, with far less focus on identification and development of positive emotions in animals. Consequently, there is a need to identify novel biomarkers of emotional states in animals. These measures must be robust, objective, and easily collectable. Furthermore, they must have the ability to distinguish valences of affect.

Recently, miRNAs have emerged as diagnostic markers for a number of neurological conditions, including those associated with emotional processes. These molecules are ubiquitous throughout the body and can be readily measured in a range of matrixes, including tissue, blood, saliva and urine. In the periphery, circulating miRNAs have been used as indicators for various disease states, including psychological conditions such as anxiety and depression. Therefore, miRNAs have the potential to be biomarkers of the neural activity associated with emotional processing. Here, I hypothesised that miRNAs would be expressed differentially in the brain and blood during different affective states in pigs. Furthermore, I anticipated that the expression of circulating miRNA could be used as a biomarker for the

neural activity associated with emotional processes, thus providing a proxy marker of emotional states in pigs. Through this body of work, three experimental chapters were conducted where I aimed to identify miRNA-based biomarkers for both negative and positive emotional states.

In Chapter 3, miRNA expression was investigated as a potential biomarker for pain in piglets in response to tail-docking, with and without anaesthetic. It was hypothesised that 1) the pain in response to tail docking would result in differentially expressed miRNAs in both the blood and brain of piglets, and that this expression would be modulated by local anaesthetic, and 2) the expression of miRNAs would be similar between blood and brain, indicating that circulating miRNAs could be used as a reliable proxy marker of brain miRNA expression associated with pain processes. The results of this study revealed two miRNAs (namely, miR-412 and miR- 7a) were differentially expressed within the brain following treatment. Specifically, miR-412 was significantly downregulated in the PAG in both TD and TDA treatments compared with the control animals. However, no difference in miR-412 expression was observed between TD and TDA treatments, suggesting the anaesthetic did not modulate the pain response. Furthermore, miR-7a was downregulated in the PAG of animals in TD treatment compared with animals in TDA treatment, but this relationship was not reflected between TD vs control treatments or TDA vs control treatments. Lastly, there was no significant effect of treatment on miRNA expression within the AMY or in blood at any timepoint.

A number of factors may account for the results observed in this study. In relation to mirR-412 expression, it is a likely possibility that the effects of the anaesthetic, and thus its pain modulatory response, were ineffective, or at least diminishing in effect, at the time of tissue collection. This may account for the lack of difference observed between TD and TDA treatments. The use of an extended-release form of anaesthetic, or an additional dose of anaesthetic closer to the time of tissue extraction, would be beneficial to incorporate in future studies to ensure the pain response from the TD treatments is sufficiently blocked at the time of tissue collection.

Tissue and nerve injuries as a result of amputation can lead to the formation of traumatic neuromas that reflect neuropathic or phantom limb pain. Previously, differentially expressed genes have been implicated in neuropathic pain conditions, including gene families linked to inflammation, macrophage, neurohormone and opioid peptide activity in response to tail-docking in pigs. Here, miR-7a was differentially expressed between TD vs TDA animals, suggesting that miR-7a may be implicated in the above processes. However, we would also have expected miR-7a to be differentially expressed between TD and TDA vs control animals. Given this was not the case, there may have been direct anaesthetic effects on the miR-7a response, including anti-inflammatory effects, which have been observed previously in humans. A limitation in this study was not including an additional measure of inflammation (i.e., pro- and anti-inflammatory cytokines), in order to elucidate the potential role miR-7a plays in inflammation processes.

A further impediment to the study design may be the time of blood sampling. In this study we observed no difference in miRNA expression in the blood at any timepoint, thus were unable to correlate changes in brain miRNA expression with changes in blood. It may be that the study design did not allow the identification of any potential acute changes in expression as the first blood sample was taken 1 hour following treatment. Previously, differentially expressed miRNA expression was observed in saliva 30-45 minutes following tail docking and castration in pigs. Given the results from this study, and the fact that miRNAs can respond rapidly (i.e., < 30 min in circulation) in response to pain processes, additional sampling times closer to the time of treatment are required to determine if any response in miRNA was missed. Furthermore, the process of blood collection has previously been shown to induce stress in animals. If this were the case here, there is a potential for any changes in miRNA response to be masked by the stress of handling/restraint and/or pain from the needles. Future research would benefit from investigating miRNA in samples collected from a less invasive method (i.e., via saliva), especially when acute changes in miRNA in saliva have previously been observed in response to pain processing.

Following a noxious stimulus, nociceptive signals transmit to brain areas including the somatosensory cortices and limbic structures associated with the sensory aspect of pain (i.e., intensity and location) and the affective aspects of pain (i.e., perception and attention), respectively. Consequently, it would be expected that differential expression of miRNA would be observed in both PAG and AMY, providing evidence of emotional processing in response to pain signals. Here, no difference in miRNA expression was observed in the AMY tissue, which it makes it difficult to determine if expression of miR-412 and miR-7a were implicated in the affective dimension of pain, or merely associated with other pain processes, including inflammation. It would have been useful to incorporate behavioural indices into the experimental design (i.e., specifically operant behavioural tests or conditioned place avoidance paradigms, providing information on an animal's learned responses related to the emotive attributes of a noxious stimuli). This then may allow any differences in the brain to be validated against the behavioural outputs that are used to infer emotional valence. Additional measures of autonomic functioning, including heart rate, respiration rate, body temperature and inflammatory cytokines would further assist in teasing apart the miRNA response observed in the PAG and should be considered in future.

In order to validate novel molecular measures (i.e., miRNA), of emotion in pigs, it was first necessary to ascertain that pharmacologically induced activation of specific neural circuits were possible in pigs, in particular those circuits associated with emotional processes. In humans, SSRIs are commonly prescribed to patients suffering from psychological conditions such as anxiety and depression, where these compounds act to increase serotonergic tone throughout these pathways. Previously, the neural networks and regions of the brain involved in serotonergic transmission have been identified, and these pathways can directly innovate brain structures associated with endocrine modulation, including activation of the HPA axis associated with glucocorticoid synthesis. In Chapter 4, the circulating cortisol response was used as a biomarker of effective delivery of a single intravenous dose of the SSRI, fluoxetine hydrochloride, to the pig brain. The outcomes of this study suggest that administration of the SSRI resulted in increased mean circulating cortisol profiles in the pigs, thus providing evidence that

activation of the HPA axis had occurred via effective delivery of SSRI to the serotonergic reward pathway. Despite these findings, investigation of individual cortisol profiles in response to SSRI treatment revealed high interindividual variations, with pigs displaying either the expected, unorthodox or no response. Whilst a number of factors are known to influence the cortisol response (i.e., sex, diurnal rhythm, feed intake, level of exercise, body weight), these factors were standardised in the study design and thus should not have impacted the results. Previous evidence in humans identifies genetic, cognitive, neuroendocrine, as well as personality factors linked to variations in SSRI efficacy. Thus, individual genetic differences relating to 5-HT transmission (i.e., receptor number and type, receptor function, polymorphisms in transporter genes, and polymorphisms in genes encoding 5-HT synthesis) (Kroeze et al., 2012; Lin et al., 2014), as well as environmental factors (i.e., stress) (Kovacs et al., 2014; Bi et al., 2021) may account for the variations observed in this study. Environmental factors, including human handling during the experiment or perhaps the confined and isolated housing conditions employed in this study, may have been considered stressful events by some of the pigs, thus potentially influencing HPA activity.

Although the mean data suggest the circulating cortisol response can be used as a biomarker of effective delivery of fluoxetine hydrochloride to the pig brain, future research requires that inter-individual differences are taken into account. Research that incorporates larger sample sizes may aid in attenuating the observed variations in cortisol response. Longer habituation periods with increased positive human-animal interactions, as well as reducing the time animals are kept separated from pen mates, may minimise any environmental effects on HPA activity in response to potential stress. Genetic factors known to influence SSRI efficacy (mentioned above), including HPA axis reactivity, could not be accounted for in this study. The animals used in this study were selected at 18 weeks old and born from varying maternal and paternal lines. Future research should consider selecting animals from the same litter to reduce genetic diversity between individuals and minimise the potential for genetic variability in SSRI response. Furthermore, measurement of 5-HT concentrations in brain regions (i.e., raphe nuclei, amygdala, and hypothalamus), as well as measures of neuroendocrine function associated with cortisol

synthesis (i.e., ACTH concentrations) may allow further distinctions between individual variations in cortisol responses.

The assessment of emotional or affective states in animals can be challenging, particularly the assessment of positive emotions, given there is a scarcity of identified behavioural or physiological biomarkers specific to these states. Currently, the most commonly-applied measures used to infer positive emotional states in animals include a number of behavioural responses (in particular behavioural tests such as judgment bias tests), as well as markers of activation of brain regions associated with reward processing (i.e., neurotransmitters such as 5-HT and DA). To date, judgement bias tests have been successful in determining the valence of affect in a number of species, including rodents, sheep, dogs, poultry, cattle and pigs. The assumption underlying these tests is that following discrimination training between positive and aversive stimuli, under ambiguity, animals that behave in a manner normally associated with a positive stimulus are said to have enhanced expectation of a positive outcome and this implies a positive emotional state. The opposite applies for negative states. Although these tests are considered effective in determining valences of affect in animals, they often require lengthy training times and thus are not suitable for a production environment. Furthermore, the necessity for extraction of brain tissue and complex laboratory analysis to measure levels of neurotransmitters, again renders this method of assessment impractical for production settings. Despite this, behavioural tests such as judgement bias and other biological markers of emotion (i.e., neurotransmitters), have the potential to be used as corroborating measures when attempting to validate novel biomarkers of emotion. Thus, in Chapter 5 I investigated the efficacy of miRNAs in brain and blood as biomarkers of positive emotional states in pigs. I anticipated that husbandry practices known to result in positive welfare outcomes (highly enriched housing) would lead to a more positive emotional state in the animals compared with practices known to compromise welfare outcomes (barren housing). Additionally, I expected that miRNA expression in the blood would be similar to that in the brain, and thus circulating miRNA could be used as a proxy-marker of positive emotions in pigs. The level of brain neurotransmitters (5-HT, DA and their

retrospective metabolites) as well as judgment bias were used as corroborating measures to infer the emotional status in pigs. The findings of this study identified that enriched housing resulted in elevated DA and its metabolite DOPAC within the striatum of pigs, but this change was not reflected in the Amygdala or PFC. Conversely, treatment had no effect on 5-HT concentration, behaviour during the JBT, or on miRNA expression in the blood or brain.

In regards to the null difference in miRNA expression between treatment groups, the result can be interpreted as either A) miRNAs are not likely to be valid biomarkers of positive affective states, or B) that the treatments employed were not sufficient to modify affective states in the test pigs. In relation to the first interpretation, the current literature on pigs has identified differentially expressed miRNA profiles in response to various stress events such as weaning stress, heat stress, castration and tail docking. These events likely trigger the body's stress systems, which ultimately results in the activation of neurological, endocrinological and immunological responses. Perhaps in contrast with the current literature investigating miRNA efficacy, particularly in negative states, it could be assumed that in this study the environmental change employed to induce positive states had a relatively lower impact on the pathological processes and/or stress systems by comparison. MiRNAs may therefore only be useful biomarkers as a consequence of events or treatments that are relatively invasive in nature, or impact on physiological systems, including those associated with stress. Further study would be necessary to confirm by means of investigating miRNAs in response to additional assumed positive events that have been shown to activate stress systems (i.e., during mating or social play (Koolhaas et al., 2011; Villalba and Manteca, 2019)).

In Chapter 5 it was further hypothesised that pigs housed in enriched conditions would experience a more positive affective state and result in positive bias in the JBT. However, no difference in behaviour was observed between enriched and barren treatments, indicating that either the treatment was insufficient to alter the affective state or perhaps factors relating to training/testing design compromised the JBT results. A number of issues relating to the housing conditions, including the type of enrichment

provided, potential incidences of aggression between individuals in enriched housing, as well as potential of rebound behaviour during the JBT in pigs exposed to barren conditions, may have been confounding factors that compromised the behavioural outcomes in the JBT. Furthermore, a number of problems may have arisen during testing which could have affected latency outcomes, including a loss of ambiguity towards the ambiguous cue, as well as pigs learning to associate the ambiguous stimulus with an unrewarded outcome. In order to overcome these issues in future research, I put forward a number of suggestions that may help to minimise these effects. It would be beneficial if future studies include the provision of different types of enrichment, including natural substrates assumed to be pleasurable and promote biological functioning (i.e., straw, wood shavings, and bark) (Bracke and Hopster, 2006). Furthermore, incorporation of additional behavioural observations in enriched housing treatments, including the level of activity towards enrichment, as well as an account of behaviours considered to reflect both positive (i.e., play), and negative (i.e., aggression, or tail biting), states will ensure that the positive effects of enrichment are not being overcome by negative behavioural events. Additionally, during testing of pigs, a design that incorporates either a secondary reinforcer (i.e., a clicker or auditory cue used in conjunction with the food reward in the positive stimulus and again in conjunction with the non-rewarded ambiguous stimulus), or a partial reinforcer (i.e., reducing the number of times the positive stimulus is rewarded), may maintain pig responsiveness towards the ambiguous stimulus (Cardoso et al., 2009; Keen et al., 2014).

Lastly, although DA concentration significantly increased in the striatum of enriched pigs, the lack of difference in DA concentration in the other brain regions (i.e., the Amygdala and PFC) was surprising. Given these brain regions have high interconnectivity in their neural networks and are all known to be implicated in emotional regulation, it makes it difficult to conclude the change in striatal DA was a direct response to emotional processing alone, so perhaps another biological process (i.e., movement, attention or arousal) was involved. Moreover, DA is implicated in behavioural control and considered essential for reward-related processes such as reward learning, and thus I would have expected this difference to be

observed in the behavioural data. Given this was not the case, it makes it difficult to draw conclusions regarding the affective state of the pigs based on the DA data alone.

Conclusions and future directions

The ability to identify the welfare of animals accurately is critically dependant on our ability to assess their constituent affective states. This is because it is now widely recognised that animals are sentient, having the capacity to experience both positive and negative emotions. Traditional measures used to infer the emotional states in animals include a range of behavioural and physiological indices, likely derived from the biological functioning of the animal as it attempts to cope with challenges, including neurological, physiological and behavioural components. Unfortunately, these measures, especially when used in isolation, lack specificity, are unable to determine valences of affect, or are not suitable for a production environment. As a result, the development of novel ways in which we can assess the emotions of animals accurately has gained momentum in the realm of animal welfare research. However, in order to develop new ways in which we can assess the emotions of animals, an understanding of the neurobiological processes involved in emotions, and how these processes impact other biological systems (i.e., behaviour and physiology) is paramount. Furthermore, novel measures of emotion must be robust, have the ability to differentiate between valences of affect, as well as be validated against existing behavioural and physiological parameters.

This thesis describes an investigation into miRNAs' efficacy as biomarkers of emotional states in pigs. Differentially expressed miRNAs were observed in the brain in response to pain, suggesting a potential for their application as markers of negative emotional states. However, this difference was not reflected in blood and thus the functionality of circulating miRNA as a biomarker for pain processing is arguable in this case. Nonetheless, additional sampling of circulating miRNA will allow any acute response in miRNA expression to be captured. Further investigation is also required to determine what role these miRNAs play in the pain response, in particular, their targets and functions. This may allow for

differentiation between the neural process pertaining to negative emotional processing or perhaps other responses to tissue damage including inflammation. Furthermore, the efficacy of miRNAs as biomarkers of negative emotional states still requires validation via corroboration with other physiological or behavioural parameters. It is also important to mention the potential genetic differences in miRNA genes between individuals. Variability in miRNA genes that result in polymorphisms in miRNA genes, and thus their function, may have accounted for some of the variability observed in these studies.

In this body of work, the investigation into miRNAs' efficacy as biomarkers for positive emotional states revealed inconclusive results. No difference in miRNA expression was observed in the brain or blood in animals exposed to environments presumed to elicit positive affective states. As mentioned, a number of factors relating to the study design may have compromised the study's outcomes and, given the absence of an adjunct measure indicative of the valence of response (i.e., behavioural or physiological indices), I am unable to confirm the validity of miRNAs as biomarkers of positive emotional states. The outcomes of this study are therefore not agreeable with the null hypothesis, at least under the type of conditions employed in this study. However, given their promise in the literature on humans, as well as the necessity to develop measures of emotions in animals, I recommend further investigation into their utility in this endeavour is warranted.

Supporting Documentation

Contextual Statement

The assessment of animal welfare has evolved to include measures of positive welfare states, which is now considered an important aspect of animal welfare thinking. To date, the majority of animal welfare assessment tools tend to rely on physiological and behavioural measures indicative of negative welfare, including measures of negative affective (emotional) state. In comparison to these measures, assessment tools that can accurately identify positive emotions in animals are underdeveloped, particularly where physiological indices taken, are unable to differentiate between positive or negative effect. As a consequence, the most practical and meaningful measures used to assess positive emotions in animals regularly rely on behavioural assessment.

The supporting documentation provided below reviews the current literature pertaining to behaviours used to assess positive emotions in animals. The documentation discusses the various behavioural indices previously used in the assessment of positive emotions and highlights potential limitations with their use, including issues with interpretation of behavioural data, observer bias and behaviour subjectivity.

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Name of Principal Author (Candidate)	Alexandra Whittaker		
Contribution to the Paper	Conceptualisation, reviewed literature, performed analysis of literature, wrote manuscript.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	25/10/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Laura Marsh		
Contribution to the Paper	Conceptualisation, supervised student, assisted in data collection, assisted in statistical analysis of data, interpreted data, edited manuscript drafts		
Signature		Date	25/10/21

Body of Work

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The role of behavioural assessment in determining 'positive' affective states in animals

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Abstract

The assessment and promotion of positive affective states as opposed to consideration of negative states alone has now become an accepted component of animal welfare thinking. However, methods for assessment of positive affective state are comparatively underdeveloped compared with their negative counterparts. Due to the current lack of established physiological methods for assessing positive effect in animals, behavioural measures are likely to be the closest proxies. In this paper, we broadly review spontaneous behavioural measures that have been utilized in mammals to determine positive affect. This includes luxury behaviours, facial expression, vocalizations and laterality. Play and anticipatory behaviours expressed in animals have received greatest research attention. However, these methods require care in interpretation due to the influence of age and other factors, such as social status on their performance. These methods may also be subjective if descriptions of individual behaviours are not well defined and validated. Methods that are amenable to automation may reduce subjectivity. Automation will also aid in more widespread implementation as an on-enterprise welfare assessment tool. There is a pressing need to develop simple and objective indices of positive affect. The use of vocalizations and facial expressions may fulfil these criteria. Future research attention should be directed towards further characterising these methods.

Keywords: Positive welfare, Behaviour, Emotional valence, Facial expression

Review Methodology: We searched the following databases: CAB Abstracts, Web of Science, PubMed and Google Scholar. The Keyword search terms used were 'positive welfare' AND 'animals', 'affective states' AND 'animals', 'welfare assessment', 'facial expressions AND 'animals', 'lateralization' AND 'animals'. In addition, we used the references from the articles obtained by this method to check for additional relevant material.

Introduction

Over the last few decades, there has been a renewed interest in the School of Animal Welfare Science which prioritizes the assessment of how animals' 'feel', over the other schools encompassing biological functioning and 'telos' [1]. This is because it is now universally recognized that animals are sentient, meaning that they have sufficient functional brain sophistication to transduce sensory and nerve impulses into experienced sensations [2]. In concert with this change in emphasis, there has been an increased importance attributed to the evaluation of positive affective states [3], which has resulted in animal welfare scientists moving away from their traditional focus on the minimization of negative experiences. These concepts have now advanced to the point where Mellor regards the consideration of positive affective states as an entrenched element of animal welfare thinking [3].

Animal welfare is an encompassing term which refers to a long-lasting state comprising the summed experiences of an individual [4]. Welfare is generally defined in terms of affective states and their relative weighting over time [5]. Affective states are feelings, emotions or moods which include amongst others, joy, happiness and fear [1]. There is, however, still great debate as to what animal emotional experiences consist of [1], and whether feelings of consciousness are present in non-human animals, for example, do they experience the 'feeling' of being afraid

in the same way as humans do [6]. Affective states are regarded as comprising subjective, behavioural, physiological and cognitive components [7, 8] that may be characterized according to their two main dimensions (1): arousal (bodily activation or excitation; e.g. calm versus excited) and valence (negative or positive; e.g. sad versus happy). Affective states can also vary in their time course ranging from short-term states such as acute pain, to more persistent states like depression. It has been proposed that these longer-term states arising from short-term emotional responses make up 'mood', and may have an adaptive function in guiding decision making [9]. When discussing animal welfare, scientists ascribe good welfare to animals that, on balance, generally experience positive affective states such as happiness, and poor welfare to animals mainly experiencing states such as fear. Neutral welfare applies when positive and negative states are experienced equally [10].

In recognition of the shift in animal welfare science thinking, Mellor and Beausoleil proposed the Five Domains model [4]. This model considers four physical or functional domains which includes nutrition, environment, health and behaviour, and a fifth domain of mental state. Mental state represents the affective domain which results from the factors inherent in domains 1–4. It is these affective experiences that make up the animal's welfare state [11]. Mellor and Beausoleil's adapted model (2015) considers some physical states in domains 1–4 with concomitant positive affects, which can be assumed to be welfare enhancing [4]. The Five Domains model therefore represents a framework to grade the impact of procedures or environments on affective experiences to come to a determination of whether an animal is in a welfare-compromising or welfare-enhancing situation [4]. However, use of this assessment tool still requires evidence of positive experiences, and methods of measuring these need to be defined and validated for the species of interest. This brings us to a fundamental concern with the now prevalent viewpoint, that positive, as well as negative mental state should be considered in animal welfare assessment. That is, whilst there is a range of well-validated methods to assess pain and suffering in animals [1], methods to assess positive welfare states are comparatively underdeveloped.

The brain is organized into discrete regions and neuronal systems that regulate positive emotions, such as post-consummatory satisfaction reward and contentment, and negative emotions, including pain, anxiety and fear. Plentiful evidence suggests that lower rather than higher brain regions are responsible for generating core affective states [12] These lower regions include brainstem and diencephalon, with higher regions represented by the cerebral cortex [13]. The most controversial question regarding animal emotion is how emotions and cognition are linked via higher brain centres. That is, can non-human animals consciously evaluate and reflect on their emotions, or 'feel' them, enabling variability and flexibility in their behavioural response to the emotion? Due to the involvement of higher brain centres, these emotions are referred to as secondary emotions [14].

In terms of poor welfare, a failure to cope with the environment and difficulty in coping have traditionally been regarded as indicators [15]. A comprehensive assessment of the welfare of an animal can then be made through examining physiological, neurological, immunological, endocrinological and behavioural responses that comprise various coping mechanisms in animals. For instance, an antibody or cell-mediated immunity response reflects impaired immune system function which may arise from husbandry factors, or adrenal cortical responses can be used to indicate acute or chronic responses to 'stress' which often indicate a failure to cope [15]. Positive emotional states will similarly cause a range of bodily responses that can theoretically be measured. Endogenous opioids may be released as a result of reward, dopaminergic systems can be activated, and neuroendocrine and immune activation may occur [1]. However, in spite of good knowledge on the neurobiology of positive experiences, experimental evidence on the physiological changes occurring remains scarce [1]. Furthermore, proposed measures are perhaps relatively more difficult to investigate than those measures of negative affect. Challenges in validation of measures may occur for several reasons. Firstly, although knowledge of the neuronal systems that regulate emotions exists, it is difficult to assess these mechanisms without extraction of brain tissue and complex laboratory analyses [16]. Additionally, the use of various imaging technologies (e.g. functional magnetic resonance imaging and electroencephalogram), can provide information on specific brain activity within the regions of the brain associated with emotions; however, these are not suitable for a production environment [17, 18]. Secondly, activation of responses may result from the experience of all emotional triggers, requiring discrimination of valence of affect, although this of course applies equally to their use in assessment of negative states [1]. In spite of these issues, measures such as heart rate variability and immunoglobulin-A levels have been proposed as worthy of further study [1].

In light of the relative lack of physiological proxies of positive states, this review will broadly examine behavioural methods of positive affective state assessment, currently reported in a range of mammalian species. This choice of subject matter is not in any way an attempt to disregard the importance of physiological biomarkers of positive state, and the need for further research in this area. We do not intend in this paper to present a comprehensive review of all the literature in this area but to consolidate techniques investigated to date, and suggest future areas of study which may be applicable across a range of species. Focus will be on measures developed since publish of several substantial reviews on this area [1, 4, 9]. We review spontaneous behaviours that have been proposed as proxy measures of positive affective state. These include luxury behaviours, facial expression, acoustic signals and laterality.

We additionally discuss kinematics as an applied and potentially objective measurement technique for assessing animal postural change.

Behavioural Methodologies for Assessment of Positive Affective State

Whilst use of animal-based indicators for welfare assessment, as opposed to characterization of resources or inputs, can be conceptually and methodologically challenging, and requires considerable time investment [19], it is essential to validate the impact of resources supplied by animal carers on affective state, and consequently welfare state. At the current time, with limited accepted physiological markers of positive state [20], behavioural measures are likely to be the closest proxies of positive affective states in animals [19].

It is well established that there are a range of behaviours that are more likely to be performed when an animal is in a state of positive affect [20]. However, corroboration of claims for new measures of positive affective state is problematic when compared with the well-recognized behavioural indicators of negative states, such as pain and distress. This largely arises because of the lack of physiological markers with which to correlate the proposed behaviours [20]. Furthermore, design of experiments to create oppositely valenced responses, with identical levels of emotional arousal, is challenging [9, 21]. Nevertheless, alternative methods for confirming the validity of these behaviours for assessment are available. For example, the behaviours may be connected to satisfaction of a motivation, for example, sexual gratification, correspond with other behavioural markers, or be similarly observed when mood-altering drugs, such as anti-depressants, are administered [20]. This substantiation is an important consideration for scientists involved in researching positive affect in animals.

Spontaneous behaviours

In the context of this review, 'spontaneous' refers to behaviour occurring naturally in the animal's living situation being unprovoked by humans. The caveat to this being that human caretakers can still influence these behaviours by failing to provide resources that allow these behaviours to occur, for example, provision of nest-building material.

Luxury behaviours

Dawkins' consumer demand paradigm allows the animal to rank activities into those which regulate deprivation ('necessities' such as feeding) and those which have little regulatory basis ('luxuries') [22]. Using the economic theory, whilst there may only be a small 'cost' attached to the non-performance of luxury activities, this implies that welfare must be high when animals can engage in these activities at high frequency [23]. These behaviours are therefore almost exclusively taken as measures of positive affective state. These behaviours include play, affiliative behaviours and unique species-specific behaviours.

Play. Play behaviour has been widely proposed as an indicator of positive emotion [1]. 'Play' encompasses functional behavioural elements such as fighting, fleeing and predatory behaviours, in addition to behaviours unique to play. However, when at play the functional elements are amplified and repeated, and of course do not result in the final intended act [1]. Play is a characteristic of juvenile stages of development and is missing or rare in most adult wild animals [24]. Interestingly, it has been reported that higher rates of adult play are observed in domestic animal species, perhaps as a consequence of neotenic retardation [24]. Play behaviour has consistently been shown to decrease in situations associated with poor welfare (see e.g. [25–27]). Play behaviour therefore may provide an indicator of welfare-compromising situations, in addition to those situations where welfare might be enhanced [28]. In addition, play has been proposed to have short-term and long-term effects, thus triggering welfare improvements, as well as reflecting them [28]. However, the situation in adult animals where play is rare indicates that it may have a different functional significance in comparison to juveniles, requiring differences in interpretation. For example, instead of play diminishing in the face of stress, there is a suggestion that play may actually regulate stress through an endogenous opioid mechanism akin to that triggered by stereotypic behaviours [29]. Other theories for its performance, which suggest its linkage with welfare-compromising situations, include that it may relieve the sensory deprivation resulting from poor environments [29], or that it occurs as a rebound effect following restriction in order to remove toxic substance build-up through stimulation of muscular reactions [30].

Both social and locomotor play have been investigated in a wide range of species including cattle [31, 32], pigs [33, 34], sheep [35], rodents [36, 37], cats [38] and dogs [39]. A comprehensive review of the many studies investigating play as an indicator of positive emotion is beyond the scope of this article and have been extensively reviewed elsewhere [28].

Play behaviour is a useful candidate for positive welfare assessment since it is easy to recognize, performed across the mammalian taxa, and can be measured non-invasively [40]. However, care is required in using play behaviour as a positive welfare assessment method. As discussed above, adult play may actually indicate welfare-compromising situations. Furthermore, play may actually result in fighting (an assumed negative situation), especially in juvenile animals [34]. Furthermore, there is much inter- and intra-species variability in propensity to play and the nature of it [28]. Some elements of play behaviour may be better indicators than others [25], and identifying play patterns in general, as well as these specific elements in the

species, and age of interest, will be important to progress this research area.

Affiliative behaviour. Affiliative behaviour typically involves maintaining proximity to, or providing food, protection or grooming to conspecifics [1], or engaging in maternal care and sexual activity [41]. Affiliative behaviour may play a critical role in attaining positive affective state in animals by reducing agonistic interactions [42–44], reinforcing social bonds [45] and having an arousal-reduction effect with long-term benefits [1]. From a physiological standpoint, benefits may result from the actions of neuroactive agents such as endogenous opioids, oxytocin, vasopressin and noradrenaline [46].

Allo-grooming has been particularly studied for socio-positive effects. The behaviour is particularly prevalent in non-human primates [45], and has been documented in cattle, pigs, horses and infrequently in sheep [1]. In addition to long-term arousal-reduction effects, short-term rewarding effects include a reduction in heart rate [47–49] and increased levels of circulating β-endorphin [50].

Allo-grooming appears to be a promising indicator of positive emotion in those species which frequently engage in it. However, considerations in interpretation are needed. Firstly, threat might increase the proximity between animals, and therefore care needs to be taken in measuring inter-animal distance [1]. Secondly, whilst benefits for the receiver seem clear, the benefit for the performer may be less clear [51]. Furthermore, social status of the receiver may modify the benefits received, with subordinate animals actually experiencing stress if licking is from a dominant counterpart [1, 52]. There may also be variations in the behaviour occurrence dependent on both animal and external factors, for example, season [53], reproductive state [54] and social status [52, 55] may modify expression. A further consideration is that allo-grooming can manifest as a redirected behaviour when another behavioural need is not met [56]. Alternatively, allo-grooming may develop into redirected behaviours, for example, feather pecking at the end of preening, which causes impaired welfare in the receivers [57]. Taking into account these limitations, affiliative behaviours and especially allo-grooming are promising candidates for behavioural measure of positive emotion.

Species-specific luxury behaviours. A range of species-specific luxury behaviours have been proposed as an indicator that important needs of the animal are being met, and that can provide an indication of positive affect. A number have been documented and only a few are included here. Self-grooming occurs across several taxa, whilst others are limited to certain species [1]. A range of these behaviours have been described for pigs, cattle, birds and rabbits amongst others, and include dust bathing, beak wiping, preening, wing and leg stretching, wing raising, wing flapping, body shaking, rubbing, ground scratching, water bathing, hoof stamping, wriggling and tail wagging [58, 59] In rodents, nest building and burrowing have been proposed to represent such behaviours in mice, and rats to a lesser extent [60]. Activities may involve interactions with the environment, for instance dust bathing and beak wiping, or comprise body movements or self-directed behaviours.

Use of these behaviours for welfare assessment is complicated. Performance of the behaviours may be modified by environmental resource availability, a condition dictated by human caretakers. Additionally, function of behaviour may differ depending on context, for example, preening may occur during an aggressive social encounter in hens. Care also has to be taken in regarding an increase in behaviours as indicating an improvement in welfare, for example, hens with parasites will engage in more scratching and preening, but their welfare is not improved [59]. Researchers also need to characterize the behaviour in the species, breed/strain of interest and with reference to life stage. For example, there are strain differences in performance of nest building in mice, and the motivation for maternal nest building is different [60].

Luxury behaviours have been extensively investigated as measures of positive affective state. However, there are some key issues in employing them as practical tools which have been highlighted above. The main considerations relate to knowledge awareness about the impact of the human-provided environment on the physical ability to perform the behaviour, inter-individual variability in expression and the relative infrequency of behaviour occurrence even when welfare is optimal [59]. In light of this, newer tools such as facial expression scoring or vocalizations may be more scientifically sound measures.

Facial expression and postural analysis

The communicative function of facial expressions is still open to debate, especially in animals. Darwin proposed that they were universal, and that emotions and their expressions were biologically innate, and evolutionarily adaptive. He also proposed that there were phylogenetic similarities among species [61]. However, in species that primarily use non-visual methods of communication, such as olfaction or vocalizations, the adaptive value of facial expression could be called into question [62]. Yet there is evidence to suggest that mice may modulate pain behaviour depending on pain status of conspecifics [63], and that primates and dogs selectively pay attention to certain facial expressions [63–65]. Facial expression may therefore indeed represent an emotional display [66]. An alternative explanation is that the animal may gain a benefit from performing the expression, for example, fear expressions with the characteristic widening of eyes may increase sensory exposure [67]. The expression of positive emotions through facial expression may provide similar adaptive value as in negatively valenced states, for example, through sensing whether a conspecific will engage in play behaviour [62], or through the relaxation of facial muscles inducing a hedonic state in a similar vein to the human smile [68].

Animal facial expressions have mainly been investigated using Facial Action Coding Systems (FACS) and Grimace Scales. FACS was originally developed in humans [69], and uses action units caused by contraction or relaxation of muscles to determine facial expression. The approach has been mainly used for recognising pain and depressive states in humans [70], but has recently been applied to a number of non-human species including primates [71], dogs [72] and horses [73]. Facial expression changes occurring as a result of pain have been categorized using grimace scales across a range of species including mice [74], rats [75], rabbits [76], horses [77], sheep [78], cattle [79], pigs [80] and cats [81]. However, only in recent times have researchers begun to use facial expression changes as an indicator of positive emotional states.

Earlier studies focused on specific areas of the face to determine distinguishing features of positive emotional expression. The percentage of visible eye whites has been examined in cattle in a number of studies [82–84]. The presumptive basis for this technique being that visible eye white increases when the upper eye lid is lifted, and that this occurs as a result of sympathetic stimulation of the muscle lifting the lid [85]. It is therefore postulated that a high percentage of eye white indicates frustration or fear, and a low percentage, satisfaction or pleasure. This was in fact determined to be the case in the assumed positive, low arousal experience caused by stroking of cattle [84]. However, findings, at least in cattle, are not this clear-cut. Sandem *et al.* demonstrated that eye white percentage actually decreased below the baseline upon cessation of the negative stimulus and on initiation of a positive stimulus [82]. Similarly, eye whites increased during a positive experience elicited by feeding preferred fodder [83]. These seemingly contrasting findings may be attributed to the specificity of the eye white measurement as a response to either emotional valence or arousal [83]. A relatively low drop in emotional arousal was likely to have occurred in the stroking study, in comparison with the high arousal likely from a favoured feed [83]. To add to the controversy surrounding this measurement technique, eye white was not found to change with emotional state in sheep [86].

Eye wrinkling, which is arguably a modified variant to the measurement of eye whites, has been proposed in horses as an indicator of emotion [87]. Eye wrinkles are caused by contraction of the inner eyebrow raiser, and their increase has been anecdotally regarded as an indicator of 'worry' by horse owners [87]. Eye wrinkle expression was assessed using a set of six measures which contribute to the 'wrinkle' look. Whilst there was suggestion of a narrowing of the angle between the highest wrinkle and the eyeball when in a positive state, other outcomes were largely unchanged [87]. It therefore remains inconclusive at this time as to the validity of this approach for affective state assessment.

Ear position is consistently a feature of the 'grimace scales' but its utility as a measure of positive state is largely undefined. This technique may have value over general facial expression changes in animals, such as sheep, where there is a limited network of superficial facial muscles, thus reducing diversity of facial expression [88]. In sheep, frequent ear-posture changes were demonstrated in negative states, with a high proportion of passive ear postures occurring in conditions inducive of positive states [89]. Similar findings were observed in dairy cattle with passive postures indicating positive states [90, 91]. However, in horses, ears were shown to be comparatively more forward leaning in a positive state, than negative [92]. In another sheep study, specific differentiation of ear posture allowed the authors to conclude that horizontal ear posture corresponded to a neutral state, backwards posture implied unpleasant, uncontrollable situations, and ears pointed up were observed in unpleasant but able to be controlled situations [88]. Backwards oriented ears have also been associated with negative situations in dogs and pigs [93, 94]. However, ears in a forward position have been described in mice [95] and dogs [96], proposed as part of a vigilance posture adopted in response to a stressor [95]. Frequency of ear posture changes may also be of value in differentiating valence of responses, although this area has received less study. In a cattle study, in contradiction to earlier findings in sheep, the number of ear posture changes increased during the assumed positive, low arousal state of stroking [91]. Drawing on the limited studies performed to date, it can be concluded that whilst some general patterns appear to be emerging regarding ear posture and valence of emotional state, these patterns are far from clear or uniform, and there may be considerable species-specific variation in performance. Ear position therefore needs extensive further investigation before it can be recommended as a tool for affective state assessment.

In a recent study in rats, the authors observed a number of features that appeared to co-occur with the induction of a positive affective state [62]. The positive state being induced through tickling, and established by the emission of positive ultrasonic vocalizations. Widening of ear angle (relaxed ears), and ear colour becoming pinker occurred in a positive state. However, other quantitative and qualitative measures, including eyeball height to width ratio, eyebrow height to width ratio, eyebrow angle, visibility of the nictitating membrane, and the Rat Grimace Scale failed to distinguish between groups. A later study similarly assessed multiple factors for their reliability in detecting positive emotion in horses [97]. Measures included ear position, lip tension, neck height and eye opening. Horses assumed to be in a positive state demonstrated raised necks, eyes half closed, with their ears pointing backwards. Surprisingly, in the longitudinal study component, similar facial expressions performed a year later suggest that horses had a memory of the positive experience and that this was expressed through facial expression. Facial expression may therefore be a sensitive and long-lived measure of emotional valence.

In conclusion, based on the limited literature available, facial expression does appear to be altered in response to

positive affective states with aspects of eye and ear position being the most reliable measures to consider. However, there is much needed further research to validate these measures and investigate species differences. Furthermore, attention needs to be given to whether these responses are sensitive to valence or arousal of state or a combination thereof.

Kinematics

The astute reader will have gathered from the above discussion that behavioural assessment of animal affective state not only relies on observing which behaviours an animal performs, but how it performs them, or the quality of the movement [98]. This 'movement quality' has been assessed using a technique termed 'whole body' or qualitative behavioural assessment [99, 100]. In fact experienced animal carers are frequently undertaking this to draw conclusions about their animals' health and wellbeing [101], yet may not be able to pinpoint the features that led them to their decision. Kinematics is a field of mechanics that can be used to quantify this assessment. The technique considers the movement of points on the body based on their co-ordinates and tracks their change in space and time [102]. Measurement methods range from the simplistic, for example, manually superimposing points of interest onto recorded video footage, to use of optical capture systems or force-plate technology [98].

Kinematic methods have been widely used to characterize gait and assess pathology [103, 104]. These outcomes are often being linked with management conditions or use, especially in working animals such as horses. For example, LeSimple *et al.* found using kinematic methods, that leisure horses had 'sounder' spines than riding school horses [105]. Kinematics has recently been proposed as a possible method of assessing animal affective state based on the characteristic behavioural patterns seen in animals believed to be experiencing particular emotions [98]. To date few studies have actually done this so most of the literature is speculative. As an example, anticipation behaviour has been considered an indicator of positive affect, potentially demonstrating 'joy' [106–108], although noting that anticipation has also been discussed as driving stereotypic behaviour [109]. In dogs experiencing expectation of an assumed reward, it has been observed that the head is held high with the mouth open, ears upright and tail wagging [98]. These behavioural elements are all amenable to kinematic analyses.

The technique could also be used in concert with other analysis methods to determine body patterns when an animal is engaging in known positive experiences, for instance when a rat is being tickled and emits positive USVs [110], or when cows are being brushed [111]. Furthermore, the technique could build on facial expression measures such as those mentioned above to provide further objectivity in assessment, and pick up subtle changes through increased levels of automation. In the study by Finlayson *et al.* described above, kinematic methods were used to examine rat facial characteristics, including ear angle [62].

In conclusion, whilst this technique is in its relevant infancy and requires knowledge of behaviour elsewhere described for its use, it potentially offers a viable method for objective analysis of affective states in animals. Current barriers to its implementation are the lack of studies investigating kinematic methods and emotional correlates, and technology barriers particularly in small animals. With reference to the latter, in the Finlayson *et al.*'s study on facial expression, animal movement during tickling precluded the capture of high-resolution video images during the treatment. Higher frame capture rates or camera stabilization systems may resolve this issue. However, given the increased focus on automation and sensing systems in agriculture for assessment of many production and health aspects of husbandry, it is not so far-fetched to imagine that these technologies could not be adapted for affective state measurement [98].

Acoustic signals

Acoustic signals could provide an indication as to emotional state of the caller since emotional state alters muscle tone and action in the vocal apparatus. These changes consequently modify various parameters of the vocalizations emitted [112]. Research on vocal communication in animals has principally focused on easily discernible parameters such as calling frequency, duration, the occurrence of vocalization types and energy distribution (see [112]). Whilst these methods have been proven to be useful indicators of emotional arousal and valence, some of the newer research methods developed from human linguistics studies could allow for a greater depth of understanding of the emotion behind such calls [112]. These methods consider vibrations of the vocal folds and their subsequent filtering in the vocal tract to determine fundamental frequency of the call (the source-filter theory) [113]. Through increased collaboration between ethologists and human linguistics researchers, the benefits of these advanced methods might be realized in animal studies.

Akin to facial expressions, vocalizations have been used widely in mammalian species to indicate responses to pain or stress (see [114] for further discussion). Most of these studies have considered arousal rather than valence of emotions [112]. An increase in emotional arousal in animals tends to cause vocalizations which are longer, louder and harsher, of higher, more variable frequencies and produced at increased rates (see [112] for review). There is also suggestion in horses that specific acoustic parameters may play contrasting roles in determining whether emotional valence or arousal is being communicated; whinny duration being suggestive of valence [115].

Fewer studies have successfully linked acoustic indicators with positive emotions. Collecting data on vocalizations

in positive states is challenging for two reasons. Firstly, vocalizations during positive states actually occur infrequently and were probably a later evolutionary adaptation to aid social communication [112]. Secondly, in order to study valence, researchers need to compare positive and negative scenarios where the level of arousal is equivalent. Negative emotional responses are often more highly valenced than their positive correlates [1]. As a corollary, it is perhaps not unsurprising that some of the published studies report events which are arguably not positive, but reflect anticipation of favoured events, implying an ambiguous affective state [115].

There is some evidence of a shift towards higher frequencies in a positive state. For example, dogs at play produced barks of shorter duration with a wider range in volume, decreased interval between barks and a reduction in frequency changes [116]. Similarly in cats, vocalizations in assumed positive affiliative situations showed wider energy distributions and increased peak frequencies [117]. Positively-conditioned pigs produce fewer vocalizations, fewer low-frequency grunts and more high-frequency grunts [118].

Alternately, shifts towards low-frequency vocalizations have been documented in some species. Rhesus monkeys and grey mouse lemurs produced different types of call during positive situations than negative, incorporating low-frequency coos and purrs into their vocal repertoire [119, 120]. African elephants and silver foxes showed decreased peak frequencies in an assumed positive situation [121, 122], although the studies may have actually been measuring arousal more so than valence.

In rodents, there is a substantial body of literature examining ultrasonic vocalizations (see [123] for review). In rats, it is well established that vocalizations of 50 kHz are emitted in positive situations, such as during play, or in response to tickling [110], and they have been considered a primal form of laughter [124]. Linkage between ultrasonic vocalizations and products of the cholinergic and dopaminergic systems provides compelling evidence for their association with negative and positive affective states [125]. Positive vocalizations are characterized by shorter durations, higher-peak frequencies and wider frequency ranges than positive ones [125]. In contrast to rats, mouse ultrasonic vocalizations have not been shown to indicate negative or positive affect [123].

There has been relatively little research attention directed towards non-vocal communication forms, such as the cat purr or equid snorts in the context of emotional correlates. Snorting signals have in fact been shown to reflect positive states in rhino [126], tapir [127] and horses [128], and negative emotional states in tapirs [129]. The characteristics of this modification being an increase in frequency with positive states [128].

In summary, whilst it appears that vocalizations in positive situations are shorter in duration, few other conclusions can be drawn due to the contradictory evidence and relative dearth of studies investigating valence of response. There may also be considerable variation in arousal according to the positive situation studied, rendering interpretation problematic. This area of positive state assessment is however objective, fairly simple to measure and is an area worthy of further research.

Laterality

It is well established in a range of species that the left and right sides of the brain process information in different ways and control different categories of behaviour [130, 131]. The left hemisphere controls behaviours occurring in non-stressful situations, whereas the right hemisphere processes unexpected stimuli and invokes fleeing and other emergency responses [132]. Davidson's laterality-valence hypothesis proposes a sharp distinction between the two hemispheres such that negative emotions are controlled by the right hemisphere and positive emotions by the left hemisphere [133]. In counterargument to this hypothesis, there is evidence of humans processing both negative and positive emotions in the right hemisphere [132].

There have been a large number of studies investigating laterality and emotion conducted over the last 30 years. These studies have tended to use lateralized behavioural expression of a motor or sensory response as the outcome of interest, considering that responses are contralateral to the dominating hemisphere [134]. The majority of research into lateralization in vertebrates focuses on negative emotions such as fear and anxiety (not discussed here). However, there have been studies examining positive experiences, including food presentation and positive social interactions.

The mammalian literature suggests a right-hemisphere dominance for cerebral processing in response to food rewards. This was evidenced in three primate studies where there was a left-hemimouth dominance in production of food calls [135] and a left-eye dominance when watching food [136, 137]. Sheep responded to a better-than-expected food reward and food disappointment with the left ear more forward pointing than the right, implying right-hemisphere dominance but showed left laterality for an expected food reward [89]. Dogs initially used their right nostril for smelling novel stimuli with the left nostril/left hemisphere taking over once the stimuli became known and routine (olfactory information is processed ipsilaterally) [138]. In the alternative, marmosets displayed right-eye preferences for observing familiar food [139]. Horses showed a left-hemispheric preference for familiar neighbour calls, with no preference for group member and stranger calls [140]. Whilst these results appear unsupportive of the laterality-valence hypothesis, they can be reconciled by considering the nature of the experimental design in the studies undertaken. In two of

the primate studies, the experiment involved observing food rather than eating it and this could have evoked the negative emotional response of frustration [136, 137]. Furthermore, in the sheep study, the better-than-expected food may have been fear-inducing as a result of its novelty [89]. In contradiction, horses have been shown to preferentially view unfamiliar objects with the right eye [141]. It may however be problematic to compare asymmetries in olfactory processing versus visual processing due to the differing value assigned to these forms of sensory processing, dependant on species. These findings highlight the importance when designing studies of considering the impact of familiarity on animal behavioural responses, since positive, unfamiliar situations may be perceived as less positive than familiar positive situations.

Lateralization in positive social situations has been studied rarely. In general, the evidence for side dominance is somewhat contradictory. A right-hemimouth dominance was seen to produce social contact calls in marmosets [139], but in chimpanzees and rhesus macaques, left-side dominance was observed [142, 143]. Dogs showed a more rightward tail wagging in response to seeing their owner, implying left-hemisphere dominance [144], but they also showed a rightward bias when presented with an unknown human and cats. Dog play calls were responded to with a right ear response [145]. In Californian sea lions, no significant effect was observed for motor laterality when meeting familiar trainers, compared with exposure to unfamiliar trainers or objects [146]. However, this study may have been underpowered and it is unclear whether positive emotions were actually induced. In sheep, it was discovered that when animals needed to pass a central obstacle to return to familiar animals, they passed on the right side. This was proposed to indicate left bias, and right-hemisphere dominance since there was a preferential turning of the head to the right to fixate the flockmates in the left hemifield [147].

Whilst laterality as a measure of emotional state could provide a simple and objective measure of emotional valence, there may be a causality dilemma in interpretation. The above-mentioned studies, and others, have investigated the effects of emotional valence on laterality response. However, temperament [148, 149], sex [150, 151], genetics, epigenetics and the environment [152, 153] may also influence laterality. There is also widespread debate about the existence of motor asymmetries at a population level [154]. Therefore, there is a risk of ambiguity in interpretation of laterality results, in that they may not truly represent an animal's current affective state, or provide evidence of a state that is able to be modified. As a consequence, there may be more value in using laterality as a selection tool rather than welfare assessment tool, since preferential limb use could assess vulnerability to stress in animals [132], and their adaptability to certain circumstances, for example, predicting success in training programmes [155].

Conclusion

Within welfare science, and indeed in the animal industries, there has been a shift in focus from evaluation of animals' negative experiences towards promoting animals' positive experiences. In this review, we have summarized behavioural-based methods for affective state assessment that have been examined. It is clear that the majority of research to date has focused on evaluation of spontaneous behaviours, such as the luxury behaviours. These methods, whilst better characterized, may be subjective in nature, and animals' propensity to perform them may vary based on characteristics such as age and health status. Whilst not described in this review, there are a number of tests, utilising provoked or learned behaviours that have been proposed to indicate affective state, for instance the judgement bias test (see e.g. [156, 157]). These tests may find utility in academic research, in determination of conditions likely to lead to contrasting affective states. However, at the current time, they are not feasible for wide-scale use in a production or laboratory setting. There are however moves to automate these methods [158], and the reader is encouraged to keep abreast of advances in these techniques.

The development of simple and objective indices of positive affect is paramount. We have highlighted some methods which hold promise in this regard, for example, vocalizations and facial expressions. Research attention should be directed towards further characterising these methods, ensuring that they are true measures of emotional valence, and identifying confounding influences. Additionally, effort should be directed towards the automation of such monitoring methods so that they can have practical effect. We hope that this review stimulates such research across a range of species and scenarios.

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