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Validating attention bias as a novel measure of affect in sheep

A thesis submitted by

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Certification of Dissertation

I hereby certify that the content of this thesis has not been and is not being submitted for any other degree to this or any other university. I also certify that the work contained in this dissertation is my own and that that all help received in preparing this thesis and all sources used have been duly acknowledged.

30/05/2019

Signature of Candidate Date

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Preface

This thesis is being submitted as a thesis by publication. Therefore, some of the chapters contained in this thesis are written in journal article format. Each will contain an introduction, methods and materials, results and discussion. The format of each article will be presented according to the journal that they were submitted to or are intended to be submitted.

Chapter 3:

Monk, J., Doyle, R., Colditz, I., Belson, S., Cronin, G., & Lee, C. (2018). Towards a more practical attention bias test to assess affective state in sheep. *PLOS ONE*, *13*(1), e0190404. doi: 10.1371/journal.pone.0190404

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No Proof of publication could be located for the following chapters:

Chapter 6:

Repeatability of an attention bias test for sheep

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Summary of thesis

To facilitate the understanding and enhancement of animal well-being in livestock production systems, there is a need to develop robust methods which can assess the emotional or affective states of animals, as an aspect of their welfare.

Chapter 1 of this thesis discusses some current methods used for the assessment of animal affect, highlighting the key limitations of each approach. The potential role of cognitive methods for welfare assessment is introduced, with a particular focus on attention bias tests. The aim of this thesis was to further develop and validate a novel attention bias test for the assessment of affective states in sheep.

Chapter 2 examines the impact of pharmacologically-induced stress on judgement bias and attention bias in sheep. The study finds no clear indication that elevated cortisol concentrations impacted on cognitive biases in sheep.

Chapter 3 refines the attention bias test methodology to remove a habituation period, improving the practical application of the test. The study demonstrates that pharmacologically-induced anxiety-like and calm-like states impact on attention bias in sheep.

Chapter 4 introduces a modified method for the assessment of attention bias, which is shown to assess and differentiate pharmacologically-induced anxiety-like and depression-like states in sheep.

Chapter 5 examines the potential influence of pharmacologically induced positive affective states on the modified attention bias test, finding no clear effect of positive states on attention bias in sheep. It is suggested that external factors may have confounded results of this study.

Chapter 6 examines repeatability of the attention bias test to gain insight into the factors influencing animal behaviour during the attention bias test. Key measures of attention had low repeatability and are suggested to be readily influenced by emotions and moods. Measures of vigilance and zones crossed had moderate repeatability and are suggested to be more heavily influenced by temperament or personality traits.

Finally, chapter 7 concludes that the attention bias test developed throughout this thesis may be a useful and practical tool for the assessment of negative affective states in sheep.

Chapter 1

An introduction to assessing affect in sheep: current methods and pathways moving forward

1.1. Introduction

> "the lower animals, like man, manifestly feel pleasure and pain, happiness and misery" (Darwin, 1872)

The notion that non-human animals experience emotions was long ago described by Charles Darwin in *The Expression of the Emotions in Man and Animals* (Darwin, 1872). However, this early work was heavily criticized, labelled as anthropomorphic and remained largely unrecognised for the better part of a century (Grassé, 1977; Jacobs, 1998). After this, animal emotions were long considered as something which were non-existent, irrelevant or which simply could not be studied scientifically (Veissier et al., 2009). Acceptance of the view that animals experience emotions has since been slow to develop. Nevertheless, there is now (re)emerging interest in the study of animal emotion as an aspect of animal welfare, and the notion that animals have some form of emotional experience has become widely accepted (Hemsworth et al., 2015; de Vere & Kuczaj, 2016).

Animal welfare is of immense importance within livestock industries as consumers, producers, industry bodies and governments demand greater standards of welfare within animal production systems (Australian Government, 2008; European Union, 2008; Kauppinen et al., 2010; Red Meat Advisory Council Ltd, 2015). With growing recognition of animal sentience and animal emotions, it is important that tools are available for researchers and producers to assess and benchmark the emotional or affective states of animals as part of a comprehensive welfare assessment. However, it is currently impossible to directly assess the conscious experience of emotion in another living being. While verbal communication allows us to gain a good understanding of the emotional experience of humans, the task of scientifically assessing emotional states in non-verbal beings is certainly much more challenging, but not impossible.

The purpose of this thesis is to further develop and validate novel cognitive methods for assessing emotional states in sheep. The following review of the literature outlines some of the ways in which affective states have been conceptualised in animals. It will discuss some of the methods currently used by researchers to gain insight into the affective states of livestock, highlighting the limitations of these approaches. The review will describe a relatively new area of animal welfare science which shows promise for the

assessment of affective states in animals; the assessment of cognitive biases. Finally, it will outline some key areas of further research required to better understand the potential for attention bias tests to be used as measures of affective state in sheep and other livestock species. It is these key areas of research which form the basis of this thesis.

1.2. Context

Much of the animal welfare research conducted to date has focused on the reduction of negative welfare states and suffering (Broom, 1991; de Vere & Kuczaj, 2016). However, it is now widely accepted that the promotion of positive welfare is also important (Boissy et al., 2007; Yeates & Main, 2008; Hemsworth et al., 2015). Previously, definitions of animal welfare have largely focused on the physical health and biological needs of an animal (Broom & Johnson, 1993; Mendl, 2001). Over time, definitions of welfare have also considered the 'naturalness' of an animal, whereby a greater ability to express natural behaviours is associated with improved welfare (Špinka, 2006). Now, definitions of animal welfare also consider what an animal wants and how an animal 'feels' (Désiré, Boissy & Veissier, 2002; Dawkins, 2004, 2006, 2008; Mendl, Burman & Paul, 2010). This consideration of how an animal feels has led to an emerging area of research into animal affect.

1.3. Conceptualising affective states

The term 'affect' describes an individual's behavioural and physiological responses, that can vary in terms of intensity and pleasantness or unpleasantness (Paul, Harding & Mendl, 2005). Affective or emotional states include both emotions and moods. Emotions are short term affective states generated in response to a specific event or stimulus, while moods are longer term affective states which are not directed at a specific event or stimulus (Paul, Harding & Mendl, 2005). A 'feeling' is the conscious experience of an affective state (Paul, Harding & Mendl, 2005).

Much of the research conducted on affect in humans and animals has focused on the existence of discrete emotional systems such as fear or joy (Darwin, 1872). Thus, many of the methods used to assess affective states in animals, and which are discussed in the following section, were developed within this context. These basic emotional systems are thought to form the foundation of all emotional responses, and are each supported

by specific brain regions and neural circuits to serve particular adaptive functions (Ekman, 1992; Panksepp, 2011).

Affect has also been conceptualised as a location within a 2- or 3- dimensional space, emphasizing the temporal relationships between different types of emotional experiences (Russell & Barrett, 1999; Watson et al., 1999; Carver, 2001; Russell, 2003). The dimensions typically describe whether an affective state is positive or negative (valence) and the degree of activation, motivation, intensity or arousal associated with the state (Kuppens et al., 2013). Dimensional theories often suggest that it is an animal's position within the affective space that forms the foundation of all emotional responses (Watson et al., 1999; Carver, 2001; Posner, Russell & Peterson, 2005; Barrett, 2006).

An integrative framework for the study of animal emotions was proposed by Mendl et al. (2010), based on similar theories to those used in the study of human emotion (Izard, 2007; Panksepp, 2007). Briefly, it was suggested that the affective state of an animal depends on its position within a core affective space, delineated by the axes of valence and arousal (Figure 1.1). The location of an animal's state within the affective space is determined by the animal's mood and their responses to short term emotion-inducing events. Specific events generate discrete emotions, which function to recruit physiological resources and motivate behaviours for survival. Discrete emotions work together with other types of emotional states, such as sensations or motivations, to generate short-term changes to an animal's location within the core affective space. Moods are longer-term, background states which can be thought of as the "running mean" of the animal's position within the affective space (Figure 1.1). Each of these systems interact with one-another, ultimately functioning for the animal to avoid punishments and acquire rewards for the enhancement of the animal's fitness for survival. Affective states may or may not be accompanied by conscious feelings.

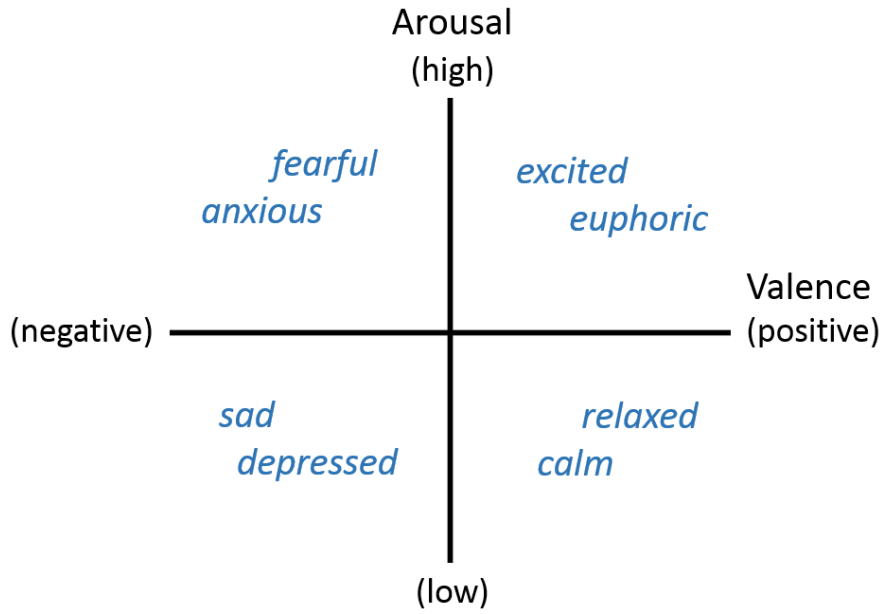

Figure 1.1. Core affective framework proposed by Mendl et al. (2010) and adapted from Russell (Russell & Barrett, 1999) and Panksepp (Burgdorf & Panksepp, 2006).

Image adapted from Mendl. et al. (2010).

An individual's susceptibility to or propensity to be in positive and negative affective states has been linked to personality or temperament assessments in humans (Gross, Sutton & Ketelaar, 1998). Concepts of personality and temperament refer to the consistency of an animals behavioural or physiological responses across time and situations or contexts. More specifically, Finkemeier et al. (2018) defined personality as a correlated set of behavioural and physiological traits, while temperament was defined as inherited and early appearing tendencies which serve as the foundation for personality. In human literature, it is suggested that temperament is primarily determined by innate neurobiological mechanisms that are subject to slow changes due to maturation and genotype-environment interactions (Strelau, 2001). Such systems may determine an animal's sensitivity to reward and punishment, driving individual differences in approach and avoidance behaviours (Hampson, 2012). It then follows that an animal's temperament influences their position within the core affective space, by impacting on their sensitivity to emotion-eliciting stimuli.

The different theories of emotional states remain highly debated, as does the ability of animals to experience subjective and conscious 'feelings' (Ekman, 1992; Izard, 2007; Panksepp, 2007). Hereafter, this thesis will focus on the integrative framework proposed by Mendl et al. (2010). The affective state of an animal will be considered as

a positive or negative state which can generate adaptive behaviours, to avoid punishment and acquire rewards (Mendl, Burman & Paul, 2010). This thesis will use labels such as “anxiety” or “happiness” to describe an animal’s potential affective state. However, this thesis will largely set aside the debate as to whether such affective states also manifest as a conscious feeling which would typically be associated with such terms. Instead, these terms should be considered as labels to describe an animal’s expected position within the affective space (Figure 1.1), which may or may not be accompanied by a conscious experience or feeling. It should be noted however that the potential for animals to experience conscious feelings largely drives concerns for animal welfare.

1.4. Understanding animal responses

Affective states function to recruit physiological resources and motivate behaviours, which means that specific affective states are often intimately linked with a certain set of physiological, behavioural and cognitive responses. These responses are often measurable and can be used as indicators of when an animal may be in a particular affective state. Before developing test paradigms to measure such responses, it is important to first gain an understanding of how an animal naturally responds to its environment. This allows researchers to design paradigms suitable for the species being tested and to more correctly interpret their responses. The basic physiology and ethology of sheep has been well documented in the literature (Arnold & Dudzinski, 1978; Lynch, Hinch & Adams, 1992; Sherwood, Klandorf & Yancey, 2005). The following sections of this thesis will only briefly summarise important responses relating to the attention bias test paradigm, highlighting how such responses relate to affective state. This includes the physiological stress response and behavioural responses related to threatening stimuli.

1.4.1. The stress response

One of the most commonly used indicators of negative affective states in the literature has been the presence or intensity of a physiological stress response (Paul, Harding & Mendl, 2005). The stress response serves as a defence mechanism for the body to respond to environmental stimuli or stressors which may disrupt homeostasis (Möstl & Palme, 2002). Stress responses are generally triggered by aversive stimuli and have been associated with negative affective states and mood disorders in humans (Lupien et

al., 2007). Stress responses involve activation of the hypothalamic-pituitary-adrenal (HPA) axis, which leads to a series of highly conserved neuroendocrine reactions. Corticotropin-releasing factor (CRF) is produced by the hypothalamus, triggering the release of adrenocorticotrophic hormone (ACTH) from the pituitary gland, which then triggers the synthesis and secretion of glucocorticoids such as cortisol from the adrenal cortex (Sherwood, Klandorf & Yancey, 2005). Together, these hormones allow animals to physiologically, behaviourally and psychologically respond to a given stressor to maintain homeostasis (Chrousos, 2009; Hermans et al., 2014).

The stress response is typically considered to be adaptive, allowing animals to appropriately respond to stressors. However, if a stressor is prolonged or repeated, the stress can become chronic and lead to dysregulation of the HPA axis. Chronic stress has many detrimental effects to animal health, welfare and production. For example, in sheep, chronic stress has been associated with increased aggressive behaviours (Sevi et al., 2001), poorer reproduction (Ehnert & Moberg, 1991; Sabrah et al., 1992; Sarma, 2011), reduced intake of feed and water (Parrott et al., 1987; Abdel-Rahman, Ahmed & Sotohy, 2000) and reduced growth rates (Thompson et al., 1995; Abdel-Rahman, Ahmed & Sotohy, 2000). Links between stress, affective states and immune function have been established, so there is also potential for immune function to act as an indicator of stress and welfare more broadly (Colditz & Hine, 2016). Chronic stress can also reduce an individuals' ability to respond to future stressors, due to their increased effort to maintain homeostasis (allostatic load) (Daniels-Severs et al., 1973; Hashimoto et al., 1988; McEwen, 2000; McEwen et al., 2012). Importantly, in humans, chronic stress is also associated with mood shifts and the development and maintenance of depressive and anxious emotional states (Southwick, Vythilingam & Charney, 2005; Beauchaine et al., 2011).

1.4.2. Sheep ethology

Sheep can be described as a fearful, gregarious, grazing ruminant species (Hinch, 2017). Their behavioural characteristics largely reflect a need to cover large areas while grazing for food, as well as avoidance of predators. Sheep spend considerable portions of their day grazing and are highly motivated to feed, particularly when access to food is restricted or when their diet is nutritionally poor or unbalanced (Doughty et al., 2016).

Sheep are highly fearful of unknown or predator species and are generally fearful of anything novel (Hinch, 2017). This high level of fearfulness serves as a predation

protection strategy. While grazing, sheep constantly scan their environments. They have a wide field of vision of approximately 290º and quickly respond to movement within their field of view (Piggins & Phillips, 1996; Kendrick, 2008). When threatened by predators or the unknown, sheep become highly vigilant and take up an alert posture, with a stiff gait and raised head (Stolba et al., 1990). Sheep will typically orient their heads and ears towards the potential threat so that it is situated within their narrow field of binocular vision (Kendrick, 2008). Sheep will generally flee predators, however some will display freezing behaviour and others may exhibit defensive behaviours such as foot stamping (Wemelsfelder & Farish, 2004). Sheep may also vocalise, although this more often occurs when sheep are isolated to try and re-establish the location of the flock (Hinch, 2017). Flocking is perhaps the most important behaviour displayed by sheep for the detection and evasion of predators (Dwyer, 2004).

Sheep are highly socially motivated although some breeds, such as Merinos, are more socially motivated than others (Hinch, 2017). The capacity for individual recognition of others is important for the social organisation of sheep. A number of studies have demonstrated the ability of sheep to discriminate visual images of conspecific faces (Kendrick, 1994; Kendrick et al., 1995, 1996). Further, sheep have shown preferences for photographs of calm conspecifics compared to stressed or anxious conspecifics (da Costa et al., 2004). These studies among others demonstrate sophisticated social and emotional recognition skills (Tate et al., 2006; Kendrick, 2008).

1.5. Assessing affective state

1.5.1. Physiological measures

Hormones which are secreted under the influence of the HPA axis are commonly assessed in the literature for the quantification of a stress response. The most commonly analysed hormones in the literature are glucocorticoids such as cortisol (Cook et al., 2001; Touma, Palme & Sachser, 2004; Doyle et al., 2010; Hawken, Fiol & Blache, 2012; Verbeek, Ferguson & Lee, 2014). Cortisol, or the metabolites of cortisol can be measured in the blood, saliva or faeces to quantify a stress response (Cook et al., 2001; Touma, Palme & Sachser, 2004; Doyle et al., 2010). However, cortisol responses can be difficult to interpret due to factors such as the influence of handling and blood sampling (Sevi et al., 2001), an animal's diurnal patterns in secretion (McNatty & Young, 1973) or the impact of previous stress on baseline concentrations or

responsiveness, particularly as stress becomes chronic (McNatty & Young, 1973; Rhodes, Nippo & Gross, 1994; Dwyer & Bornett, 2004; Mormède et al., 2007).

During chronic stress, glucocorticoid concentrations usually decline, even though the animal continues to show behavioural signs of stress. Therefore, glucocorticoid concentration alone may not be able useful for assessing chronic stress. However, some methods also allow differentiation between acute and chronic stress, by assessing dysregulation of the HPA-axis. Stimulation tests involve administration of exogenous hormones like CRF and ACTH, then the assessment of a following endogenous ACTH or glucocorticoid response thereafter (Mormède et al., 2007). Chronic stressors have been shown to alter the responsiveness of the adrenals by either increasing or decreasing cortisol responses relative to control animals (e.g. Bowers et al., 1993; Verbeek et al., 2019).

Body temperature can also be used as an indicator of physiological stress. During a stress response, different parts of the body can increase or decrease in temperature as blood flow is redistributed to areas of importance, such as skeletal muscle, to aid fight-or-flight responses (Hsieh et al., 1990; Sherwood, Klandorf & Yancey, 2005). For example, the core body temperature of sheep is shown to increase during an acute stress response (Sanger et al., 2011), whereas peripheral body parts, such as the tails and paws of rats, show a decreased temperature response during stress (Vianna & Carrive, 2005). Body temperature can be measured using small logging devices such as iButtons, that can be inserted intra-vaginally or rectally for accurate assessment of body temperature in the field (Lea et al., 2008). Other less invasive methods, such as the use of infrared thermography (IRT), can also allow for external temperature assessment. IRT assessment of the lacrimal caruncle of the eye has been shown to correlate well with core body temperature in cattle (Church et al., 2014). Although, it is noted that a range of environmental factors can reduce accuracy of IRT recordings, such as camera-to-object distance, wind and solar loading, which may limit the practical application of such measures in the field (Church et al., 2014).

Importantly, spikes in hormone concentrations and body temperature can also occur when animals are in positively-valenced or neutral affective states, such as during sexual arousal, while playing or in anticipation of feed (Dawkins, 2003; Mason & Mendl, 2007; Travain et al., 2016). As such, it is suggested that many physiological measures give information on the arousal component of affective state but do not

necessarily indicate valence. Nevertheless, they still provide valuable information on the affective state of an animal, especially when examined in conjunction with other behavioural or cognitive responses.

1.1.1. Behavioural measures

A number of reviews have discussed the use of behavioural responses when measuring the welfare and affective states of livestock (Wemelsfelder & Farish, 2004; Boissy et al., 2007; Forkman et al., 2007; Yeates & Main, 2008; de Vere & Kuczaj, 2016). Once again, the literature has largely focused on the presence of negative affective and welfare states, with a particular focus on fear or fearfulness (Forkman et al., 2007). Common responses assessed during behavioural studies include measures of locomotion such as zones crossed, vocalisations, vigilance, eliminations, defensive behaviours such as foot stamping, affiliative or social behaviours such as grooming and investigative behaviour such as sniffing. However, interpretation of these behaviours is not always straight-forward and there are many inconsistencies in how these behavioural responses have been interpreted in the literature.

Inconsistencies within the literature largely stem from the fact that the same or similar behavioural responses can be driven by different underlying motivations and can vary both between and within individuals at different times or within different contexts. For example, increased locomotion can occur when an animal is exploring or fleeing, while decreased locomotion can occur when an animal is content, injured or exhibiting a freezing response (Désiré, Boissy & Veissier, 2002; Wemelsfelder & Farish, 2004; Boissy et al., 2007; Reefmann, Wechsler & Gygax, 2009; Pedernera-Romano et al., 2010). As a result, researchers must rely on understanding the context in which a behaviour is exhibited, as well as the accompanying physiological and behavioural responses, to interpret an animal's affective or welfare state.

1.1.2. Cognitive bias

When the affective state of a human or animal alters the way in which it processes information, this is called a cognitive bias (Mendl et al., 2009). In the human literature, specific affective states are shown to alter people's processing of information and consequently their behavioural or physiological responses to that information in predictable ways. Therefore, researchers can assess the way in which people respond to certain types of information to infer their underlying affective states. The potential for

using cognitive methods to assess affective states in non-human animals has been noted by a number of authors (Paul, Harding & Mendl, 2005; Mendl et al., 2009). These cognitive approaches can potentially offer a better way to assess the valence of affective state, compared to behavioural or physiological approaches alone. There are many types of cognitive biases but the two key types which have been explored in animal welfare science thus far are judgement biases and attention biases.

1.1.1.1. Judgement bias

A judgement bias occurs when the affective state of an individual alters the way in which it interprets and responds to ambiguous information (Harding, Paul & Mendl, 2004; Marchant-Forde, 2015). It has been widely demonstrated that people in negative affective states are more likely to judge ambiguous information as being negative (pessimistic), while people in positive affective states are more likely to judge ambiguous information as being positive (optimistic) (e.g. Eysenck et al., 1991; Haselton & Nettle, 2006; Mogg, Bradbury & Bradley, 2006). A judgement bias task was first adapted for use in animals by Harding et al. (2004). Since then, judgement bias tasks have been widely used in the animal welfare literature across a range of species, including sheep (Bethell, 2015). Judgement bias tasks typically involve training animals to reliably discriminate and respond to a positive and a negative cue. Animals are then presented with an ambiguous cue, often intermediate between the trained cues, and their responses are recorded. Animals which exhibit the trained positive response are thought to have interpreted the ambiguous cue in an optimistic way, while animals which exhibit the trained negative response are considered pessimistic. It is suggested that judgement bias tasks allow us to gain information on the mood of an animal, by testing their responses to stimuli that are not emotion-eliciting (Mendl, Burman & Paul, 2010). Although, it is worth noting that some studies suggest judgement biases are also influenced by temperament (Asher et al., 2016; Barnard et al., 2018).

In sheep, pessimistic biases have been observed after negative treatments such as exposure to unpredictable and uncontrollable aversive husbandry procedures (Doyle et al., 2011b; Destrez et al., 2013) or pharmacological depletion of serotonin to induce a depression-like state (Doyle et al., 2011a). Optimistic biases have been displayed after repeated exposure to positive events (Destrez et al., 2014) and after administration of opioids to induce positive states (Verbeek et al., 2014). However, some judgement bias results have been inconsistent with the prior hypotheses. For example, some studies

aiming to induce acute or chronic stress have observed unexpected optimistic judgement biases (Doyle et al., 2010; Sanger et al., 2011). While judgement bias tests show promise as measures of affective state, the occurrence of unexpected results demonstrates a need to exercise caution when interpreting animal responses during tests.

Judgement bias tests also have a number of limitations to their practical application which have been discussed extensively in the literature (Baciadonna & McElligott, 2015; Bethell, 2015; Doyle, 2017). For example, judgement bias tests typically require lengthy training periods before animals can discriminate the positive and negative cues. Further, animals which do not meet the learning criteria cannot be assessed (Doyle, 2017), thus biasing sampling within populations. Given these limitations of judgement bias tests, there is a need to explore more practical methods which can be used to assess affective states in animals.

1.1.1.2. Attention bias in humans

An attention bias is the tendency to process certain types of information before others (Bar-Haim et al., 2007). This manifests as the preferential allocation of attention towards certain types of information over others. Attention biases are determined by the salience of the information presented, or its perceived importance to the individual, and can be influenced by an individual's affective state. In the human literature, attention biases have largely been examined in relation to anxious states and anxiety disorders. It has been shown that individuals in anxious states consistently pay more attention towards threatening information compared to non-anxious individuals (Bradley et al., 1995; Bradley, Mogg & Lee, 1997; Bar-Haim et al., 2007). Attention biases are observed across a range of different anxiety disorders and are suggested to be an intrinsic component of trait anxiety (Cisler & Koster, 2010).

While attention bias has largely been studied in anxious individuals, there is some evidence that attention biases occur among other affective states as well. For example, there is some evidence that depressed individuals display attention biases away from positive stimuli and towards dysphoric stimuli (Peckham, McHugh & Otto, 2010; Armstrong & Olatunji, 2012). There is also some evidence of attention biases towards threatening information in depressed individuals (Mogg, Bradley & Williams, 1995; Mathews, Ridgeway & Williamson, 1996), although this has not been consistently shown in all studies (see reviews; Armstrong and Olatunji, 2012; Peckham et al., 2010).

It was suggested by Peckham et al. (2010) that biases in attention appear to be a characteristic of negative affective states in general rather than markers of clinical disorders.

More recently, studies have shown that people in positive affective states display attention biases towards positive stimuli (Tamir & Robinson, 2007; Caudek, Ceccarini & Sica, 2017). Further, in attention bias modification studies, training people to attend to rewarding stimuli has been shown to increase positive affect (Grafton, Ang & MacLeod, 2012; Morales, Fu & Pérez-Edgar, 2016). In young adults, mood-congruent attention biases towards positively or negatively valanced stimuli have been observed after positive and negative mood induction (Isaacowitz et al., 2008; Caudek, Ceccarini & Sica, 2017). However, mood-congruent attention biases were not consistently observed across all populations, as older adults displayed mood-incongruent processing of the stimuli, possibly as a mechanism to regulate mood (Isaacowitz et al., 2008).

1.1.1.3. Measuring attention bias

A range of different tasks have been developed to assess attention bias in humans. One common example is the Stroop task, which asks a subject to name the colour of a word while ignoring the content of the word itself (Stroop, 1935). Individuals with known anxiety disorders take longer to name the colours of threatening words compared to neutral or positive words, indicating that the threatening information captured their attention away from the designated task (Williams, Mathews & MacLeod, 1996; Mathews, Mackintosh & Fulcher, 1997). In Stroop tasks, and in similar tests such as the dot-probe task (e.g. Mathews et al., 1997, 1996), attention bias is assessed as the degree to which a distractor cue captures an individual's attention away from a given task. These types of assessment methods have been adapted for use in non-human primates (King et al., 2012; Parr et al., 2013). A similar task has also been adapted for use in sheep (Verbeek, Ferguson & Lee, 2014). In this study, a salient food-related cue distracted food-restricted animals from a trained task, which was interpreted as an attention bias towards the food-related information.

Another common method for the assessment of attention biases in humans is to measure looking time as an indication of attention. Looking time tasks typically measure gaze patterns towards one or more valenced stimuli. These tasks were originally developed for preverbal infants and they have recently been adapted for the use in animal species such as non-human primates (Bethell et al., 2012). Looking time tasks for the

assessment of attention bias have also been developed for use in sheep. Vogeli et al. (2014) examined the effect of barren, unpredictable housing in Lacaune sheep on attention paid towards videos of conspecifics engaged in aggressive, affiliative or non-social behaviours. All tested animals showed increased attention to the aggressive videos; however, the unpredictably-housed sheep spent longer orienting towards the videos overall compared to sheep housed in enriched, predictable conditions. This was interpreted as a possible attention bias towards social information. Lee et al. (2016) exposed Merino sheep to a dog for 10 s in a specialized arena, then the dog was removed and sheep behaviours were recorded for 3 mins. Sheep which were in a pharmacologically-induced anxious state spent more like looking towards the previous location of the dog (threatening stimulus), were more vigilant and displayed a longer latency to eat a feed reward which had been placed in the arena, relative to saline controls. This was interpreted as an attention bias towards the threat in anxious sheep. This attention bias test had been based on a method developed for starlings, which also examined latency to feed after exposure to a threat (alarm call), but used vigilance behaviour alone as a key measure of attention towards the auditory threat (Brilot & Bateson, 2012).

1.1.1.4. Benefits of attention bias tests and outstanding questions

Attention bias tests potentially have a number of advantages over their judgement bias counterparts. Attention bias tests do not require that animals meet specific learning criteria in order to be tested, so the tests can be used to assess the entire population of interest. Additionally, the test developed by Lee et al. (2016) was rapid, only requiring a brief habituation period and taking 3 min per animal to conduct. However, the use of attention bias tests in livestock is currently very new and there are many remaining questions as to whether these types of tests can be useful for assessing the valence of affective state. Studies to further improve the practical application of these tests and to better understand their implications for welfare assessment are necessary.

While judgement bias is suggested to be a more general measure of animal mood, attention bias tests appear to be more context specific, depending on the salience of the information presented during testing. This may mean attention bias tests can provide information on more specific affective states than judgement bias tests. Further studies examining the influence of different types of affective states on attention biases in sheep

will aid the interpretation of animal responses during testing. Additionally, studies in humans have shown that attention biases are influenced by trait anxiety, but potentially also to shorter-term emotions and moods. Studies to determine which of these aspects of an animal's affective state are primarily driving behavioural responses during an attention bias test will again aid interpretation of responses and help researchers to better understand the potential role of this method in welfare assessment.

1.2. Using pharmacological models to study affect

Experimental validation of novel behavioural and cognitive tests is important to ensure the tests are working as expected, before applying them in a practical setting. To validate a new test, researchers can experimentally induce an affective state, then apply the test to ensure it discriminates the treated animals from untreated controls. Affective states can be experimentally induced using environmental or pharmacological manipulations. Environmental manipulations have the advantage of more closely matching the natural occurrence of an affective state, which likely involves activation across a range of neurophysiological pathways. However, the effects of environmental treatments are not always maintained throughout the testing period and it can often be difficult to identify the state an environmental manipulation has induced (Doyle et al., 2010; Sanger et al., 2011).

Pharmacological models generally target a limited number of neurophysiological pathways and may not give a true reflection of naturally occurring states. However, this can be beneficial by giving detailed information on the specific pathways that are involved in the formation of a particular behavioural or cognitive response. Further, pharmacological models can be applied in a more standardised and repeatable manner than environmental manipulations, can remain active for the duration of testing and can be readily matched with appropriate control treatments (Mendl et al., 2009; Doyle et al., 2015). The following section outlines some key pathways and pharmacological agents which may be useful for the validation of an attention bias test in sheep.

1.2.1. Pharmacological models of affect in sheep

Administration of exogenous ACTH and glucocorticoids have been used to model stress responses in sheep. The drugs can be administered once to model acute stress or can be repeatedly administered as a model of chronic stress. For example, Henry et al. (2010)

and Clarke et al. (2016) each used daily injections of synthetic ACTH (Synacthen) to model stress in sheep, while Schlink et al. (2002) administered exogenous cortisol to model stress. These studies examined the impacts of stress on factors such as food intake or wool quality. To our knowledge, no studies have used pharmacological models of stress in sheep within the context of studying affective state.

Several studies in sheep have used pharmacological agents to target the serotonergic system to modulate affect. For example, the anxiogenic drug 1-*m*-chlorophenylpiperazine (mCPP) has been used a number of times in sheep to model anxiety-like affective states (Drake, 2006; Doyle et al., 2015; Lee et al., 2016). The drug mCPP has a varied effect on different serotonergic receptors, acting as both a serotonin reuptake inhibitor and a serotonin releasing agent, although it largely acts as Serotonin agonist (Feuchtl et al., 2004). The drug has been shown to have an anxiogenic effect in humans (Benjamin, Greenberg & Murphy, 1996) and in sheep, is associated with an increase in fear related behaviours such as increased agitation during isolation and potentiation of a startle response (Doyle et al., 2015). On the other hand, depletion of serotonin has been clearly linked with depression in humans (Nemeroff & Owens, 2009). In sheep, the drug (pCPA) has been used to deplete serotonin concentrations as a model of depressed states and was shown to induce a pessimistic judgement bias, consistent with a negative mood (Doyle et al., 2011a).

GABAergic systems have also been targeted for the manipulation of anxious states. The anxiolytic drug diazepam acts as a GABA agonist and is associated with a reduction of fearfulness and anxiety in humans (Roy-Byrne, 2005). This drug has also been used to reduce anxiety and model calm-like states in sheep (Drake, 2006; Destrez et al., 2012; Doyle et al., 2015; Lee et al., 2016). Although, responses to this drug in sheep have not always been consistent (Drake, 2006; Doyle et al., 2015). Opioid systems can be targeted for the modulation of pain, but also have a key role in the mediation of euphoric effects of recreational drugs such as morphine and heroin (Corbett et al., 2009). In sheep, the drug morphine was shown to enhance an optimistic judgement bias after consumption of a feed reward (Verbeek et al., 2014), suggesting this system may be useful for the modelling of positive affect in sheep.

It is important to note that the use of pharmacological agents to model affect comes with some limitations. For example, drugs may have unwanted effects, either due to the additional action of the drug on neurophysiological pathways unrelated to emotion, or

due to adverse reactions to the drugs that can vary between individuals. Thus, some degree of caution is required when interpreting animal responses after administration of these pharmacological agents. Nevertheless, the use of pharmacological models can be a good first step to better understanding the mechanisms driving behavioural or physiological responses in animals and to validate novel measures of affective state.

1.3. Aims and hypotheses

The primary aim of this thesis was to further develop and validate a novel method for the assessment of attention biases in sheep, as a measure of animal welfare. The present research begins with the following set of hypotheses:

1) An attention bias test, based on that developed by Lee et al. (2016), can be used in sheep to assess and differentiate different types of positive and negative affective states.
2) The method developed by Lee et al. (2016) can be further refined to improve its practical application and to better facilitate interpretation of animal responses.
3) Behaviour in the attention bias test is primarily driven by short-term emotional states and moods, but some aspects of animal behaviour during testing are also influenced by underlying personality or temperament traits, which remain stable over time.

The following section of this introduction briefly outlines how these hypotheses were tested in the experimental chapters of this thesis.

1.4. Outline of experimental chapters

Chapter 2 examines the effect of pharmacologically induced stress on judgement biases and attention biases in sheep. The data presented in this chapter were generated in 2013, prior to the commencement of this doctoral degree. The version of the attention bias test applied in Chapter 2 is similar to that described by Lee et al. (2016), but used a longer duration of exposure to the threatening stimulus.

Chapter 3 aims to modify the attention bias test method presented by Lee et al. (2016) to remove the habituation period and shorten the test duration, making the test more practical to apply to larger groups of animals or in an on-farm setting. This chapter also more directly examines the influence of the threatening stimulus on animal responses during testing.

Chapter 4 examines the effect of pharmacologically induced anxious and depressed states on attention biases in sheep, to determine whether these negative states can be assessed and differentiated using a modified test method. The method is modified in this chapter to remove the potential confounding effects of appetite, thus facilitating a clearer interpretation of animal responses during testing.

Chapter 5 examines the effect of pharmacologically induced positive states on attention biases in sheep, to determine whether the test can assess positive affective states and differentiate these from negative states. This chapter uses the modified method presented in chapter 4.

Chapter 6 examines the repeatability of an attention bias test across 3 trials. This chapter examines whether animal responses during testing are influenced by transient affective states or temperament traits. The data presented in this chapter were generated as part of a larger experiment, which was conducted over 1 year in 2016 to 2017. This chapter uses the test method presented in chapter 3.

The thesis concludes by discussing how the attention bias test developed throughout this thesis may relate to affective states and animal welfare more broadly.

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Chapter 2

Pharmacologically-induced stress had minimal impact on judgement and attention biases in sheep

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2.1. Abstract

The emotional impact of exposure to stressors has not been well quantified in animals. We hypothesised that exogenous induction of stress in sheep would induce a pessimistic judgement bias and increased attention towards a threatening stimulus, suggestive of a negative emotional state. Stress was induced pharmacologically by administering synthetic adrenocorticotropic hormone. Judgement bias was assessed using a spatial go/no-go task after exposure to acute stress (one injection), chronic stress (21 daily injections) and acute-on-chronic stress (2 min isolation after 28 daily injections). Attention bias was assessed during chronic stress only (22 daily injections). In contrast with our hypotheses, there was no strong evidence that Synacthen administration altered judgement bias or attention bias at any stage of the experiment. Stressed sheep were more likely to approach ambiguous locations than saline Control animals, however, statistical evidence for models fitting treatment group was very weak. Overall, our findings suggest that elevated levels of cortisol may not fully explain changes to judgement bias observed in previous studies after environmentally-induced stress. Further studies are required to better understand which aspects of environmentally-induced stress alter judgement bias and to further validate cognitive methods of assessing affect in sheep.

2.2. Introduction

Domesticated animals regularly experience stressful events and environments which may impact their physical and mental well-being. However, the impact of stress on the affective (or emotional) states of animals is not fully understood. The stress response involves activation of the hypothalamic-pituitary-adrenal (HPA) axis, which leads to a series of highly conserved neuroendocrine reactions allowing animals to physiologically, behaviourally and psychologically respond to homeostatic threats[1,2]. This response is typically considered to be adaptive, however when a stressor is prolonged or repeated, the stress can become chronic and may lead to dysregulation of the HPA-axis and maladaptive stress responses[3,4]. In humans, this can lead to mood changes and has been implicated in the development and maintenance of depressive and anxious emotional states[3,5]. In addition to the impact of chronic stress itself, an increased effort to maintain homeostasis (allostatic load) can reduce the ability of animals to cope with and adapt to additional acute stressors, and may also lead to further shifts in

affective state[4,6–10]. Thus, the potential impact of stress, particularly chronic stress, on the mental well-being of animals and their ability to cope with additional stressors deserves further attention.

A key limitation for the study of affective states in animals has been a lack of quantitative assessment methods. To address this limitation, an increasing body of literature has assessed emotional states via their impact on an individual's cognitive processing of information, termed cognitive bias. Many of these studies have focused on sheep, which are farmed globally in large numbers and are used in biomedical research[11–14]. The type of cognitive bias most widely studied in sheep is judgement bias, where affective state can alter an individual's interpretation of ambiguous situations[15,16]. Individuals in positive affective states typically make more positive judgements about ambiguous information (optimism), while individuals in negative affective states typically make more negative judgements about ambiguous information (pessimism)[14]. Judgement biases can be assessed using a range of test paradigms. For example, in a go/no-go spatial discrimination task, subjects are trained to discriminate two locations, such that they approach the rewarded location (go) and avoid the negative or non-rewarded location (no-go). Subjects are then presented with an ambiguous, intermediate location and their go or no-go response is interpreted as the subject perceiving the ambiguous cue to be positive or negative respectively. In sheep, animals treated with an anxiolytic drug or an opioid, to induce positive affective states, were more likely to approach ambiguous locations during a go/no-go spatial discrimination task, suggesting they were more optimistic[17,18]. Sheep treated with a serotonin inhibitor to induce a depressed state were less likely to approach ambiguous locations[19]. These studies show that judgement bias tests may be used as a measure of affective states in sheep.

Another type of cognitive bias which has been more recently studied in animals is an attention bias, where affective state can alter an individual's allocation of attention towards different types of information[20]. For example, humans in anxious states pay more attention towards threatening information than non-anxious individuals[21–23]. Tests for attention bias are potentially a more rapid and practical option for assessing affective states in animals, compared to typical judgment bias test paradigms. In sheep and cattle, a novel attention bias test has been developed and pharmacologically validated as a potential measure of anxiety-like states, where animals given an anxiogenic drug spent more time looking towards a threatening stimulus and displayed increased vigilance behaviour compared to control animals[24–26]. Further, behavioural responses in a

modified version of the test have also been shown to reflect pharmacologically-induced depression-like states in sheep[27]. Together, these studies show that attention bias tests may also potentially be used to assess affective states in sheep.

A number of studies in sheep have examined the impacts of environmental manipulation to induce endogenous acute and chronic stress on cognitive biases, however the results have not always been consistent between studies. Using a go/no-go spatial discrimination judgement bias task, pessimistic judgement biases were observed in sheep after 3 weeks[28] and 9 weeks[29] of unpredictable, uncontrollable exposure to aversive husbandry procedures, expected to have induced chronic stress. Two further studies found several months of unpredictable, stimulus-poor housing only had a weak effect on judgement bias, with one study finding a slight pessimistic bias[30], while the other found a slight optimistic bias[31]. In contrast, three days of restraint and isolation stress induced an optimistic bias in sheep[32], as did an acute shearing challenge[33]. Most recently, Verbeek *et al*.[34] examined the impact of chronic stress induced by 9 days of lying deprivation on both judgement and attention bias in sheep. Further, they examined the impact of an additional acute stressor (shearing) on judgement bias. Prior to the shearing challenge, chronically stressed sheep appeared to be more optimistic during judgement bias testing and showed an attention bias away from a threat when compared to control animals, consistent with a more positive emotional state. After shearing, no differences in optimism were evident between the groups. It has been proposed that the unexpected optimistic judgement biases observed in many of these studies may reflect a positive emotional state caused by release from the imposed environmental stressors. This highlights a key challenge in using environmental manipulations to alter the state of an animal, as it can be difficult to identify which state the manipulation has induced and to maintain the state throughout the testing period[32,33].

Pharmacological treatments can alter the state of an animal in a more standardised and repeatable manner than environmental manipulations, while remaining active for the duration of testing[35,36]. Further, pharmacological treatments can be readily matched with appropriate controls (e.g. saline injections). A number of studies have examined the impact of pharmacological treatments on cognitive biases in animals, with most pharmacological agents aiming to alter the affective states of the subjects[19,24,27,37,38]. Few studies have examined the impact of exogenous induction of stress on the affective states of animals through pharmacological manipulation. The link between stress and affective states is well established in humans and the mechanism of action is well

described, with exogenous glucocorticoids shown to readily cross the blood-brain barrier to access the emotion related parts of the brain, including the amygdala. Adrenergic receptors have been found in the amygdala and play an important role in fear processing and memory for emotionally relevant information[39]. In addition, the link between mood disturbances, such as psychosis, and glucocorticoid administration has been confirmed in humans[40]. In animals, two studies have investigated the impact of pharmacologically-induced stress on judgement biases. In rats, injection of corticosterone and a noradrenaline reuptake inhibitor, to mimic the early stages of the acute stress response, caused a pessimistic bias in an audio discrimination judgement bias task[41]. In chickens, seven days of corticosterone administration, expected to induce chronic stress, caused a pessimistic bias during a spatial discrimination judgement bias test[42]. Conducting a similar study in sheep, stimulating the HPA axis via exogenous induction of stress, could help to better understand the impact of stress on cognitive biases and affective state. Previous studies in sheep have developed a model using daily injections of Synacthen, a synthetic adrenocorticotropic hormone, over a period of 4 weeks to chronically elevate plasma cortisol concentrations and induce a stress response[43,44]. Synacthen may therefore be a suitable candidate drug to pharmacologically validate the effect of acute and chronic stress on the affective states of sheep.

The aim of the current experiment was to determine the impact of stress on the emotional states of sheep, examining the effects of acute, chronic and acute-on-chronic stress. The study followed a similar design to Verbeek *et al*.[34], assessing emotional state using judgement bias and attention bias tests, however acute and chronic stress states were induced pharmacologically using Synacthen, instead of using environmental manipulations. We hypothesised that sheep given one injection of Synacthen (Stress; acute stress stage) would be more pessimistic than control animals given one saline injection (Control; acute stress stage) in a judgement bias test. Further, we hypothesised that continued daily injections of Synacthen over a 3 week period (Stress; chronic stress stage) would induce a more pessimistic judgement bias and an increased attention bias towards a threat compared to animals given daily saline injections (Control; chronic stress stage). Finally, we hypothesised that an additional acute stressor (2 mins of isolation) would induce a more pessimistic judgement bias in the chronically stressed animals compared to the control animals exposed to an acute stressor only (acute-on-chronic stage). It was expected that the Stress group would become increasingly more

pessimistic between the acute, chronic and acute-on-chronic stress stages. Plasma cortisol concentrations were assessed during the experiment to confirm the effect of Synacthen administration on HPA-axis responses.

2.3. Results

2.3.1. Judgement bias

One animal from the Stress group failed to go to the Positive (P) location on day 1 of judgement bias testing, but approached the P location on the other test days. Data for that sheep were retained for analyses. The other 26 sheep approached the P location on all test days.

The maximal model fitted to the judgement bias data included fixed effects of location, treatment, test day and all interactions. A subset of the best ranked models from the "dredge" function in R ($\Delta i < 4$) are presented in Table 2.1. The null model consisting of the intercept only had a high Δi AICc of 173 and Δi BIC of 157.

Table 2.1. Factors influencing animal responses during judgement bias testing.

Estimated information criterion were obtained using the "dredge" function in R, where the maximum generalised linear mixed effects model included fixed effects of treatment, location, day and all interaction terms. Random effects were day nested within animal ID for each model. Models are listed in order of increasing Δi BIC. Only models with a Δi BIC or Δi AICc < 4 are presented. The highest ranked models are highlighted in green.

Model	df	Δ*i* BIC	w_i BIC	Δ*i* AICc	w_i AICc
location	7	0	0.76	8.71	0.01
location + treatment	8	2.37	0.23	7.16	0.02
location * treatment	12	10.79	0.0	0	0.77
location * treatment + day	14	21.39	0.0	2.87	0.18

Δ*i*; differences in information criterion values compared to the optimal model (lowest BIC or AICc) within the given set of models. w*i* BIC and w*i* AICc: Schwarz and Akaike weights respectively, indicates relative weight of evidence for each model within the given set of models.

All models listed in Table 2.1 included location as a fixed effect. The best-ranked models by AICc also included treatment as a fixed effect, however support for a treatment effect was considerably weaker when considering BIC (Table 2.1). Model predictions from three of the best-ranked models using BIC are given in Figure 2.1.

Predicted values from each of these models suggest that sheep from the Stress group were more likely to approach the ambiguous locations than Control animals. This is particularly demonstrated by the observation that the Stress group were approximately 3 times more likely to approach the Near-Negative (NN) location than the Control group (raw proportion of go responses 0.63 and 0.19 respectively, Figure 2.1).

Evidence that test day had an effect on approach was negligible**.** One of the models fitting day as a fixed effect had a Δi AICc < 4, however the Δi BIC value for this model was greater than 20 (Table 2.1). When day was included in the model fitting treatment and location, proportion of go responses shifted upwards between days 1 and 21 (more likely to go) then down on day 28 (less likely to go) (Figure 2.2). However, the predicted values for each day of testing remained within the 95% confidence intervals of predictions from the same model excluding day as a fixed effect (Figure 2.2).

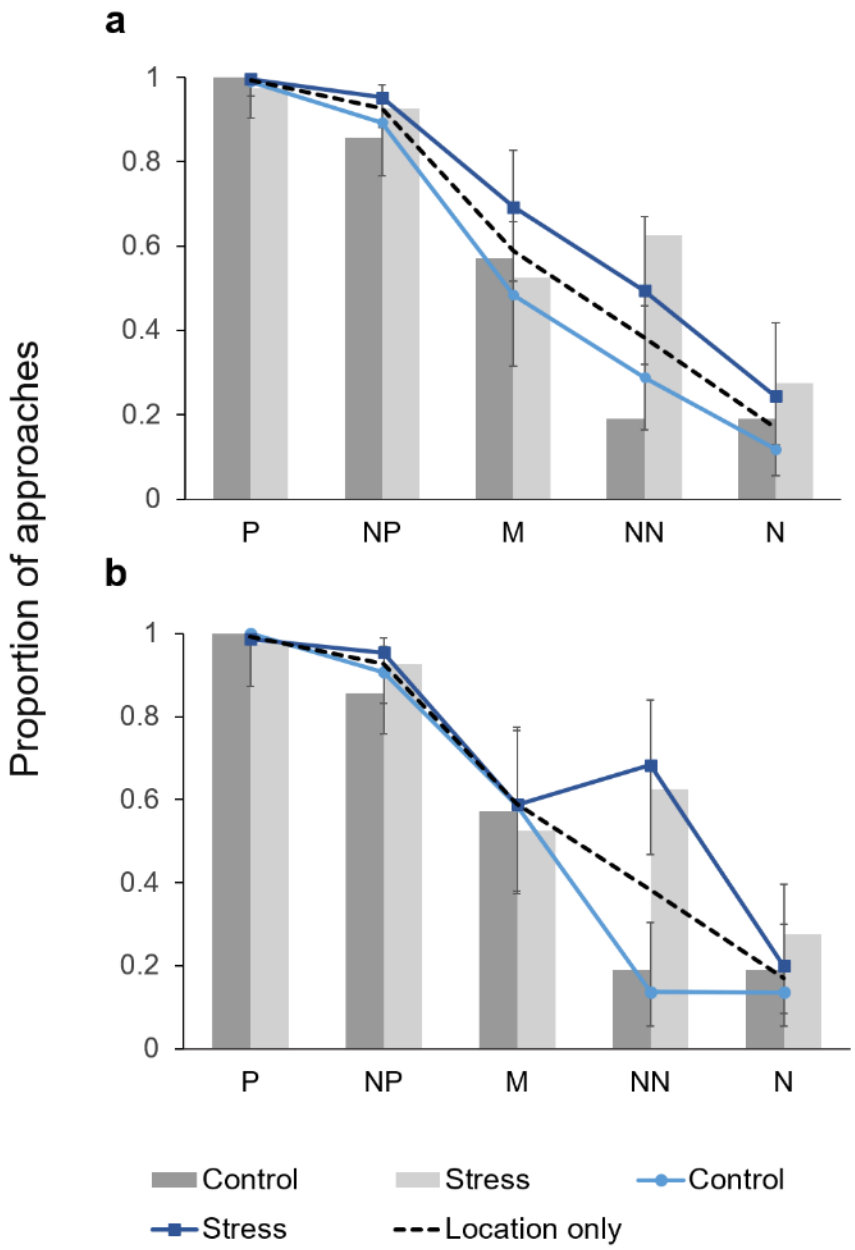

Figure 2.1. Mean predicted values ± 95% CI from generalised linear mixed effects models including fixed effects of (a) treat + location and (b) treat + location + treat:location during judgement bias testing.

Predicted values from the model fitting location only are represented by the black dashed line on both (a) and (b). For each model, test day nested in animal ID was included as a random effect. The bar charts on both (a) and (b) show the total number of go responses as a proportion of the number of entries into the judgement bias test for each location across all test days (P, positive location; NP, near positive, M, middle; NN, near negative; N negative location).

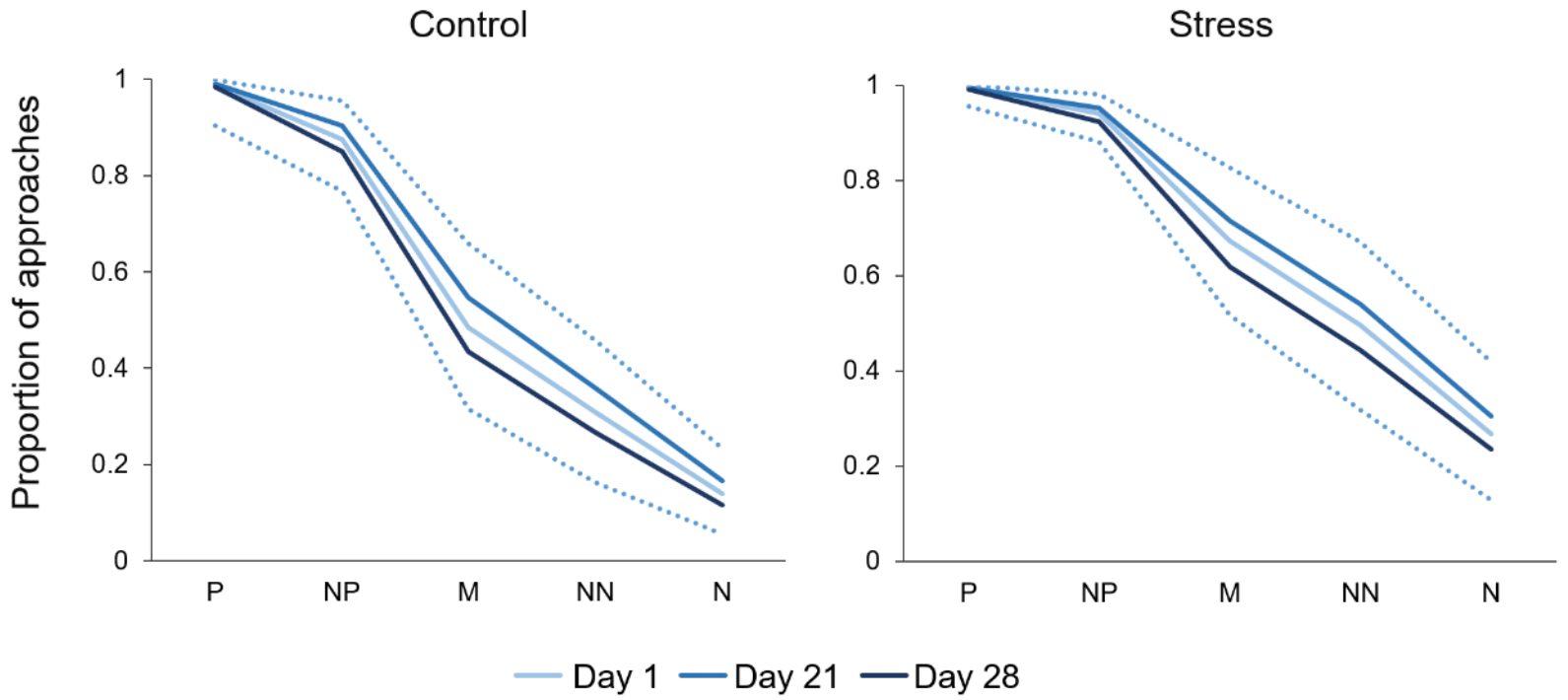

Figure 2.2. Mean predicted values of proportion of approaches in the judgement bias tests for the Control and Stress treatment groups on days 1, 21 and 28 of testing.

The generalised linear mixed effects model fitted treatment group, location and day as fixed effects and day nested in animal ID as random effects. The dotted lines represent the 95% CI for the same model after removing day as a fixed effect. The predicted means from the model including day lie within the 95% CI's of the model fitting fixed effects of treatment and location only (P, positive location; NP, near positive, M, middle; NN, near negative; N negative location).

2.3.2. Attention bias

Control animals had a greater latency to eat than Stress animals (89.6 and 52.5 s respectively), however this difference was not significant in the Cox proportional hazards model (Log likelihood ratio (1) = 1.83, P=0.2; Figure 2.3). All other variables examined were best described by a model fitting the intercept only, as opposed to models fitting treatment group (Table 2.2). For amount of food eaten and time spent immobile, models fitting treatment group also had some evidence.

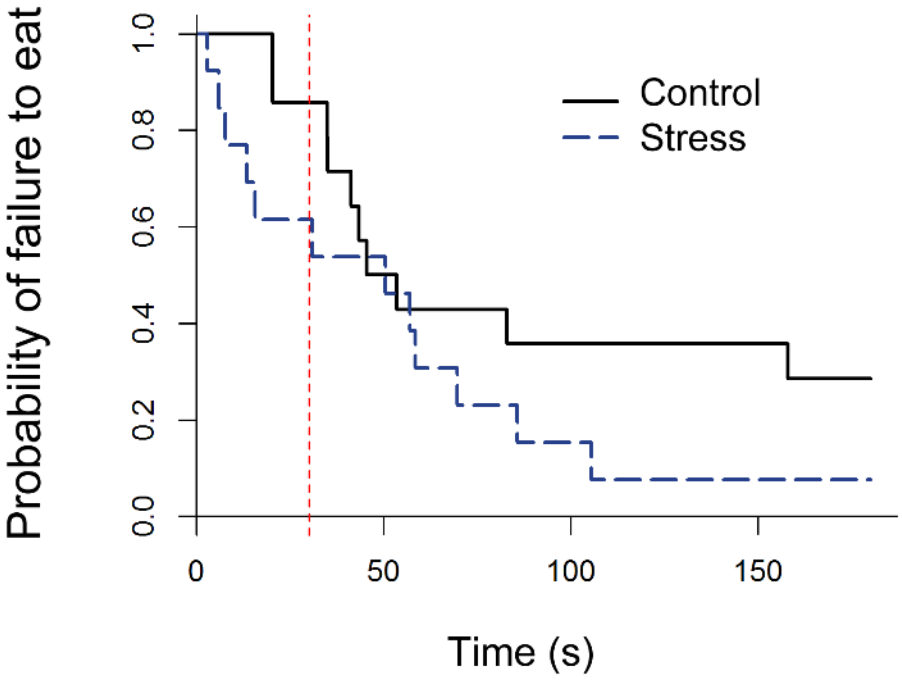

Figure 2.3. Kaplan-meier curves for latency to eat in the attention bias test for the model fitting treatment group.

The red dotted line indicates the time at which the dog window was covered during testing. For each time an animal ate the food, the probability on the Y axis drops.

Table 2.2. Predicted means and estimated information criterion for behaviors in the attention bias test.

Parameters were obtained using the "dredge" function in R, comparing a generalised linear model fitting the intercept only (Int) with a model including Treatment as a fixed effect. Models with a Δi < 2 are highlighted green.

Variable	Predicted means [95% CI]		Treatment model			
	Control	Stress	w*i* BIC	Δ*i BIC*	w*i* AICc	Δ*i AICc*
Attention to dog (s)	22.8 [14.8; 35.4][&]	18.6 [11.8; 29.3][&]	0.20	2.82	0.26	2.07
Vigilance (s)	123.1 [109.6; 136.5]	117.5 [103.5; 131.4]	0.19	2.92	0.25	2.16
Food eaten (g)[$]	70.9 [29.3; 112.4]	93.5 [50.4; 136.5]	0.21	2.65	0.28	1.90
Time immobile (s)	3.3 [1.0; 8.2]	1.4 [0.1; 4.2]	0.27	1.95	0.35	1.20
Zones crossed (*n*)	14.6 [9.2; 22.7]	14.7 [9.2; 23.2]	0.16	3.3	0.22	2.54

[&]predicted means and CI calculated on the log scale, back-transformed values are presented. [$]variable was analysed for the entire test period (with and without dog), all other variables were analysed for the period without the dog present only. w*i* BIC and w*i* AICc: Schwarz and Akaike weights respectively, indicates relative weight of evidence for each model within the given set of models Δ*i*; differences in information criterion values compared to the optimal model (lowest BIC or AICc) within the given set of models

2.3.3. Cortisol response

There was strong evidence for the model fitting time, treatment and their interaction for cortisol response on day 1 (Table 2.3). For cortisol response on day 29, there was strong evidence for the model fitting treatment only (Table 2.3). Animals treated with Synacthen showed a greater cortisol response than Control animals on both days of testing (Figure 2.4). Control animals showed little deviation from the baseline cortisol concentration on either test day (Figure 2.4).

Table 2.3. Influence of time and treatment on cortisol responses after injection of Synacthen on days 1 and 29.

Estimated information criterion were obtained using the "dredge" function in R, where the maximum generalised linear mixed effects models included treatment, sample time and their interactions as fixed effects and sheep ID as a random effect. Models are listed in order of increasing AICc. Models with a Δ*i* < 2 are highlighted in green.

Day	Model	df	Δ*i* BIC	w_i BIC	Δ*i* AICc	w_i AICc
1	treat*time	7	0	1	0	1
	intercept	4	187.0	0	194.1	0
29	treat	5	0	0.90	0	0.70
	treat+time	6	4.66	0.09	2.22	0.23
	intercept	4	16.3	0	18.8	0

Δ*i*; differences in information criterion values compared to the optimal model (lowest BIC or AICc) within the given set of models. w*i* BIC and w*i* AICc: Schwarz and Akaike weights respectively, indicates relative weight of evidence for each model within the given set of models.

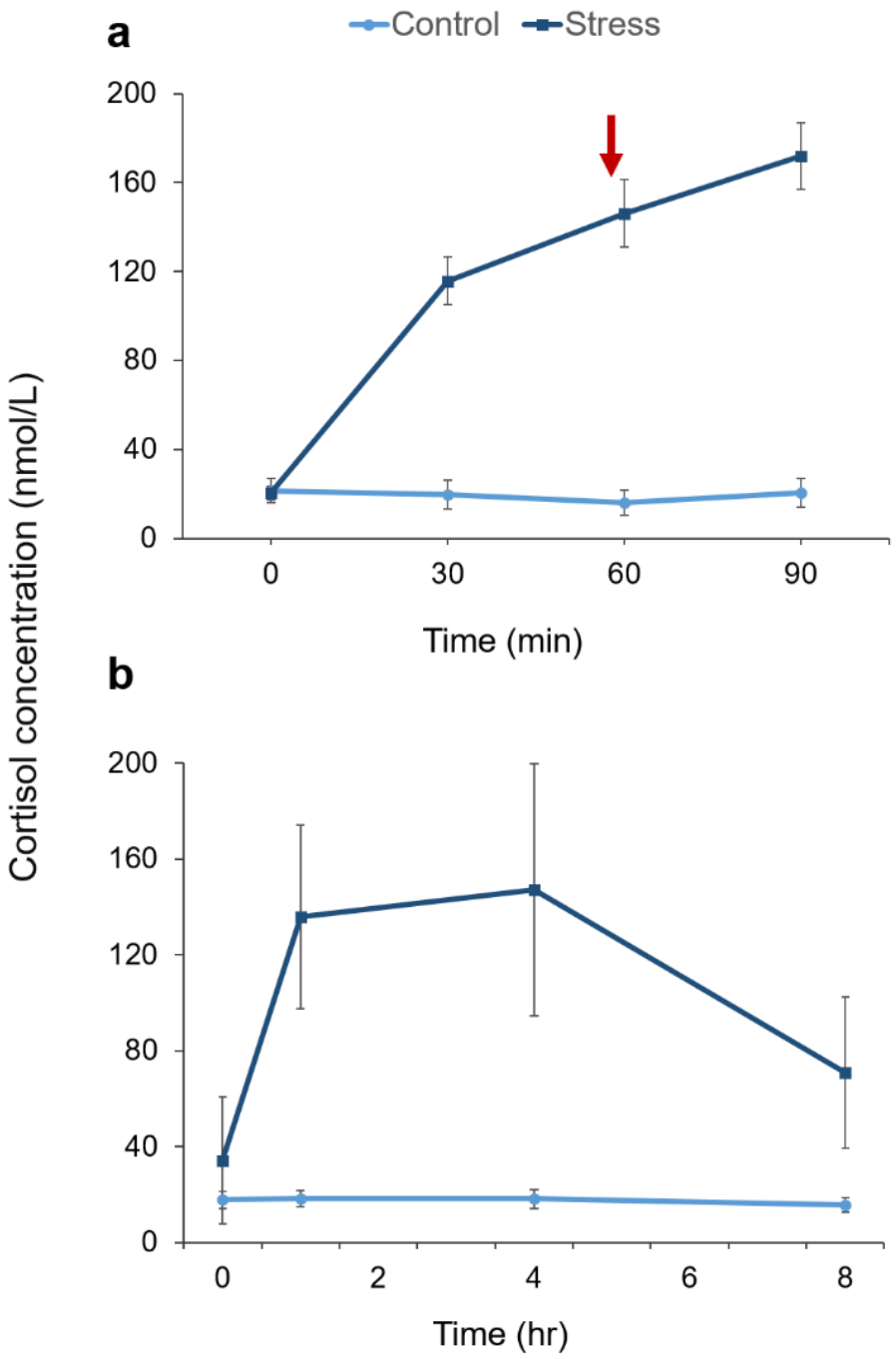

Figure 2.4. Cortisol response on days 1 (a) and 29 (b) of the trial after injection of Synacthen (Stress) or Saline (Control).

Predicted means ± 95% CI from a linear mixed effects model are given for each time point. The baseline blood sample was taken immediately prior to injection (Time 0). The red arrow indicates time of judgement bias testing on day 1.

2.4. Discussion

In contrast with our hypotheses, administration of an exogenous glucocorticoid (Synacthen) to induce stress had no strong effect on judgement bias or attention bias in sheep, relative to the control animals. Further, the results do not support our hypothesis that the judgement biases of stressed sheep would change between the acute, chronic and acute-on-chronic stress stages. There was some evidence for an optimistic bias in the stress animals at all time points, which is consistent with some[32–34], but not all previous studies[28,29,45]. However, statistical evidence for this optimistic bias was weak. The effect of Synacthen on the Stress animals was confirmed by an increased plasma cortisol concentration on days 1 and 29. This response is similar to that observed previously in sheep administered Synacthen daily for 4 weeks[43], and provides physiological evidence that the induced cortisol responses persisted for the duration of judgement bias and attention bias testing. A lack of effect on cognitive bias therefore suggests that elevated levels of cortisol cannot fully explain changes to judgement biases observed in previous studies after environmentally-induced stress[34]. Alternatively, these results may suggest that the cognitive bias tests used in this study could not detect changes to affective state caused by increased circulating cortisol concentrations. Further studies are therefore required to better understand which aspects of environmentally-induced stress may be contributing to an altered judgement bias in sheep, and to further validate cognitive bias tests as measures of affective state.

It is possible that administration of exogenous glucocorticoids did not impact on the affective states of sheep. A lack of effect could be due to the use of an exogenous hormone to induce stress, which may not equate to the stress response caused by an environmental manipulation, that impacts on an array of neurophysiological pathways. Alternatively, it may suggest that glucocorticoids do not have a role in modulating affective state in sheep, regardless of their origin. Previously, environmentally-induced elevations in cortisol have been observed without alterations to judgement bias in pigs[46], and altered judgement bias has been observed without evidence of changes to endogenous cortisol concentrations in sheep. However, studies in humans have established a clear link between administration of exogenous glucocorticoids and affective states[39]. Further, direct relationships between exogenous glucocorticoids and judgement bias have been demonstrated in chickens and rats[41,42]. We therefore suggest

our inability to replicate these results in sheep may be due to the limitations of the cognitive bias tests we used and our study design, as discussed below.

It is also possible that the cognitive bias tests were unable to detect a change in affective state in the Stress group, due to the confounding effects of feeding motivation or sensitivity to reward, as discussed by Verbeek at al.[34]. Glucocorticoids have been shown to increase the salience of pleasurable and compulsive activities in humans and rodents, such as ingestion of sugars, fats and drugs of abuse[47–50]. Increased motivational behaviours for rewarding stimuli and ingestion of "comfort foods" are thought to reduce the negative consequences of stress by downregulating HPA-axis activity[51,52]. Further, it has been proposed that animals may seek positive or rewarding experiences to counteract negative experiences[53]. In sheep, cortisol responsiveness has been linked to metabolic and behavioural traits associated with obesity[54]. As such, an increased motivation to eat palatable food rewards in the Stress group may have masked a potential change in judgement bias and attention bias, and may explain the observation of a weak optimistic bias during the current study.

Another factor which may have limited the ability of the judgement bias test to detect a response is the presence of olfactory cues from the dog and food during testing. Ideally, testing of ambiguous locations would occur in the absence of any cues[55], however this is often difficult to achieve in a practical setting. Instead, both positive and negative cues can be kept in place throughout all training and testing, as to not include an additional olfactory cue which may signal a particular outcome during ambiguous trials. In the current study however, the scent of the positive location changed between the positive and negative training sessions as, while some food was present next to the arena at all times, this scent may have been stronger during positive trials when there was food located inside the arena. This means the strength of the scent of food during the ambiguous trials was the same during negative training trials, potentially providing an additional cue that the outcome would be negative. If this were the case, we might expect a low proportion of go responses across all treatment groups to the ambiguous locations, however this was not observed, which suggests the presence of olfactory cues had minimal impact on results. Nevertheless, it cannot be ruled out that elevated cortisol levels enhanced the animals' perception of the scent of the food outside of the arena during ambiguous trials, such that they were more likely to expect a positive outcome. Further studies should be aware of and control for this potential effect during judgement bias tests as described by Verbeek *et al*.[34].

An effect of Synacthen on the Stress group may not have been detected if the Control animals had an equivalent stress response, due to the impact of the treatment and testing procedures. This is not supported by the cortisol responses for the Control animals that remained very low on days 1 and 29 during judgement bias testing and blood sampling. Further, sheep often voluntarily moved into the testing areas during the experiment, being led by a familiar handler with a feed bucket. Therefore, it seems unlikely that the Control animals found the treatment and testing procedures to be highly aversive. Nevertheless, the judgement bias and attention bias testing procedures involved isolation, novelty and exposure to predators, which are innately stressful for sheep[56–58]. Thus, it cannot be ruled out that the treatment and testing procedures impacted on Control animal responses during cognitive bias testing, so that they could not be distinguished from the Stress group.

Regardless of an impact on affective state, it makes biological sense for an animal which has been recently threatened and that is undergoing a physiological stress response to be more vigilant and pay more attention towards additional threats[59,60]. A lack of response during attention bias testing in the chronically stressed animals may therefore suggest that the dog and novel environment during attention bias testing were not perceived to be threatening and therefore did not elicit an additional response from the Stress group. Although as discussed previously, this seems unlikely given that isolation and predators are innately stressful for sheep. Another possibility is that the Stress group perceived the dog as a threat more strongly than Control animals, but failed to respond accordingly with an appropriate behavioural response, potentially demonstrating a maladaptive response associated with chronic stress and increased allostatic load[4,6–10]. Finally, a lack of response may again indicate that the pharmacological model of chronic stress was not effective as a manipulation of affective state, or that the attention bias test was not sensitive enough to detect an effect of the drug on the animals' behaviour. Further studies examining the impact of stress on the attention bias test only, using naive animals, would provide further insight into these issues.

It is important to consider the potential impact of repeated assessments on animal behaviour during testing, both between and within the two experiments[34]. The attention bias test is still relatively new and the effects of repeated testing on animal behaviour have not yet been explored. Therefore, exposure to the attention bias test during the previous study may have altered animal responses during the current study. Additionally, while a different dog was used in the attention bias test than for judgement

bias training and testing, repeated exposure to dogs may have reduced the animals' responses to this potential threat during the attention bias test. Another potential limitation of the study was that it only applied one test session at each stage of the experiment, with a single exposure to each ambiguous stimulus per session. As animal responses can vary considerably between trials due to day to day variation, this may have limited our ability to detect an actual effect in the study. On the other hand, there are also limitations to repeated judgement bias testing as animals learn the ambiguous cues are not rewarded upon repeated testing, and begin to approach ambiguous cues less often[61–64]. In the current study, we observed no effect of day on judgement bias test responses. Further, the study design was balanced so that each treatment underwent the same number of tests. As such, we do not expect repeated testing within the current study to have affected the differences in judgement bias between treatment groups.

Animals allocated to a stress treatment during the previous study[34] were balanced between the Control and Stress groups in the current study, however, it is important to acknowledge the potential effect of earlier stress-inducing treatments on animal responses during the current study. Early life stress or neglect has been shown to impact judgement biases later in life in rats and female goats[65–67]. Even prenatal stress has been found to influence judgement bias in lambs[68]. Thus, it would not be surprising if the treatments applied to animals in the previous study impacted their responses in the current study. However, due to the small number of animals used in the current study, we were unable to further investigate these potential effects. Using the same sheep between studies reduced the time taken for animals to reach the necessary learning criteria for judgement bias testing. However, due to the potentially confounding effect of previous experiments on animal responses, through earlier treatments and habituation to the test procedures, it is suggested that future studies use naive animals for cognitive bias testing, unless it relates to the hypotheses being tested.

It has been proposed that the differences in results between previous judgement bias studies may be due to the duration of the applied stressors, where chronic stress has more consistently caused a shift towards pessimism, while acute stress has yielded mixed results[14,69]. This theory is not supported by the findings of the current study, which found no evidence of a pessimistic judgement bias after chronic stress, or an optimistic bias after acute stress. Further, this is not supported by the results of Verbeek et al.[34], which observed an optimistic bias after chronic stress. However, it should be noted that the durations over which chronic stress have been induced vary greatly

between studies[28–31,34], and that studies intending to induce chronic stress may not have done so successfully. Alternatively, it has been suggested that previous judgement bias studies may have incorrectly inferred the affective states induced by the given treatments, by failing to consider mismatches between the animals' perceived expectations and outcomes during affective state induction or testing [70]. However, this theory is also unsupported by the current study, as well as a recent meta-analysis which aimed to determine whether such mismatches could predict judgement bias test outcomes more accurately than the inferred affective states[71].

The differing effects of stress on judgement bias responses in previous studies could also potentially be explained by differences in assessment methods or the breeds of sheep used in each study. Most studies in sheep have used a spatial discrimination task to assess judgement bias, however the types of positive and negative reinforcers have varied. Each of the studies which found an optimistic bias in stressed animals tested Merino sheep using a dog to reinforce the negative location[32–34]. Studies which found a pessimistic bias tested Romane sheep and used an air blower to reinforce the negative location[28,29,45]. Two further studies which found no effect on judgement bias tested Lacaune sheep using either an air blower[30] or presentation of straw instead of feed[31] as a negative reinforcer. A range of fear studies have observed differences in behavioural reactivity between sheep breeds[56,57,72]. Further, sheep have been shown to exhibit context specific patterns of behaviour, depending on the fear-eliciting stimuli presented[56,57,72]. Together these findings suggest the type of reinforcer and/or breed of sheep used in the experiments may have impacted the direction of the bias observed during previous judgement bias studies. Further studies aiming to examine the differences in judgement biases between sheep breeds or comparing different types of positive and negative reinforcers may shed light on the discrepancies observed between studies.

Overall, the results from this study do not support our hypotheses that pharmacologically-induced acute and chronic stress would cause pessimism and an attention bias towards threatening information in sheep. Further, our results show that increased circulating cortisol concentrations may not fully explain the responses observed during previous chronic stress studies, although the observation of a weak optimistic judgement bias is consistent with some previous results[32,34]. We suggest these findings should not be taken to say that chronic stress does not negatively impact on animal affective states. Instead, we suggest researchers should be cautious in their

interpretation of cognitive bias test results in sheep until the methods are further validated. The study of affective states in livestock is a field of research in its infancy. Contradicting results between studies of judgement bias in particular show that tests for affective state require continued refinement and validation, before they can be considered robust measures of affect as an aspect of animal welfare.

2.5. Methods

2.5.1. Ethical statement

The protocol and conduct of this study were approved by the CSIRO McMaster Laboratory Animal Ethics Committee (Animal research authority #12-30), under the New South Wales Animal Research Act 1985. The protocol was carried out in accordance with all relevant guidelines and regulations. All animals were closely monitored for health and welfare during and after the experiments.

2.5.2. Animals and management

Thirty-two maiden Merino ewes (18-19 months of age, average bodyweight 50.8 ± 3.9 kg) were used in this experiment, which was conducted in 2013 between April and May. All ewes were born on the same experimental farm in Armidale, Australia and were reared as one group after weaning. During training and testing, animals were grazed on pasture located approximately 50 m away from the handling and testing facilities. Sheep were walked between the pasture and testing facilities by a familiar handler. Sheep were fed a familiar supplementary ration of pelleted, lucerne-based concentrate each afternoon following training.

All of the ewes in the current study had previous experience with judgement bias and attention bias testing during the experiment conducted by Verbeek *et al.*[34]. Sheep were returned to the farm flock between the previous and current studies, for a period of 10 months, during which the sheep were managed as per normal farm practices. Readers are asked to refer to Verbeek *et al.*[34] for further details of the previous experiment. Briefly, in the earlier study all ewes were trained to judgement bias testing over a 2 month period, then 30 of the sheep were randomly allocated to control and chronic stress treatment groups (n=15 per group). The chronic stress group was subjected to social isolation and 18hrs of lying deprivation per day over 9 days, while the control animals were housed as a group in a paddock over the same time period. On day 9, all sheep were exposed to an additional acute shearing challenge. All sheep underwent judgement

bias testing twice during the experimental period, before and after shearing. All sheep underwent attention bias testing once prior to shearing.

2.5.3. Experimental design

A summary of the experimental protocol and timeline is given in Table 2.4. Details of each procedure are given in the following sections. As a general overview, sheep were retrained over a period of one month to undertake a judgement bias test. Twenty-eight sheep which successfully reached the training criteria were then pseudo-randomly split into two groups; Stress and Control treatments, balancing for positive location side and cue colour, as well as treatment allocation in Verbeek *et al.*[34] (see below for details). All sheep underwent once daily injections from days 1 to 29 with saline (Control) or synthetic ACTH (Stress). On day 1, the first injection was expected to induce an acute stress state in the Stress group. By days 21 and 22, ongoing injections were expected to have induced a chronic stress state in the Stress group. On day 28, all animals underwent 2 mins of isolation to induce acute stress in the Control group and acute on top of chronic stress (acute-on-chronic) in the Stress group. Judgement bias was assessed at each of these stages. Attention bias was assessed during the chronic stress stage as an additional assessment of affective state. Plasma cortisol response to the injection was assessed on days 1 and 29 of the experiment. Due to unforeseen circumstances, one animal in the Stress treatment group was removed from the study due to injury between days 1 and 21. Data from this animal were removed from the study. Only data for the remaining 27 animals were analysed.

Table 2.4. Experimental design and timeline.

Day	Procedure	Stage of experiment
-20	Begin daily judgement bias training	Training
0	Complete judgement bias training	
1	Begin once daily injections	Acute stress
	Judgement bias test 1	
	Blood sampling	
21	Judgement bias test 2	Chronic stress
22	Attention bias test	
28	Final daily injection	Acute-on-chronic stress
	Isolation box (2 min)	
	Judgement bias test 3	
29	Blood sampling	Blood sampling

2.5.4. Judgement bias arena

The current study used the same judgement bias arena described by Verbeek *et al.*[34], adapted from Verbeek *et al.*[73]. Testing occurred in a 3 x 3 m arena surrounded by 1.5 m high wooden walls (Figure 2.5). Sheep entered the arena through a start box centred along the front wall of the arena and exited through a door on the right (Figure 2.5). The back wall of the arena was divided into five sections with wood panels 40 cm high (hereafter locations). Removable panels were located at the front of each location. A single location was made accessible to the test sheep for each trial or training session, by removing one of the front panels prior to testing. Sliding vertical doors were located behind the two most outer locations, which could be lifted to reveal a dog, lying down quietly (negative reinforcer). Dummy sliding doors identical in appearance to the actual doors were placed behind the three middle locations (ambiguous locations).

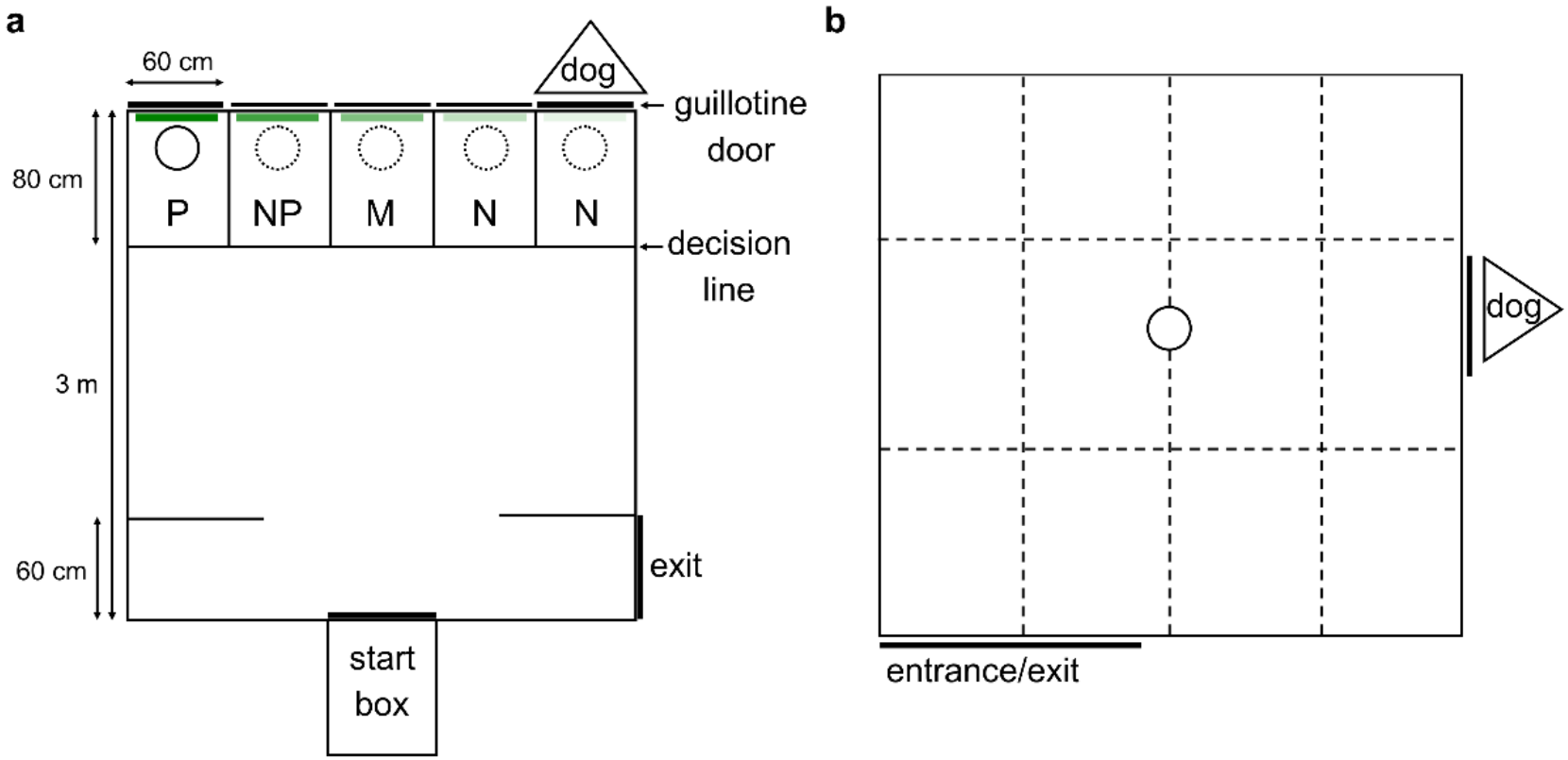

Figure 2.5. Schematic diagrams of the judgement bias (a) and attention bias (b) test arenas.

The judgement bias test arena (a) had five potential bucket and cue locations; (positive (P), near positive (NP), middle (M), near negative (NN) and negative (N)). Only one location was accessible with only one bucket and coloured cue card present at a time during training and testing. The circles indicate the potential positions of a bucket which either contained feed (solid line) or was empty (broken lines). The diagram depicts the set up for sheep assigned to a Positive location on the left with a 95% colour density. The diagram was adapted from Verbeek et al.34. For a photograph of the arena, see Verbeek et al.34, however note that the bucket locations differed between studies. The attention bias arena (b) contained a familiar bowl holding feed (circle). A dog was visible through a window for the first 30 s of the test, then the window was covered and dog was removed.

To facilitate discrimination between locations, five green coloured cue cards which differed in colour density (0, 25, 50, 75 and 95%) were created by adjusting the transparency of the same colour green in Microsoft PowerPoint[73,74]. The cues, printed on A3 paper and laminated, could be attached to the doors at each location in the judgement bias test. Therefore, a total of five cues and locations were used; positive (P, location of a bucket containing feed), near positive (NP), middle (M), near negative (NN) and negative (N, location of the dog). Only one location was accessible (front panel removed), with only one corresponding cue card present at a time during training and testing. Only the accessible location contained a bucket, which was placed in front of the closed sliding door, visible to the sheep. The bucket either contained a small feed reward (approximately 100 g of pelleted lucerne based concentrate, P location) or was empty (all other locations). The bucket was located inside the arena during the current study, while the previous study positioned the bucket outside the arena behind the vertical sliding door at the P location[34] . This change in bucket location was made to better facilitate learning, by providing an additional visual cue for the location being tested.

2.5.5. Judgement bias training

For two days before beginning judgement bias training, sheep were moved into the arena in groups of two for 5 min to re-habituate to the arena, with no buckets or cue cards present. Sheep then underwent one judgement bias training session per weekday for 4 consecutive weeks, following the procedure described by Verbeek *et al.*[34]. During retraining, each sheep was assigned the same Positive location (either the outer left or outer right location in the arena) and Positive cue card (0 or 95% colour density) assigned to them in the previous study. Sheep were first trained to approach their assigned P location, containing a bucket with a small feed reward, while all other locations were inaccessible. Once a sheep left the start box, they were given 3 min to step over the P location decision line with both front feet (go response) and begin consuming feed (Figure 2.5). The decision line was physically visible to the sheep. The sheep were let out of the arena after consuming the feed, or if 3 mins had elapsed without the sheep crossing the decision line (no-go response). Positive training was conducted over the first 2 training sessions. During the first session, sheep entered the test arena 3 consecutive times. During the second session, and for all subsequent training and testing days, sheep entered the test arena 5 consecutive times per session.

During the remaining training sessions, sheep were exposed to the P location for 3 of the arena entries and the N location for 2 of the entries in each training session. The sequences in which P and N locations were presented were randomised between sessions. During training to the N location, an empty bucket was placed in the corner location opposite the assigned P location (right or left) with an alternate cue card (95 or 0% colour density). If a sheep crossed the N location decision line (go response), the sliding door was opened to reveal a live dog sitting quietly. Once the sheep retreated from the bucket, the door was lowered and the sheep was let out of the arena. If the sheep did not approach a location within 30 s of exiting the starting box, the sheep was let out of the arena.

A sheep was considered to be trained when it correctly responded to 14 out of 15 consecutive positive locations and to 8 out of 10 consecutive negative locations. A 'positive response' was considered correct when the sheep stepped over the P decision line (go response) within 10 s of exiting the starting box. A 'negative response' was considered to be correct when the sheep did not cross the N decision line within 30 s of exiting the starting box (no-go response). During training, 4 sheep failed to reach these criterion and were removed from the study.

2.5.6. Pharmacological treatments

All animals received an intramuscular (i.m.) injection once daily for 29 consecutive days (Table 2.4). Control animals were administered 0.5 ml of BP Saline while Stress animals were administered 0.5 ml of Synacthen Depot (0.5mg of tetracosactrin zinc phosphate complex, Mallinckrodt Pharmaceuticals, UK). Synacthen has been used in sheep previously at this dose rate to chronically elevate plasma cortisol concentrations, mimicking the physiological response of the HPA axis to stress[43].

Injections for each animal were staggered by approximately 10 mins on days 1, 21, 22 and 28 to allow for judgement bias and attention bias testing, beginning and ending at approximately 8:00 AM and 1:00 PM respectively. On all other days, injections were administered to all animals at 8:30 AM. The injection site was rotated each day between the rump, thigh and dorsal muscles on the left and then right sides of the animals.

2.5.7. Blood sampling and analysis

Blood sampling occurred on days 1 and 29 for assessment of plasma cortisol concentration as an indicator of HPA axis response. Blood samples were collected via jugular venepuncture into heparinised vacutainers on each of the sampling days at four consecutive time points. On day 1 (acute stress), samples were taken at time 0 (baseline prior to Synacthen injection) then at 0.5, 1 and 1.5 hrs post injection to evaluate the acute cortisol response to the Synacthen injection. Judgement bias testing occurred immediately prior to collection of the 1hr post injection blood sample. On day 29 (chronic stress) samples were taken at time 0 (baseline prior to Synacthen injection) then at 1, 4 and 8 hrs post injection to evaluate the cortisol return to baseline.

Blood tubes were kept on ice until they could be processed. The blood samples were centrifuged at 2000x *g* for 15 min at 5°C, then the plasma was distributed into 2ml aliquots that were stored at -80°C until analysis for cortisol concentration. Plasma cortisol concentrations were measured using a commercial radioimmunoassay (Orion Diagnostica, Espo, Finland), previously validated for ovine plasma cortisol[75]. The intra-assay and inter-assay coefficients of variance (CV) for quality controls containing 24.9, 51.6 and 104.9 nmol/L of cortisol were 3.4, 5.7 and 7.7% and 6.5, 4.5 and 12.6% respectively.

2.5.8. Judgement bias assessment

Judgement bias was assessed 3 times during the experiment, on days 1, 21 and 28, as described by Verbeek *et al.*[34]. Testing occurred 1 hr post injection for each given day, where treatment groups were spread evenly throughout the day. During testing, each sheep was released into the arena five consecutive times with each of the 5 locations presented once. The trained locations were reinforced with food (P) or exposure to the dog (N), however the ambiguous locations were not reinforced. Presentation of the locations occurred in one of the following orders: P, NP, M, NN, N (P first) or N, NN, M, NP, P (N first). Half the animals were pseudo randomly assigned to each start location on each day of testing, balancing between treatment groups, so that presentation order for each animal may have differed between test days. After leaving the start box, sheep were given a maximum of 30 s to respond for each location before being let out of the arena. The go or no-go responses were recorded from a video screen in real time. All tests were continuously recorded by two video cameras and a 16 channel Digital Video Recorder.

Immediately prior to judgement bias testing on day 28, each sheep was moved into an isolation box for 2 mins to induce acute isolation stress[76,77]. The isolation box consisted of a 1.5 x 0.5 x 2 m wooden box with an open roof covered by shade cloth.

2.5.9. Attention bias assessment

Attention bias was assessed as described by Verbeek *et al.*[34], using a similar method to that validated by Lee *et al.*[24]. The test was conducted in a 4 x 4 m arena surrounded by a 1.5 m high fence covered in opaque black cloth (Figure 2.5). The concrete floor was divided into 12 rectangular zones (1 x 1.2 m) marked on the ground with white paint. A familiar bowl containing 200g of concentrate pellets was placed in the centre of the arena. A small window (77 x 58 cm) was located on one wall behind which a dog was sitting quietly (different from the dog used in judgement bias training). Each sheep was tested individually for a total of 3 min. The dog was visible to the sheep for the first 30 s of testing, then the window was covered by a retractable opaque cover and the dog was removed for the remainder of the test. All tests were continuously recorded by a video camera.

The following behaviours were recorded in real time or from video footage; time spent looking towards the dog or covered window with binocular vision (attention to dog[24]), duration with the head at or above shoulder height (vigilance[24,78]), latency to eat the food, number of zones crossed with both front legs and duration spent immobile with no movements of the head or body for greater than 3 s. Latency to eat was recorded from the beginning of the test. All other behaviours were recorded separately for the durations with and without the dog present. Only data without the dog present are presented, as this is the period where responses are to a perceived threat, as opposed to an actual threat posed by the presence of a predator, and thus better reflect anxiety states assessed using attention bias[24,34]. Between animals, all uneaten food was removed and the bowl refilled with new pellets so that the amount of food eaten by each sheep could be calculated. The arena was also cleaned after eliminations to avoid the influence of odour cues on further subjects. Vocalisations, eliminations and foot stamps were also recorded, however these data were not further analysed due a low number of occurrences during the test.

2.5.10. Statistical analysis

The data were analyzed using R version 3.5.1[79]. All model residuals were checked for normality and homoscedasticity using Shapiro Wilks test for normality and visual assessment of Q-Q and residuals vs. fitted values plots where appropriate.

An information criterion based approach was used for model selection to determine which models most appropriately fit the data within a given set of models[34,80,81]. A maximal model was fitted for each analysis, then the parameters for models containing each possible combination of the predictors and their interactions were calculated using the "dredge" function from the package "MuMIn"[82]. We examined both the Akaike Information criterion adjusted for small samples sizes (AICc) and the Bayesian Information criterion (BIC), which more heavily penalizes model complexity. Due to this heavier penalisation, models selected by BIC are simpler and emphasize the key predictors, whereas model selection by AICc is typically preferred for model predictions[83]. Thus, our interpretation of results relied more heavily on BIC, however AICc is also presented. Models were selected based on Δi (AICc or BIC difference relative to the smallest AICc or BIC value in the given set of models) and w_i (Akaike or Schwarz weight), indicating the relative weight of evidence for model i being the best fit for the data within the given set of models. Where multiple models had a $\Delta i < 2$, those models could be considered equally likely to be the best fit for the data, however models with a $\Delta i < 4$ also have considerable empirical support[84].

2.5.10.1. Judgement bias testing

The go/no-go responses during judgement bias trials were analysed using generalized linear mixed effects models using the package "lme4"[85], as described by Gygax *et al.*[81]. Fitted models included all go/no-go responses across the 3 test days. The maximal model included fixed effects of location, treatment, day and all possible interactions between those variables. Location was fitted as a factor rather than a continuous variable, due to evidence of a non-monotonous pattern in go responses across locations. The random effects for each model were day nested in animal (id) to account for repeated measures across test days.

2.5.10.2. Attention bias testing

Latency to eat data were analysed using a Cox's proportional hazards model using survival analysis, as described by Monk *et al*.[25 86,87]. Animals which failed to eat within 180 s were deemed as censored results. Attention to dog, vigilance, food eaten and time spent immobile were analysed using linear models. Zones crossed data were analysed using a negative binomial general linear model, due to evidence of over-dispersion in the data. The maximal model for attention bias data fitted treatment group as a fixed effect only.

2.5.10.3. Cortisol response

Cortisol data were analysed using linear mixed effects models. The maximum models for each day of sample collection included treatment, time of sample collection and their interaction as fixed effects and sheep id as a random effect. Data from one animal in the Control group on day 1 and two animals from the Stress group on day 29 were removed from the analysis due to abnormally high baseline cortisol concentrations (>150 nmol/L). Thus, data for n=26 and n=25 animals were analysed for days 1 and 29 respectively.

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2.8. Author contributions

C.L contributed to the generation of hypotheses, design and conduct of the experiment and revision of the manuscript. C.L attained funding for the study. S.B contributed to the coordination and conduct of the experiment. J.M conducted the statistical analyses, wrote the main manuscript text and prepared the figures and tables. All authors reviewed the manuscript.

2.9. Competing interests

The authors declare no competing interests.

2.10. Data availability

The datasets generated during the current study are available in the CSIRO Data Access Portal (DAP) [https://doi.org/10.25919/5c8725f046880].

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Chapter 3

Towards a more practical attention bias test to assess affective state in sheep

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3.1. Abstract

Tests for attention bias potentially offer more rapid assessment of affective state in animals than existing cognitive methods. An attention bias test has previously been developed for sheep and validated as a measure of anxious states. The 3 minute test assessed behavioural responses of sheep in an enclosed arena after brief exposure to the threat of a dog. Experiment 1 of the current study aimed to refine the previously developed method, removing the need for a habituation period and shortening the test duration. Sheep were given either an anxiolytic drug, an anxiogenic drug or a control treatment prior to testing to induce contrasting affective states. Differences in behaviour were found between the treatment groups within the first 45s of the test, indicating the original test duration could be shortened from 180 s. During testing, 36 of 40 animals in the control and anxiolytic groups ate the novel feed offered in the test, indicating it is not necessary to habituate animals to a feed container. Experiment 2 aimed to confirm the responses measured in the test were primarily towards the dog rather than other aspects of the test environment. Sheep exposed to an empty window at the beginning of the test behaved differently to those which were exposed to a dog, indicating sheep behaviour in the test is at least partially a response to the dog. A third group of sheep were also tested with the dog immediately after having small data loggers attached to their necks. Behaviour of these sheep did not differ from the sheep tested without loggers, indicating data logger attachment did not impact their behaviour in the test. In both experiments, treatments did not appear to modify activity (zones crossed), which we propose indicates the test was primarily detecting valence of the affective state rather than arousal.

3.2. Introduction

As animal welfare becomes an increasingly important consideration for society, we need to develop more practical measures of welfare which take into account the emotional or affective states of animals. Affective state is currently understood as a position of the animal within an “affective space” delineated by axes described as valence and arousal [1,2]. Arousal describes the physiological activation of the state while valence describes whether the state is hedonically positive or negative. Negatively valenced states may be particularly strong indicators of poor welfare, however it can often be difficult to determine the valence of an affective state using behavioural and

physiological indicators. For example heart rate can be used as an indicator of arousal but increases in heart rate occur across a range of emotional valences which may be positive (e.g. meeting a sexual partner), negative (e.g. exposure to a predator) or neutral (e.g. increased locomotion) [3]. An alternative approach for assessing valence is to use a concept termed cognitive bias, where the affective state of an animal alters the way it processes information, which in turn affects the behavioural responses of the animal to its environment [3]. By measuring these variation in responses between individuals or treatment groups in defined behavioural tests paradigms we can make inferences about the underlying affective state of an animal [4]. The form of cognitive bias most widely studied in non-human animals thus far is judgement bias, in which the emotional state of the animal influences its interpretation of ambiguous situations [3]. Judgement bias tests have been used to study both positively and negatively valanced affective states in a range of animal species including rats, dogs and sheep [3,5,6]. The majority of judgement bias tests follow a paradigm developed by Harding et al. [7], which initially involves training animals to discriminate between and respond to a positive and a negative stimulus. The requirement for a training period means typical judgement bias tests are impractical as applied measures of welfare for livestock in production environments. Furthermore, not all animals successfully learn the test procedure, and thus a portion of the population of interest need to be excluded from assessment of judgement bias. A further limitation of the judgement bias methodology has been the difficulty of differentiating between the influence of arousal from the influence of valence on the response of the animal to ambiguous cues.

Another type of cognitive bias called attention bias is the tendency to process certain types of information before others [8], and may offer a more rapid method for the assessment of certain affective states in animals [3]. In human studies, individuals in high states of anxiety show greater attentional biases towards threatening stimuli than non-anxious individuals [e.g. 9,10]. Attention biases have also been found in non-human primates [11], starlings [12] and sheep [13]. Starlings denied access to water baths responded to an alarm call with increased vigilance and less willingness to feed than those which were able to bath. These findings were interpreted as birds being more anxious due to compromised flight ability and consequently directing more attention towards the threatening cue. This test was adapted for use with sheep, where the threatening cue was the presence of a dog for 10 s at the beginning of the test [13]. The test was validated as a measure of anxiety by pharmacologically manipulating the

anxious states of sheep. Sheep in an induced anxious state responded with increased vigilance, less willingness to feed and paid more attention to the previous location of the dog than sheep in a reduced anxious state. While the method was more rapid than existing judgement bias tests, it still required prior training to familiarise sheep to a feed bucket and the duration of the test was 3 min per animal which may limit its use in applied contexts. Removal of the need to train sheep to the bucket and reduction in the duration of the test would provide the basis for a more practical method, potentially applicable in a range of contexts such as on-farm. Additionally, Lee et al. [13] did not include a control group that were not exposed to the dog, and thus it was unclear whether the responses measured in the attention bias test were toward the dog or due to another aspect of the test such as isolation or novelty. It was suggested further use of the attention bias test should include a treatment without the dog to address this question.

The current study aimed to refine and further validate the attention bias test developed by Lee et al. [13], to measure affective states in sheep. Experiment 1 aimed to make the test more practical by shortening the duration and eliminating the need for training. We hypothesised that sheep would be willing to eat a novel food in a novel environment and that differences in behaviour between the treatment groups would be detectable in under 3 min. Experiment 2 aimed to confirm that the responses being measured during the test were directed towards the threat. We hypothesised that sheep exposed to an empty window would behave differently to those exposed to a dog, indicating the observed behaviours were at least partially due to the presence of a threat. A secondary aim for experiment 2 was to investigate whether attachment of small data loggers to the animals immediately prior to testing would alter their behaviour in the test. It was hypothesised that behaviour of animals in the Logger group would not differ from the control group, creating opportunities for automation of behavioural measurement without the need for habituation. Both experiments assessed general activity of sheep as a measure of arousal in addition to behavioural measures of attention bias.

3.3. Materials and methods

3.3.1. Animal ethics

The protocol and conduct of the experiments were approved by the CSIRO McMaster Laboratory Animal Ethics Committee and the University of New England Animal Ethics Committee, under the New South Wales Animal Research Act 1985.

3.3.2. Experiment 1

3.3.2.1. Animal and treatment details

Sixty 5-month-old castrated male Merino lambs, born and raised at pasture, with average bodyweight 29.9 ± 3.3 kg were used in this experiment. Sheep had prior experience of supplementary feeding with oaten chaff and a pelleted ration containing comminuted lucerne, but had never been supplementary fed with hay. All sheep had undergone routine handling previous to the current experiment and were therefore familiar with the presence of humans. The sheep were randomly allocated to one of three treatment groups, balancing for bodyweight (*n*=20 per treatment): 1) anxiolytic (Diazepam, 0.1 mg/kg i.v.), 2) anxiogenic (meta-Chlorophenylpiperazine (m-CPP), 2 mg/kg i.m.) and 3) Control (receiving saline i.m.). These dose rates have been used on sheep in previous studies to alter emotional states with no observable adverse impact on the animals for diazepam [14] or m-CPP [13,15]. m-CPP is a serotonin-2A (5-HT_{2A}) receptor agonist [16,17] while diazepam works through activation of GABAergic receptors [14,17]. Drugs were administered 30 min prior to testing. It was expected that m-CPP and diazepam would increase and decrease anxiety respectively, causing changes in vigilance and feeding behaviours during the test, but that they would not have a sedative effect on the animals (as indicated by no differences in locomotory behaviour and vocalisations between groups).

3.3.2.2. Attention bias testing

The current study used the same testing arena (Figure 3.1 and S1 Video) and treatment injection protocols as Lee et al. [13]. The test arena comprised a 4 x 4.2 m yard with opaque walls 1.8 m high. On one side of the arena, a retractable opaque cover was placed in front of a window (77 cm x 58 cm) behind which a dog (kelpie cross border collie) was located. At the beginning of the test the dog sitting quietly was visible to the sheep through the window. Once a sheep entered the arena, the door was shut behind it, and a timer was started when the sheep made visual contact with the dog. After 3 s, the opaque cover was lowered in front of the window then the dog was removed. The cover took 3 s to lower such that the dog window was fully covered with no part of the dog visible by approximately 6 s. The test ended 3 min after the sheep had first made visual contact with the dog. Animals were tested individually in a random order, ensuring equal distribution of treatments across the day. Prior to testing, feed was withheld from

sheep overnight to minimise variation in appetite during testing, but sheep were given ad lib access to water.

The attention bias test used in the current study differed from that used by Lee et al. [13] in two key ways. Firstly, as the current study aimed to remove the habituation period used in the original protocol, sheep were presented with approximately 1.5 kg of lucerne hay at the centre of the test arena rather than a familiar bucket containing feed. It was expected sheep would be more likely to eat hay without training than pellets from an unfamiliar bucket, even though hay was a novel feed for this group of animals. Second, the period of exposure to the dog was shortened from 10 s in the previous study to reduce the total test duration. It was expected 3 s would be long enough for sheep to recognise the dog as a potential threat.

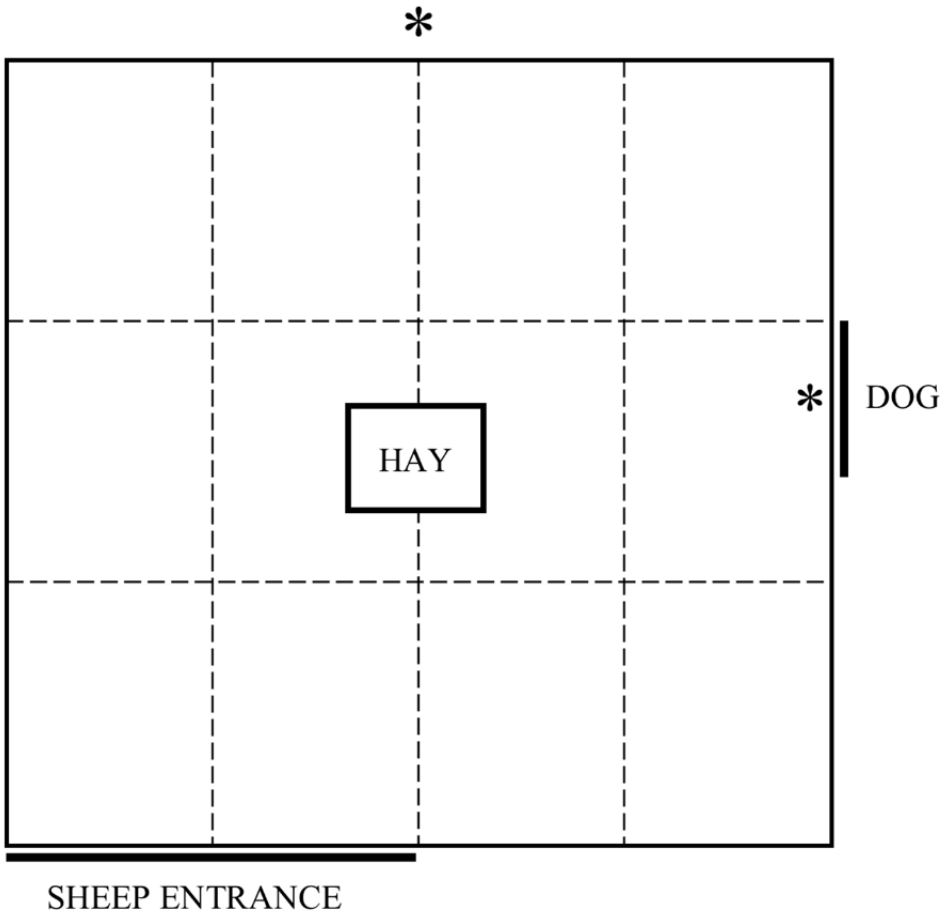

Figure 3.1. Diagram of the attention bias test arena comprising a 4 x 4.2 m yard with opaque walls 1.8 m high and hay placed in the centre.

Dashed lines represent a 1 x 1.4 m grid painted on the ground. "*" denotes the positions of 2 cameras. A dog was visible for the first 3 s of the test, then the window was covered.

3.3.2.3. Behavioural measurements

Latency to eat from first visual contact with the dog and vocalisations were recorded on the day of testing by observers blind to the treatment groups (one observer per behaviour). Duration of vigilance behaviour, duration of attention towards the window (attention to threat), total time spent eating and number of zones (grids) crossed were later collated from video footage by an observer blind to the treatment groups. To determine whether the test could be shortened, duration of vigilance, latency to eat, attention to threat and zones crossed were also determined for a 45 s time period after behavioural observations began. This time period was the shortest of 30, 45 and 60 s time periods to give significant results in preliminary data analysis.

Vigilance was defined as having the head at or above shoulder height [13,18]. If the sheep shook their head or entire body, this was not considered vigilance behaviour, regardless of head position relative to shoulder position. Attention towards the threat was defined as the amount of time spent with the head oriented toward the closed dog window. Attention to threat was measured for the first 60 s of the test [13]. An eating event started when the sheep began consuming the hay. The eating event continued while the sheep was chewing provided that the head stayed within approximately 20cm of the feed and the sheep remained non-vigilant. Once the sheep became vigilant or moved away from the hay, this was considered to be the end of the eating event, even if the sheep continued chewing. A sheep was considered to have crossed one zone (marked grid section, Figure 3.1) when both front legs were placed into a new zone or one was placed in the zone and the other was on the line. Sheep could simultaneously be vigilant, attentive to the window and crossing zones. Feeding behaviour was mutually exclusive with vigilance and zone crossing. Examples of each behaviour are given in the supporting information (S1 Video).

3.3.3. Experiment 2

3.3.3.1. Animal and treatment details

Sixty 12-month-old castrated male Merino lambs with average bodyweight 38.5 ± 3.1 kg were used in this experiment. The lambs were born and raised at pasture with minimal exposure to humans beyond routine management practices and no experience with the attention bias test. Prior experience of supplementary feeding was unknown for this group. The sheep were randomly allocated to one of three treatment groups

(*n*=20 per treatment): 1) dog in window at commencement of test as described for experiment 1 (Dog), 2) no dog in window at the beginning of the test (No-dog) and 3) dog in window at beginning of test and sheep fitted with data loggers attached to their necks (Logger).

3.3.3.2. Data loggers

HOBO® Pendant G acceleration data loggers were used (dimensions: 58 mm x 33 mm x 23 mm, weight: 18 g) (Onset Computer Corporation, Pocasset, MA, USA). The HOBO® Waterproof Shuttle and HOBOware® Pro software (version 3.7.8) were used for programming and reading the HOBO loggers (Onset Computer Corporation, Pocasset, MA, USA). Data loggers were programmed to record tilt and acceleration at a logging interval of 0.25 s (4 Hz, measurement range: ±3 gravitational force (*g*); accuracy: ±0.075 *g* at 25°C). Immediately prior to testing, loggers were activated using a magnet and then attached to the back of the neck of the sheep using a small strip of Velcro®. Velcro® provided a fast, easy method of attachment, requiring only a short period of restraint and a small point of contact with the sheep when compared to other methods such as a collar or halter. Wool on the back of the neck was parted and the logger was nestled as close to the skin as possible so that movement of wool had minimal impact on logger angle. Sheep in all groups had been shorn 5 months prior to testing. The current paper does not discuss the suitability of these data loggers for measuring vigilance, it reports on whether attachment of the loggers altered animal behaviour during testing.

3.3.3.3. Attention bias testing

The Dog group underwent the attention bias test as described for experiment 1. The No-dog group underwent the same test, however sheep were exposed to an empty window instead of a dog at the beginning of the test. The opaque cover was lowered 3 s after the sheep had looked toward the empty window, unless it was unclear when the sheep looked at the window, in which case the cover was lowered after 10 s. The Logger group underwent the test as for the Dog group with exposure to the dog, however sheep had data loggers attached immediately prior to testing. The same operator attached all data loggers, keeping the location of the logger on the neck consistent between animals. Across all treatment groups, animals were caught in a predetermined order and moved to the entrance of the attention bias test prior to testing. Sheep in the Logger group were

held at the entrance for up to 30 s longer than sheep in other treatment groups while the logger was attached. The current study therefore aimed to determine whether the combined effect of additional restraint time and presence of the logger during testing impacted sheep behaviour during the test.

3.3.3.4. Behavioural measurements

The same behaviours were measured for experiment 2 as for experiment 1, except for vocalisations which were not recorded, after vocalisations were found to be non-significant in experiment 1 (see Results section). Additionally, whether the animal sniffed the dog window was recorded. Behaviours were collated from video footage using The Observer XT 12.0 (Noldus Information Technology). Each of the behaviours were recorded by a single observer blinded to treatment group. To determine whether treatment effects were evident for a shortened version of the test, duration of vigilance and latency to eat were also determined for 60 s and 45 s time periods after behavioural observations began.

3.3.4. Statistical analysis

Data were analysed in R version 3.2 [19]. P values less than 0.05 were considered to be significant, values where $0.1>P>0.05$ were considered a tendency towards significance.

3.3.4.1. Experiment 1

Vigilance data were analysed by Kruskal-Wallis non-parametric one-way analysis of variance (ANOVA) as the parametric model residuals did not meet normality assumptions and could not be improved by transformation [20]. Normality of parametric model residuals were checked using visual assessment of normal Q-Q plots and the Shapiro-Wilk test of normality. Post hoc multiple comparison tests were performed using the package pgirmess [21]. Attention to threat data met normality assumptions and were analysed using a one-way ANOVA, fitting treatment and test order as fixed effects. Test order was not found to be a significant predictor and was subsequently dropped from the model. Zones crossed and vocalisations were analysed using generalised linear models with a quasi-poisson distribution, fitting treatment and test order as fixed effects. Test order was found to be non-significant and was dropped from both models. Zones crossed data were analysed in the same way for the 45 s time

period. Use of a quasi-poisson distribution was necessary as data were found to be over-dispersed, violating the assumptions for a poisson distribution.

Latency to eat data were analysed with Cox's proportional hazards model using survival analysis [22,23]. Any animal that failed to eat within 180 s was deemed as a censored result, recorded as a 'survival' incidence in the traditional way survival analysis is used. A two stage approach was needed because none of the m-CPP sheep ate the feed during the test, and so no hazard function could be predicted for this group. Firstly, the 'survdiff' function was used to assess differences between the survival curves for each of the three drugs. This function generates a log-rank test that compares the curves. A cox proportional hazards model was then conducted on the Diazepam and Control groups only. This method considers explanatory variables that affect the hazard of an event happening. From the fitted model, hazard ratios can be predicted to investigate the effects of different factors on whether or not an animal was likely to eat the feed. Hazard ratio values are positive values ranging from zero to infinite. A hazard ratio of >1 indicates a higher likelihood of eating the feed compared with the reference level for each categorical explanatory variable. Values between 0 and 1 indicate a lower likelihood of eating the feed compared with the reference level. Note that the use of the term hazard in survival analysis does not necessarily imply a deleterious outcome and, in this study, the hazard refers to the sheep eating the feed. The survival analyses were performed in the same way for the 45 s time period. Time spent eating was analysed by Kruskal-Wallis non-parametric one-way ANOVA.

3.3.4.2. Experiment 2

Data for vigilance, attention to threat and time spent eating were analysed in the same way as for experiment 1. Latency to eat data did not require a two stage approach and were analysed using only the cox proportional hazards model as described for experiment 1, fitting treatment and test order as fixed effects. Analyses were performed in the same way for the 60 and 45 s time periods for vigilance and latency to eat.

Zones crossed data were analysed using a generalised linear model with a quasi-poisson distribution as described for experiment 1, fitting treatment and test order as fixed effects. Test order was retained in the model. Two strong outliers were identified in the zones crossed data, one from the Dog group and one from the Logger group (80 and 96 zones crossed respectively, overall mean was 22). While these appeared to be valid

responses from the sheep, they had high leverage within the dataset and so the zones crossed data were analysed twice, once including these outliers and once excluding them. Two-sided Fisher's exact tests were conducted to compare how many animals in each treatment group sniffed the dog window during the test.

3.4. Results

3.4.1. Experiment 1

Differences were found between treatment groups for vigilance and attention to threat (Table 3.1). The m-CPP group spent more time displaying vigilant behaviour than the other groups. The Diazepam group displayed the lowest vigilance, however this was not significantly lower than the Control group (observed difference 7.2 < critical difference 13.2). The Diazepam group spent the least amount of time looking towards the dog window, while the m-CPP and Control groups did not differ. These findings were consistent for the 45 s test. There was no effect of treatment on zones crossed during the 180 s test, however animals in the Diazepam group crossed fewer zones during the first 45 s of the test (Table 3.1). There was no effect of treatment on vocalisations (X^2=3.22, df=2, P=0.2).

No sheep in the m-CPP group fed during the test, while only 2 and 5 animals failed to eat during the 180 s and 45 s time periods respectively for both the Control and Diazepam groups (log-rank P<0.001). The hazard ratios of the Diazepam and Control groups did not differ, indicating they were likely to begin eating the food at a similar rate for both the full length (180 s) and shortened (45 s) tests (Table 3.2). The Kaplan-Meier plot for the 45 s test shows the time of each animal's first feeding event and the proportion of sheep which failed to eat (Figure 3.2). The m-CPP group spent the least time eating while the Diazepam group spent the most time eating, however the difference between the Control and Diazepam groups was not statistically significant (Table 3.1).

Table 3.1. Mean ± s.e.m. behavioural responses of sheep during the attention bias test in experiment 1.

Behavioural measure	Diazepam	Control	m-CPP	Test value	P value
Vigilance (mean rank duration) (180 s test)	19.3 ± 3.6^{a} (108.4)	26.5 ± 3.2^{a} (132.6)	45.8 ± 2.2^{b} (162.6)	H=24.5	<0.001
Vigilance (mean rank duration) (45 s test)	20.7 ± 3.7^{a} (24.8)	26.2 ± 3.4^{a} (29.2)	44.7 ± 2.2^{b} (35.8)	H=21.2	<0.001
Attention to threat (s) (60 s test)	24.9 ± 2.0^{a}	34.7 ± 2.0^{b}	36.2 ± 2.0^{b}	F=9.82	<0.001
Attention to threat (s) (45 s test)	19.7 ± 1.5^{a}	26.3 ± 1.5^{b}	28.0 ± 1.5^{b}	F=8.17	<0.001
Zones crossed (180 s test)	3.1 ± 0.1 (22.6)	3.3 ± 0.1 (26)	3.3 ± 0.1 (26)	X^2=0.76	0.68
Zones crossed (45 s test)	1.4 ± 0.2^{a} (4.0)	2.2 ± 0.2^{b} (8.0)	2.1 ± 0.1^{b} (8.6)	X^2=10.9	0.004
Time eating (mean rank duration) (180 s test)	43.4 ± 3.1^{a} (54)	35.6 ± 2.7^{a} (26.7)	12.5 ± 0.0^{b} (0)	H=36.2	<0.001
Time eating (mean rank duration) (45 s test)	41.3 ± 3.5^{a} (13.9)	34.7 ± 3.2^{a} (7.6)	15.5 ± 0.0^{b} (0)	H=27.0	<0.001

Different superscripts (^{a,b}) within rows indicate a significant difference between treatments as determined using post-hoc analyses. Mean rank durations are given for vigilance and time eating, raw means (s) are given in parentheses. Least-squares means are given on the log scale for zones crossed, back-transformed means are given in parentheses.

Table 3.2. Hazard ratios for latency to eat in the 180 s and 45 s attention bias test as affected by treatment in experiment 1.

Test duration (s)	Treatment	Coefficient[1]	SE (coeff)	Hazard ratio[2]	P value
180	**Control**	**Reference**			
	Diazepam	0.04	1.04	1.04 (0.51 - 1.57)	0.9
45	**Control**	**Reference**			
	Diazepam	0.00	0.37	0.99 (0.54 - 1.60)	0.99

Hazard ratios indicate likeliness to eat the feed compared to the reference treatment. A hazard ratio <1 indicates a reduced hazard, >1 indicates an increased hazard, 1 = no effect.
[1] Regression coefficient from the Cox-proportional hazards model
[2] 95% CI in parentheses.

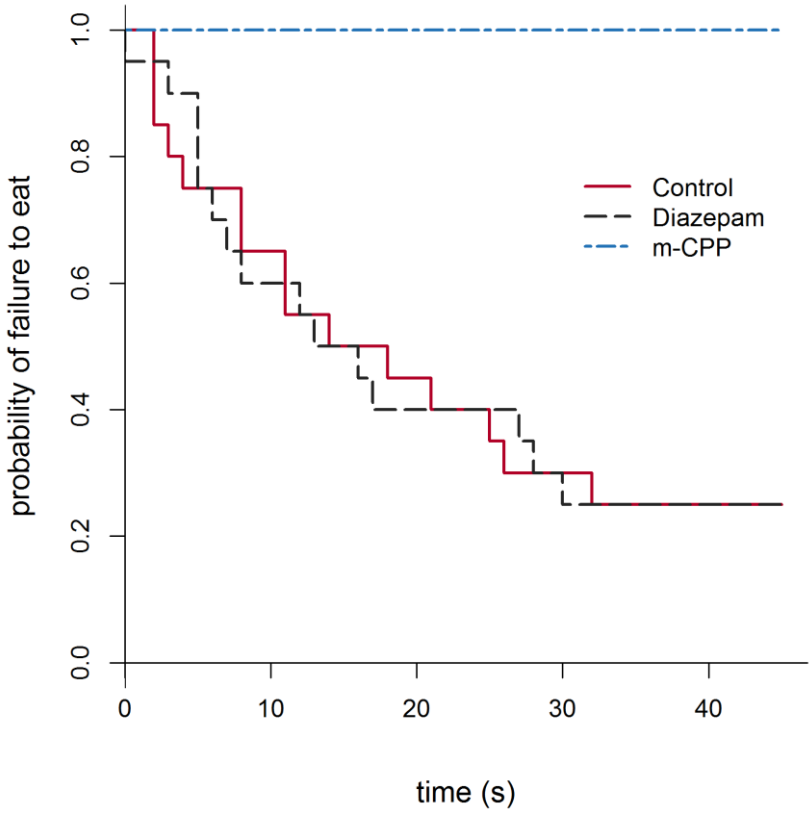

Figure 3.2. Kaplan-Meier curves for latency to eat during the 45 s time period in experiment 1.

Every time an animal initiated its first eating event, the proportion of sheep which failed to eat on the Y axis drops.

3.4.2. Experiment 2

The No-dog group spent significantly less time displaying vigilance behaviour than the Dog and Logger groups during the 180 s test (Table 3.3). However, a significant difference was no longer seen between the Dog and No-dog groups when the test was shortened to 60 or 45 s (observed differences 10.12 and 7.1 respectively < critical difference 13.22). Vigilance did not differ between the Dog and Logger groups at any time period. Attention to threat did not differ between any treatment groups (Table 3.3). Sheep in the No-dog group spent the most time eating. While an overall treatment effect was found for time eating (P=0.03), the observed differences between the No-Dog vs. the Dog (12.0) and Logger (12.7) groups were less than the critical difference (13.22) in post-hoc analyses (Table 3.3).

Table 3.3. Mean ± s.e.m. behavioural responses of sheep during the attention bias test in experiment 2.

Behavioural measure	No-dog	Dog	Logger	Test value	P value
Vigilance (mean rank duration) (180 s test)	20.0 ± 3.0^{a} (147.0)	35.7 ± 3.6^{b} (165.4)	35.9 ± 4.1^{b} (163.3)	H=11.0	0.004
Vigilance (mean rank duration) (60 s test)	22.4 ± 3.8^{a} (51.2)	32.5 ± 3.3^{ab} (56.0)	36.7 ± 4.0^{b} (56.1)	H=7.2	0.03
Vigilance (mean rank duration) (45 s test)	24.1 ± 4.1 (38.9)	31.2 ± 3.3 (42.4)	36.2 ± 3.9 (42.6)	H=5.0	0.08
Attention to threat (s) (60 s test)	38.1 ± 1.8	39.5 ± 1.8	40.7 ± 1.8	F=0.48	0.62
Time eating (mean rank duration) (180 s test)	38.7 ± 3.5 (9.6)	26.7 ± 3.7 (2.7)	26.1 ± 3.7 (3.0)	H=7.0	0.03

Different superscripts (^{a,b}) within rows indicate a significant difference between treatments as determined using post-hoc analyses. Mean rank durations are given for vigilance and time eating, raw means (s) are given in parentheses.

The hazard ratios for the Dog and Logger groups did not differ, indicating they were likely to first eat the food at a similar rate for both the full length (180 s) and shortened (45 s) tests (Table 3.4). For the 180 s test, the No-dog group was approximately 3 times more likely to eat the hay than the Dog group (P=0.007). This was also evident when the test was shortened to 60 s (P=0.042), however when shortened to 45 s there was only a tendency for the Dog and No-dog hazard ratios to be different (P=0.066). The Kaplan-Meier plot for the 60 s test shows the time of each animal's first feeding event and the proportion of sheep that failed to eat (Figure 3.3).

Table 3.4. Hazard ratios for latency to eat in the 180, 60 and 45 s attention bias tests as affected by treatment in experiment 2.

Test duration	Treatment	Coefficient[1]	SE (coeff)	Hazard ratio[2]	P value
180	**Dog**	**Reference**			
	No-dog	1.05	0.39	2.87 (1.33-6.19)	0.007
	Logger	0.03	0.43	1.03 (0.45-2.37)	0.95
60	**Dog**	**Reference**			
	No-dog	1.10	0.54	3.00 (1.04-8.64)	0.042
	Logger	-0.19	0.67	0.83 (0.22-3.08)	0.777
45	**Dog**	**Reference**			
	No-dog	1.25	0.68	3.48 (0.92-13.14)	0.066
	Logger	0.33	0.76	1.38 (0.31-6.18)	0.671

Hazard ratios indicate likeliness to eat the feed compared to the reference treatment. A hazard ratio <1 indicates a reduced hazard, >1 indicates an increased hazard, 1 = no effect.
[1] Regression coefficient from the Cox-proportional hazards model
[2] 95% CI in parenthesis.

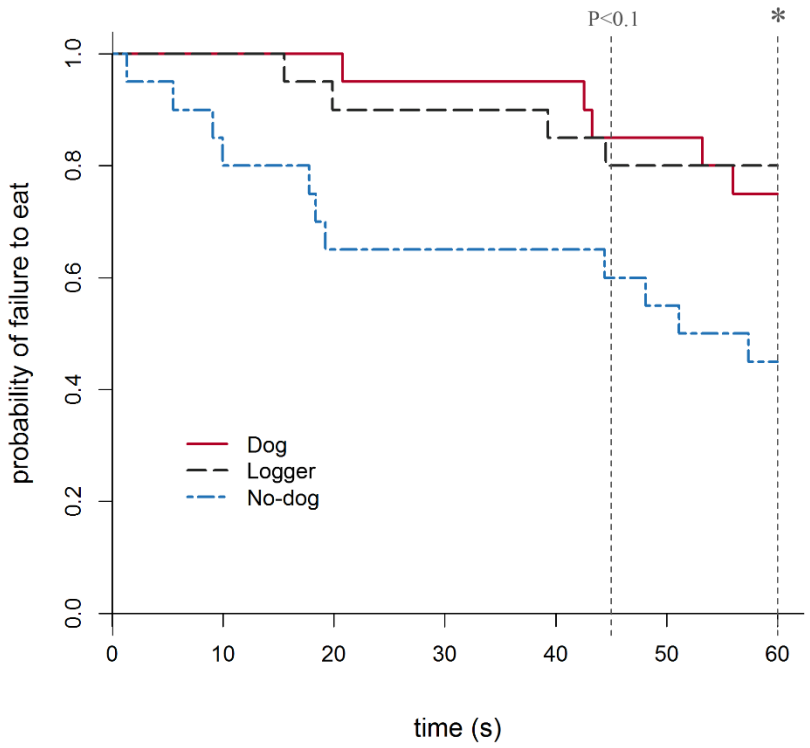

Figure 3.3. Kaplan-Meier curves for latency to eat during the 60 s time period in experiment 2.

Every time an animal initiated its first eating event, the proportion of sheep which failed to eat on the Y axis drops. The effect of treatment group was significant at 60 s (*) and tended towards significance at 45 s (P<0.1).

When excluding the two outliers, the number of zones crossed were higher for the No-dog group than the Dog group (t=2.81, P=0.007), however when outliers were included in the model this was not significant (t=1.42, P=0.16). Zones crossed did not differ between the Dog and Logger groups (t=0.54, P=0.59). More animals in the No-dog group sniffed the window cover than the Dog and Logger groups (11, 3 and 3 animals respectively, fishers exact test, P=0.008).

3.5. Discussion

The results of the current study support our initial hypotheses that 1) sheep would be willing to eat a novel feed during the test, 2) the test duration could be shortened from 180s, 3) the behaviours measured in the test were at least partially a response to the dog and 4) attachment of data loggers prior to testing did not alter animal behaviour in the test. Experiment 1 demonstrated that sheep do not require training to a feed bucket prior

to testing, as 36 of 40 animals in the Control and Diazepam groups ate the novel feed presented in the test. This means the test can be conducted in a single day, making it a more practical measure of anxious states in sheep. It should however be noted that the sheep were withheld from feed overnight prior to testing, which may be necessary so they are motivated to eat, and to reduce variability in appetite during the test. Furthermore, the treatment differences for vigilance, latency to eat and attention to threat were consistent across the 45 s and 180 s time periods, indicating the test can potentially be shortened to less than 1 min per sheep. This allows for a greater number of animals to be tested across a day, making the test more applicable to larger populations.

Sheep exposed to a dog were more vigilant and less likely to eat in the attention bias test than those which were exposed to an empty window, supporting our hypothesis that the behaviours measured in the test were at least partially a response to the dog. During the first 60 s of the test however, this effect was not significant which may indicate other stimuli, such as sudden window cover movement, contribute to the responses of sheep during the test. Given the results of experiment 1 and Lee et al. [13], which demonstrate the measures can be used to differentiate anxious states, we expect other stimuli related to the test may have also contributed to an anxious state, but to a lesser degree than the presence of a threat. Attention to threat did not differ between any treatment groups, indicating this measure did not discriminate between a response to the dog and a response to sudden window cover movement. Once again this may indicate sudden movement causes an anxious response in animals which is initially indistinguishable from that caused by the presence of a predator using this test paradigm.

There were no differences between treatment groups for zones crossed and vocalisation counts during the 180 s test, although the Diazepam group crossed fewer zones during the first 45 s of testing. Decreased activity relative to the other treatment groups may indicate Diazepam had a sedative effect on the sheep, however we expect it is more likely that animals in the Diazepam group crossed fewer zones because they chose to spend more time eating, which directly competed with total time available to cross zones during the test. Within the concept of affect being the position of an animal in a two dimensional space described by axes of valence and arousal [1,2], we propose zones crossed in this study may be closer associated with arousal than valence. If this interpretation is correct, arousal does not appear to have been strongly influenced by

pharmacologically heightened anxiety or exposure to the dog in the attention bias test. In contrast, heightened anxiety resulted in enhanced vigilance and increased latency to eat which are likely to be associated with a negatively valenced state. This finding may create the potential for the test to assess the valence of an affective state independent of arousal. Alternatively, zones crossed may not have been a suitable measure of arousal in this study, in which case we cannot be sure whether the pharmacological treatments modified arousal. Measurement of physiological responses during the test in future studies may help to better assess the arousal dimension of affective state and help determine the potential for the test to discriminate between arousal and valence.

During experiment 1, many sheep within the m-CPP treated group displayed abnormal behaviours such as head, tail and whole body shaking, indicating an adverse reaction to the drug. The same dose rate did not cause an adverse response in adult ewes [13] but has elicited abnormal behaviours in 8-month-old sheep [16], indicating this dose rate may be inappropriate for younger animals. We propose the undesirable responses in this study had minimal impact on vigilance, or alternatively may have further increased anxiety and therefore vigilance due to the compromised ability of sheep to escape [24]. In each case, vigilance should still be a valid indicator for anxiety in the attention bias test. This is supported by previous studies in starlings, sheep and humans which consider vigilance to be a key measure for attention bias [12,13,25]. Attention to threat did not differ between the Control and m-CPP groups, which could potentially be related to the adverse response to the drugs if m-CPP treated sheep were disoriented. However, this measure should still be valid for the Control and Diazepam groups which differed significantly. This is supported by the findings of Lee et al. [13], showing attention to threat may be a key measure in the attention bias test.

The drugs used in this study have been known to effect feeding behaviour, with m-CPP dose-dependently suppressing food intake in rodents and humans [26–29] and diazepam increasing food intake in birds and non-human primates [30,31]. An adverse response to the drug may have further impacted appetite and feeding behaviours in the m-CPP group. Consequently, latency to eat and total time spent eating cannot be considered reliable measures for experiment 1. This is not to say latency to eat cannot be a useful indicator in the attention bias test in the absence of drug treatments. Latency to eat was a key measure of attention bias for starlings [12] and the results from experiment 2 where no drug treatments were given indicate feeding behaviours are directly related to

the presence of the dog. If our interpretation that latency to eat is primarily an assessment of valence rather than arousal is valid, then we can conclude that latency to eat may be a key measure of valence within the attention bias test, however further validation is required to confirm this, where anxious state and arousal are independently manipulated.

The current study presents a quick, easy method of data logger attachment which does not appear to significantly impact animal behaviour in the test. As collation of behavioural data is often time consuming and labour intensive, automation allows for more rapid and practical tests including, but not limited to, the attention bias test. This is of particular importance if the test is to be applied to large groups of animals. While this study focused on automation of vigilance behaviour, data loggers can potentially be used for automation of the other key measures in the test such as attention to threat or latency to eat. Importantly, data loggers may also help further determine the role of arousal in modifying performance of animals in the test.

This study provides further pharmacological validation that the attention bias test may be useful for detecting anxious states in sheep. We have also demonstrated that the test may be useful across different ages and sexes, as the current study tested young castrated males while Lee et al. [13] tested adult ewes. While these results are promising, the attention bias test is still new and more work is required to better understand, validate and refine the test. We suggest a number of priority areas to begin further work. Firstly, while most studies in humans have only found attention biases in anxious individuals, there is evidence that attention biases also occur in clinically depressed individuals [32,33]. Further studies could assess whether other negative affective states, such as depression, can result in attention biases in animal species detectable with this test paradigm. Such studies should also help clarify the extent to which the test is primarily a measure of valence. Second, further refinement of the method is required for the test to become practical for large groups of animals or for use in an on-farm setting. Automation of behavioural measures and adapting the test to work in existing sheep handling facilities are two routes which would make this test more practical. Finally, there is potential for the attention bias test to be used as a measure of temperament as well as a measure of transient anxious states, however further research is required to explore this potential. If the test can be used to assess an anxious trait, it could be applied to larger groups of animals for estimation of genetic parameters related

to anxious behaviours and temperament. Anxious temperament is known to be influenced by genetic factors and temperament is a heritable trait [34–36]. By identifying and incorporating temperament into sheep breeding programs, we may be able to select for calmer animals that are better suited to a domestic environment, are easier to handle and have improved welfare.

3.6. Conclusions

Overall, the current study shows the attention bias test developed by Lee et al. [13] can be further refined so that it does not require training and may be shortened to less than 1 min per animal. This faster method for assessing anxious states in sheep may provide a more practical measure of affect which can be used in further animal welfare research. This study also verifies the responses being measured in the test are at least partially a response to the dog, further validating it as a measure of attention bias. The potential for the test to discriminate valence from arousal deserves closer examination. With further refinement and automation the test should be suitable for application to larger populations of animals.

3.7. Acknowledgements

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3.9. Supporting information

S1 Video. Video showing the beginning of the test and examples of key behaviours.

S1 Dataset. Raw behavioural data for experiment 1.

URL: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0190404

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Chapter 4

Attention bias test differentiates anxiety and depression in sheep

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4.1. Abstract

Negative affective states such as anxiety and depression pose a risk to animal welfare, however practical tests for assessing these states in animals are limited. In humans, anxious individuals are shown to pay more attention towards threatening information than non-anxious individuals, known as an attention bias. Previously, an attention bias test was developed and validated as a measure of anxious states in sheep, where more anxious sheep showed increased attention towards a threat (dog) and were more vigilant than Control animals. Studies in humans suggest that attention biases also occur in depressed individuals, with observations of attention biases towards threats, as well as biases away from positive stimuli. Given these findings, we hypothesised that an attention bias test for sheep could also be used to assess states of depression. We predicted that Merino ewes in pharmacologically induced Depressed (para-chlorophenylalanine) and Anxious (m-chlorophenylpiperazine) states would show greater attention towards a threat than Control animals (saline), but that the Depressed sheep would show relatively less interest in a positive stimulus (photograph of a conspecific). During testing, Depressed sheep paid more attention towards the threat and less towards the photograph than Control animals as predicted (Analyses of Variance, $P<0.05$, $n=16$ per treatment). Interestingly, anxious sheep showed an attention bias in the opposite direction, paying more attention towards the photograph and less towards the threat than Control animals ($P<0.05$). Both Anxious and Depressed sheep were more vigilant than Control animals ($P=0.002$). These results suggest the attention bias test can be used to measure and differentiate states of depression and anxiety in livestock. The bidirectional nature of the attention bias identified between treatments highlights the importance of measuring multiple behaviors in the test and considering the context in which the test is applied. This will enable a clearer characterization of the affective state of an animal, as an aspect of its welfare.

4.3. Introduction

The affective states of animals comprise an important component of animal welfare, with negative depression-like states (hereafter depression) and anxiety-like states (hereafter anxiety) potentially posing a risk to the well-being of livestock in production systems. Practical methods which can assess these negative affective states would therefore be useful for the study, assessment and improvement of animal welfare. Cognitive methods from the human literature offer the potential to assess a range of different affective states in animals (Paul et al., 2005). For example, it has been well established that humans in anxious states pay more attention towards threatening stimuli than non-anxious individuals, known as an attention bias (Bar-Haim et al., 2007; Bradley et al., 1995, 1997). Based on this concept, a test has been developed for assessing attention biases in sheep, measuring the degree to which an animal's attention is directed towards a potential threat (location recently occupied by a dog) and away from a positive stimulus (food). The test has been validated as a measure of anxious states, where animals in a pharmacologically induced anxious state spent more time looking towards the previous location of a dog, were more vigilant and were less likely to feed than calm (saline control) animals during a 3 min test (Lee et al., 2016; Monk et al., 2018). While attention biases towards threats have predominantly been associated with anxious states in the human literature, there is some evidence that attention bias towards threats also occurs in depressed individuals (Mathews et al., 1996; Mogg et al., 1995), although this has not been consistent in all studies (see reviews (Armstrong and Olatunji, 2012; Peckham et al., 2010)). However, evidence of attention biases away from positive stimuli occurring in depressed individuals is more consistent (Armstrong and Olatunji, 2012). Given these findings, we might then expect that states of anxiety and depression in animals can both result in attention biases towards threats, while depression can result in a bias away from positive stimuli, when compared to normal individuals. The potential for an attention bias test to assess not only anxious states, but also other negative affective states such as depression in livestock deserves further investigation.

Anxiety and depression in human and animal models are also associated with different sets of behavioral and physiological responses, which may help to differentiate these states during an attention bias test. For instance, key features of anxiety in humans include increased fearfulness of novel experiences or environments and exaggerated

fear responses to potential threats (Guze, 1995). These features can be measured in animals through behaviors such as reduced interactions with the novel environments and changes in locomotion (see reviews (Boissy, 1995; Dodd et al., 2012; Forkman et al., 2007)). During the attention bias test for sheep, this was also reflected by increased vigilance in anxious animals (Lee et al., 2016, 2017; Monk et al., 2018). A key feature of depressive states in humans is a loss of interest or pleasure in daily activities (anhedonia) (Guze, 1995), which has also been reproduced in rodent models of depression (e.g. (Barr and Phillips, 1999; Harrison et al., 2001; Moreau et al., 1992)). During an attention bias test, this may be reflected by reduced interactions with the positive stimulus. Physiological responses during testing may also help to differentiate anxious and depressed states. For example, stress-induced hyperthermia is shown to be reduced by anxiolytic but not anti-depressant drugs (Bouwknecht et al., 2007) and has been previously observed in anxious cattle during an attention bias test (Lee et al., 2017). By considering these key differences in behavioral and physiological responses along with the presence or absence of an attention bias towards the threat, it may be possible to differentiate states of anxiety and depression in sheep during an attention bias test.

Controlling external factors which can influence behavior in the attention bias test is essential for the standardized application of the test and effective interpretation of behavioral responses. The previously established method relies on a feed reward as a positive stimulus, however motivation to feed can vary considerably between animals and within animals over the span of a single day. Further, while pharmacological models of affective state provide a useful tool for validation of test methodology, these agents have known impacts on appetite, making interpretation of results difficult (Doyle et al., 2015). As such, it would be desirable to replace the feed reward used in the established test method with an alternative positive stimulus that can be more easily standardized and allow for clearer interpretation of behavioral responses. As sheep are social animals, a conspecific could work as an alternative positive stimulus, however addition of another live animal could again introduce unwanted variation into the test. Photographs of conspecifics have been shown to reduce fear-related behaviors of isolated sheep in previous studies, indicating they were perceived as positive by the test animals (Bouissou et al., 1996; Vandenheede and Bouissou, 1994). As such, a photograph or model of a conspecific may be a more suitable alternative positive stimulus than food

for the attention bias test which can be presented in a more standardized manner during testing.

The aim of the current study was to determine whether responses in the attention bias test for sheep may indicate affective states of depression as well as anxiety. We hypothesized that sheep in pharmacologically induced states of depression and anxiety would both show an attention bias towards the threat relative to the control animals. Further, we hypothesized that it would be possible to differentiate between states of depression and anxiety by comparing their relative attention towards the positive stimulus and by considering other behavioral and physiological responses in the test. Specifically, we expected the two treatment groups would differ in temperature response and activity during the test as well as exploration of the positive stimulus and environment. Finally, we hypothesized these attention biases could be assessed using a photograph or model of a conspecific as a positive stimulus instead of the feed used in previous studies. To test these hypotheses, states of depression and anxiety were induced through pharmacological manipulation of serotonergic pathways prior to assessment in a modified attention bias test.

4.4. Materials and methods

4.4.1. Animal ethics

The protocol and conduct of the experiments were approved by the CSIRO F.D. McMaster Laboratory Animal Ethics Committee (AEC17-25) and the University of New England Animal Ethics Committee (AEC17-126), under the New South Wales Animal Research Act 1985.

4.4.2. Pilot study

A pilot study was conducted to determine an appropriate positive stimulus to replace food in the attention bias test. A fiberglass model of a sheep and a range of photographs were trialed (see below for details).

4.4.2.1. Animal details

Fifteen, non-pregnant, non-lactating Merino ewes (2.5 years old) from the CSIRO farm flock in Armidale, NSW were used in the pilot study; five sheep during phase 1 of the experiment and ten sheep during phase 2 of the experiment. Sheep had no prior experience with the attention bias test or the positive stimuli used in this trial. Sheep had prior experience with dogs during routine farm management.

4.4.2.2. Attention bias test arena

The current study used the same testing arena (Figure 4.1) as Monk et al. (2018), which was adapted from Lee et al. (2016). The test arena comprised a 4 x 4.2 m concrete yard with 1.8 m high opaque walls. A small window was positioned on the side of the arena behind which an unfamiliar dog (Border Collie) was standing quietly. Alternative positive stimuli could potentially be placed along the walls opposite and adjacent to the dog window (Figure 4.1, see section 2.2.3 for details). The test arena was divided into a grid with 9 sections, which were overlayed on the video footage during behavioral analysis (Figure 4.1). Due to constraints of the arena construction material, the dog window was not centered along the side wall. As such, the grid was skewed so that the middle zones remained centered around the dog window (Figure 4.1). When a sheep was introduced to the arena, the dog was visible through the window for 3 s, after which the window was covered by a retractable opaque cover and the dog was removed. Exposure for 3 s was previously shown to be sufficient to affect attention behaviors in sheep (Monk et al., 2018). The 3 s interval began once the door into the arena was closed behind the sheep and a hidden observer was confident that the sheep had made visual contact with the dog. The hidden observer was positioned above the arena in a building next to the test arena, out of view of the sheep. The 3 min test interval commenced once the window was fully closed to obscure the dog. Behaviors during the test were recorded by a Sony Handicam handheld video camera (Sony, Australia, model number HDR-XR550) (Figure 4.1).

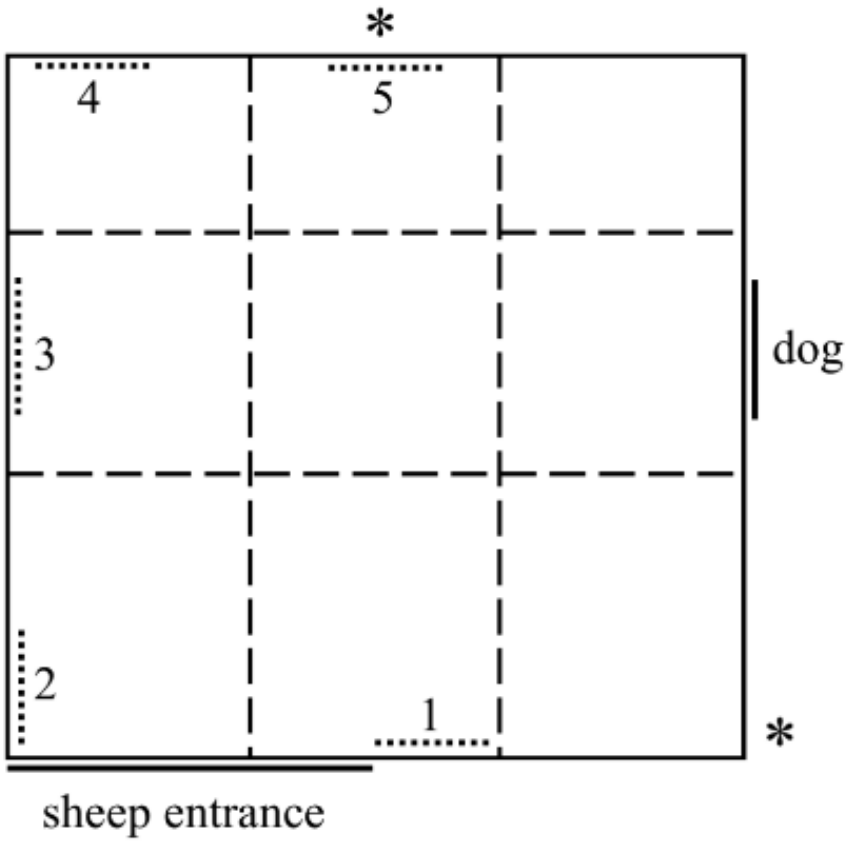

Figure 4.1. Diagram of the attention bias test arena comprising a 4 x 4.2 m yard with opaque 1.8 m high walls.

A dog was visible for the first 3 s of the test, then the window was covered. The numbered, dotted lines represent potential locations of positive stimuli during the pilot study. Only position 3 was used during the main trial. "*" denotes the positions of two cameras. Dashed lines represent a grid overlaid on video footage post-testing.

4.4.2.3. Alternative stimuli

To replace the feed reward used in the original attention bias test, five alternative stimuli were trialed during the pilot study: a fiberglass model of a sheep, two photographs (hereafter photo) of an unfamiliar Merino sheep (one side profile, one front-on) and two photos of the faces of an unfamiliar Merino sheep (one side profile, one front-on) (Figure 4.2). The model and photos were approximately life-size. All photos were printed on 200gsm matte cardstock, then were cut out and mounted to 5 mm thick black corflute board using spray adhesive. The front-on head only photo had a small amount of wool glued to the top of the head as an additional sensory cue. The wool came from an unfamiliar conspecific, collected from a shearing shed.

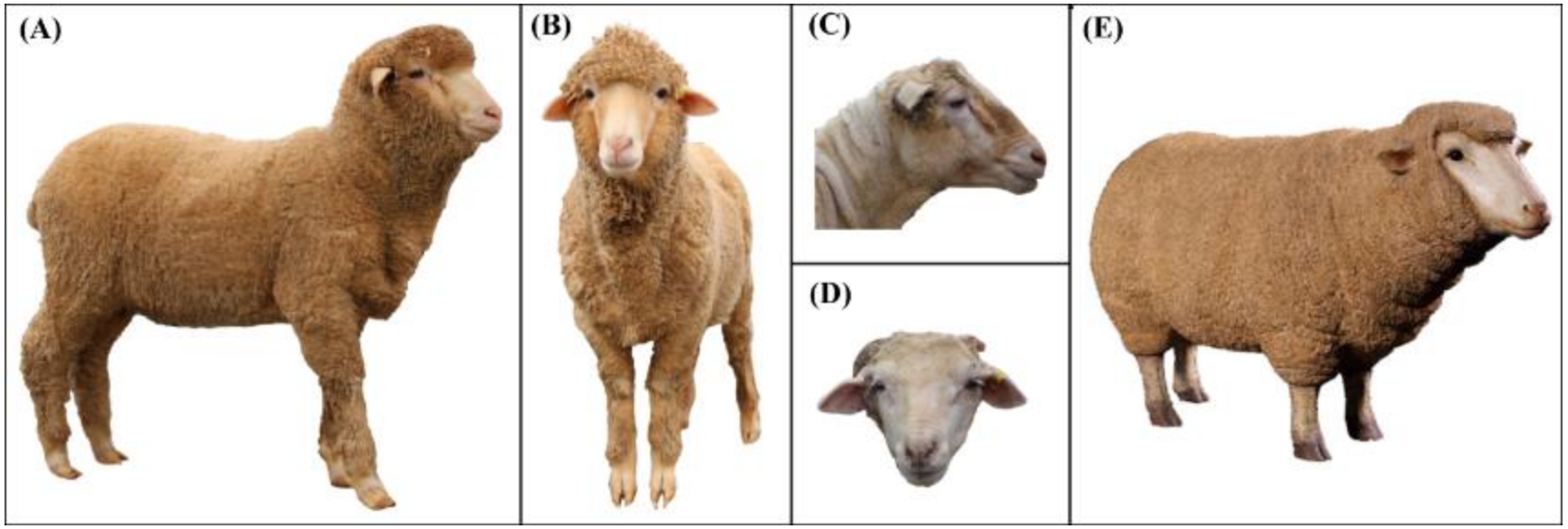

Figure 4.2. Alternative stimuli trialed during the pilot study.

Stimuli included photographs of entire sheep from the side (A) and front (B), photographs of faces only from the side (C) and front (D) and a three-dimensional fiberglass model of a sheep (E). All stimuli were approximately life-size.

4.4.2.4. Phase 1: Identification of an alternative positive stimulus

Phase 1 of the pilot study aimed to identify which of the alternative stimuli was preferred by the sheep. Animals were first tested as a group, then individually during an attention bias test.

A group of 5 sheep had numbers painted on their rumps for individual identification, then were moved into a small yard (5 m x 4 m) containing all 5 stimuli for 30 mins. The stimuli were located along the edges of the yard, spaced approximately 2m apart from one another. The number of interactions each sheep had with each of the stimuli was recorded. Interactions included sniffing or attempting to chew the stimuli. Sheep were not exposed to a dog during this time.

All 5 stimuli were then moved into the attention bias test arena together. The locations of the stimuli in the arena are shown in Figure 4.1; the stimuli were spaced approximately 1-2 m apart. Each of the 5 sheep individually underwent the attention bias test as described above, including exposure to a dog for 3 s. During the 3 min test, the number of interactions sheep had with each of the alternative stimuli was recorded. The positions of the stimuli were randomly rotated between test sheep to prevent stimulus location impacting on results.

4.4.2.5. Phase 2: Validating the stimulus during the attention bias test

Phase 2 of the pilot study aimed to validate whether the alternative stimulus selected during Phase 1 would be an appropriate replacement for food in the attention bias test. Observations during phase 1 of the pilot study indicated the fiberglass model was preferred by test sheep, however for easier replication in future studies, the side profile photo of an entire sheep (second preference) was selected for further use in phase 2.

Ten sheep, different from those used in Phase 1, were tested individually in the attention bias test as described above to determine whether the chosen stimulus and its location were appropriate. The criteria for a successful stimulus were that 1) the test sheep showed interest in the stimulus, 2) test sheep did not appear to be fearful of the stimulus and 3) test sheep were willing to move away from the stimulus during testing to explore the arena. To assess these criteria, the following behaviors were recorded during the 3 minute test; latency to sniff the photo, time spent standing within 1m of the photo, time

spent looking at the photo, time spent looking at the dog window and number of grid sections entered (Figure 4.1). The group of sheep had been briefly habituated to the stimuli for 5 mins prior to testing, to reduce the potential impact of stimulus novelty, which may induce fear (Boissy, 1995), on sheep responses during the test.

For the first 3 sheep that were tested, the front-on photo of a sheep face was also mounted next to the side profile photo as an additional attractant. The front-on photo was removed after 3 sheep were tested as it was deemed unnecessary. The other 7 sheep were tested using the side-profile photo only.

Alternate locations were trialed to ensure sheep were attracted to the photo and not just the given location. The stimulus was initially placed in the location directly opposite the window (Location 3, Figure 4.1.) After 6 sheep had been tested, the photo was moved to the wall next to the door (Location 1, Figure 4.1) and a further 2 sheep were tested. The photo was then moved to the opposite wall (Location 5, Figure 4.1) and the final 2 sheep were tested.

4.4.3. Main trial

4.4.3.1. Animal details

Fifty non-lactating, non-pregnant Merino ewes (18-20 months old) with average bodyweight of 37.9 ± 2.8 kg were used in this experiment. Sheep had undergone routine handling prior to the current experiment and were therefore familiar with the presence of humans, but had no experience with the attention bias test or photos. Sheep had prior experience with dogs during routine on-farm management. The sheep were divided into two cohorts (n=24 and 26) to be tested on separate days for logistical reasons. Treatment groups were evenly distributed between the two cohorts.

On day 0 of the main trial, all sheep were weighed and sorted into treatment groups balancing for bodyweight, then had numbers painted on their rumps for individual identification and were divided into 2 cohorts for testing. The first cohort then began pharmacological treatment the following afternoon (Day 1). The second cohort was returned to pasture and began treatment 2 days later so that attention bias testing would be staggered over 2 days. Treatment of the second cohort was identical to that described below.

4.4.3.2. Drug details

The drug 4-chloro-DL-phenylalanine methyl ester hydrochloride (pCPA; Sigma Aldrich, St. Louis, USA) was used to pharmacologically induce a state of depression. This drug has been shown to decrease brain serotonin levels and increase depression-like behaviors in a number of animal species including sheep (Doyle et al., 2011; Kubala et al., 2008; Stracke et al., 2017). The administration protocol for pCPA followed that described by Doyle et al. (2011), which caused a pessimistic judgement bias in sheep that was thought to reflect a depressed emotional state. Prior to administration, pCPA was dissolved in BP Water for Injection (Baxter, Toongabbie, Australia) at a rate of 50 mg/ml. pCPA was administered over 5 consecutive days at a rate of 40mg/kg/day, administered in half doses each morning (8:00 AM) and afternoon (4:00 PM). The first dose was given on the afternoon of day 1 so that the final dose was given on the morning of day 6 prior to testing in the attention bias test. The solution was administered intraperitoneally (i.p.), by injection through the right paralumbar fossa, midway between the iliac crest and the last rib, approximately 50 mm below the end of the lumbar processes (Hurter, 1987).

The drug m-Chlorophenylpiperazine (mCPP; Tocris, Bristol, UK) was used to pharmacologically induce a state of anxiety. This drug has been shown to induce anxious states in humans and has been used a number of times to induce anxiety-like behaviors in sheep (Doyle et al., 2015; Drake, 2006; Lee et al., 2016; Monk et al., 2018). The administration protocol followed that described by Lee et al. (2016). Prior to treatment, mCPP was dissolved in BP Water for Injection at a rate of 86 mg/ml. mCPP was administered as a single intramuscular injection into the rump of the animal at a dose rate of 2mg/kg, 30 mins prior to testing in the attention bias test.

4.4.3.3. Drug treatment protocol

Animals were distributed between 3 treatment groups, ‘Anxious’, ‘Depressed’ and ‘Control’ (*n*=16 per group) balancing for bodyweight. All sheep received an injection of either their treatment group drug, or an equivalent volume of BP saline, for 5 consecutive days prior to testing, at 8 AM on the morning of testing and 30 min before undertaking the attention bias test as outlined in Table 4.1. In total, all sheep received 10 intraperitoneal injections and one intramuscular injection prior to undergoing the attention bias test. Two spare animals were included with cohort 2 and were treated in the same way as the Control group throughout the study. Due to the presence of

abnormal stumbling behavior in some of mCPP treated sheep in mob 1, the spare sheep were also administered mCPP on the attention bias test day to ensure adequate numbers in the treatment group could be achieved if any animals had to later be removed from the study.

Table 4.1. Summary of drug treatments during the main trial.

The pharmacological agent and administration method are given for each treatment group at each time point. All sheep received a total of 10 intraperitoneal injections during days 1 to 6 and one intramuscular injection on day 6, 30 mins prior to undergoing the attention bias test.

Days	Time	Treatment group		
		Anxious (*n*=18)	**Depressed (*n*=16)**	**Control (*n*=16)**
1	4 PM	Saline (i.p.)	pCPA (i.p.)	Saline (i.p.)
2-5	8 AM	Saline (i.p.)	pCPA (i.p.)	Saline (i.p.)
	4 PM	Saline (i.p.)	pCPA (i.p.)	Saline (i.p.)
5	4 PM	ibuttons inserted Cohesive bandages applied		
6	8 AM	Saline (i.p.)	pCPA (i.p.)	Saline (i.p.)
	30 min prior to attention bias test	mCPP (i.m.)	Saline (i.m.)	Saline (i.m.)
	30 min post injection	Attention bias testing		

Between intraperitoneal injections, all sheep were returned to paddocks adjacent to the handling facilities with access to pasture and fresh water. Sheep were mustered from the paddocks to the yards prior to each injection, then were monitored continuously for 30 mins post injection before being returned to the paddocks.

On day 6, sheep were given their final intraperitoneal injection in the morning then were moved using a trailer to a second set of yards where the attention bias test was located. Sheep were left in the yards undisturbed for 2 hrs to allow them to settle following transportation. Individual sheep were given their single assigned intramuscular injection (Table 4.1) at 5 min intervals over a 2.5 hr period so that each animal received their injection 30 mins prior to undergoing the attention bias test. All injections and attention bias tests were completed within 3 hrs on test days.

4.4.3.4. Attention bias test

The main trial used the same attention bias test described for the pilot study, using a single photo of a sheep in side profile located on the wall directly opposite the dog window (Location 3, Figure 4.1). A high resolution copy of the photo is available in the supplementary materials (Supplementary Image 1). On day 6, the photo was placed in a holding yard with all sheep for a brief habituation period (5 min) approximately 30 mins before beginning any injections.

4.4.3.5. Behavioral measures in the attention bias test

The behaviors recorded in the attention bias test are summarized in Table 4.2. Open and close mouthed vocalizations were scored on the day of testing by a hidden observer. The observer was positioned behind the opaque matting near the door wall of the test arena, out of view of the sheep. The same observer also recorded the dog's posture, movements and vocalizations at the beginning of the test. Dog behavior was later categorized on a 3 point scale as: 1 – quietly standing still, 2 – lunging or crouching down or 3 – barked at the sheep with any posture. A score of 3 was given on 3 occasions. All other sheep behaviors in the attention bias test were collated from video footage using The Observer XT 12.0 (Noldus Information Technology, Wageningen, The Netherlands). To determine whether treatment effects were evident for a shortened version of the test, separate analyses were also performed for the first 60 s of behaviors recorded during the test.

Table 4.2. Ethogram of behaviors recorded during the attention bias test.

Behavior	Definition
Attention	The direction in which the sheep is looking with binocular vision (Lee et al., 2016; Piggins and Phillips, 1996). The test arena was divided into 6 areas of attention: dog window, dog wall (including the window), photo, photo wall (including the photo), door wall and back wall. Total duration of attention was recorded for each section, as well as latency to look at the photo.
Vigilance	Time spent with the head at or above shoulder height (Frid, 1997; Lee et al., 2016). Latency to become non-vigilant was also calculated.
Sniff photo	Number of times and latency to sniff the photo
Sniff environment	Number of times and latency to sniff the floor or walls of the test arena
Vocalizations	Number of open mouthed bleats and close mouthed bleats were recorded separately
Zones entered	Number of zones entered (1 to 9)
Zone duration	Total time spent in each of the 9 zones and latency to enter the zone closest to the dog
Elimination	Number of urinations or defecations

Sheep treated with mCPP were monitored for abnormal behaviors previously described by Doyle et al. (2015). These included ataxic gait, tail shaking, head shaking, body shaking and head rolling. Abnormal behaviors were observed in 14 of the 18 mCPP treated sheep. Head shaking was observed in 6 sheep and tail shaking in 9 sheep. No body shaking or head rolling was observed. Ataxic gait was observed in 5 sheep, 2 of which stumbled multiple times while the other 3 stumbled only once.

4.4.3.6. Internal body temperature

Internal body temperature was recorded using Thermochron iButtons® (Model number DS1922L-F5, accuracy 0.5°C, resolution 0.063°C, weight 3.3g) (Embedded Data Systems, Lawrenceburg, USA) which were attached to blank (progesterone-free) Controlled Internal Drug Release devices (CIDR®, Zoetis, Melbourne, Australia) using polyolefin heat-shrink tubing, as described by Lea et al. (2008). A CIDR was inserted into the vagina of each sheep one day prior to testing using an applicator lubricated with obstetrical lubricant. The iButtons were set to log at an interval of 10 s beginning at 8:00 AM on the day of attention bias testing. Data were extracted using the program eTemperature version 8.32 (OnSolution, Castle Hill, Australia). The following time points were then selected for further analysis: -40 min, -30 (time of injection), -20, -10, 0 (start attention bias test), 3 (end of attention bias test), 8, 13 and 18 mins. Data from

2 temperature loggers were missing due to technical faults and so post-attention bias test data for the last 2 animals tested each day were removed as these animals were handled soon after testing.

4.4.3.7. Data loggers

HOBO® Pendant G accelerometer data loggers were used to record steps during the attention bias test (dimensions: 58 mm x 33 mm x 23 mm, weight: 18 g) (Onset Computer Corporation, Pocasset, MA, USA). The HOBO® Waterproof Shuttle and HOBOware® Pro software (version 3.7.8) were used for programming and reading the HOBO loggers (Onset Computer Corporation, Pocasset, MA, USA). Data loggers were programmed to record at a logging interval of 0.03 s (33 Hz) when activated using a magnet (measurement range: ±3 gravitational force (*g*); accuracy: ±0.075 *g* at 25°C).

On the afternoon prior to testing, after receiving their injections, all sheep had a small silicone pad attached to the outside of their left hind leg in the middle of the cannon bone using veterinary cohesive bandage. The silicone pad had a small recess in which the HOBO loggers could be placed to keep them still and away from the leg. The following day, immediately prior to entering the attention bias test, a HOBO logger was activated using a magnet, then was tucked between the bandage and the silicone pad on the outside of the leg. All data loggers were removed at the end of the day after testing had been completed. Steps were calculated from the accelerometer data using an in-house program (CSIRO Livestock Phenomics Information System version 1.0, Bryce Little, 2015).

4.4.3.8. Additional measurements and procedures

Blood samples were taken from all sheep on days 1 and 6 of the experiment for assessment of gene expression as part of another project (not further reported on here). Sample collection on day 6 occurred after all behavioral testing had been completed. The 2.5ml blood samples were collected via jugular venipuncture using a 1” 18 g needle and Paxgene RNA protect vacutainers. On the morning of day 6 after sheep received their intraperitoneal injections, the rectal temperatures of the sheep were recorded to ensure no animals had developed a fever during the trial. No fevers were detected at this time.

4.4.4. Statistical Methods

4.4.4.1. Pilot study

Decisions in phase 1 of the study were made based on the qualitative observations of two researchers at the time of the experiment. Summary statistics were obtained from the data using Microsoft Excel 2013.

4.4.4.2. Main Trial

Data were analyzed using R version 3.2.3 (R Core Team, 2015). P values less than 0.05 were considered significant. All model residuals were checked for normality and homoscedasticity using Shapiro Wilks test for normality and visual assessment of Q-Q and residuals vs fitted values plots. Cohort (test day), test order and dog behavior score were fitted as fixed effects in all linear models and analyses of variance (ANOVA), however none of these factors reached significance and were subsequently removed from all models using a backward elimination approach. Post hoc multiple comparisons were conducted using a Tukey method for adjustment of P values.

Attention data were analyzed by one-way ANOVA. Data for attention towards the dog, photo and photo wall were log transformed to meet normality assumptions. Attention in the dog's direction included a number of outliers with high leverage on the data, therefore these data were analyzed using an ANOVA on 10% trimmed means using the package WRS2 (Mair et al., 2017). The results from the ANOVA on trimmed means were also confirmed using Kruskal-Wallis non-parametric ANOVA. Vigilance duration data were analyzed by Kruskal-Wallis non-parametric ANOVA as the parametric model residuals did not meet normality assumptions and could not be improved by transformation (Grosjean and Ibanez, 2014). Post hoc multiple comparison tests for Kruskal-Wallis ANOVAs were performed using the package pgirmess (Giraudoux, 2016).

Time spent sniffing the photo and environment required transformation to meet normality assumptions, then were analyzed using one-way ANOVA. Time spent in the zone closest to the dog did meet normality assumptions and was analyzed using one-way ANOVA. Photo sniff frequency and number of zones entered were analyzed using generalized linear models with a Poisson distribution for count data. Number of steps and sniff environment frequency were analyzed using generalized linear models with a negative binomial distribution due to evidence of over-dispersion. Due to the low

occurrence of vocalizations, vocalization data were analyzed using Fishers Exact Tests, examining the number of animals in each group which vocalized. Attention, vigilance and exploratory behaviors during the first 60 s of testing were analyzed in the same way as for the full test duration.

All latency data were analyzed with Cox's proportional hazards model using survival analysis, as described by Monk et al. (2018) (Therneau, 2015; Therneau and Grambsch, 2000). These data included latencies to look at the photo, sniff the photo, sniff the environment, enter the zone closest to the dog and become non-vigilant. Animals which failed to perform each behavior within 180 s were deemed as censored results.

Body temperature data were analyzed using a maximum likelihood multilevel linear model to account for repeated measures on the same animals at each time point, fitting treatment, time and a treatment x time interaction as fixed effects (Field et al., 2012). Changes in temperature from the beginning of attention bias testing onwards were then assessed in the same way. These data were obtained by subtracting the Time 0 (start of test) values from subsequent values for each animal.

4.5. Results

4.5.1. Pilot study: Phase 1

The test sheep interacted with the fiberglass model more than any other stimulus in both a group setting and individually during the attention bias test (Table 4.3). The life-size photo of an entire sheep from the side received the second highest number of interactions (Table 4.3).

Table 4.3. The number of interactions a group of 5 sheep had with a variety of positive stimuli during Phase 1 of the pilot study.

The sheep (n=5) were tested as a group in a small yard (Group) then individually in the attention bias test (Individual). Stimuli included a fiberglass model of a sheep and 4 life-size photos (Figure 4.2) of entire sheep or sheep faces.

Stimulus	Total number of interactions		Number of animals interacting	
	Group	Individual	Group	Individual
Fiberglass model	8	13	5	4
Body photo (side)	5	4	4	4
Body photo (front)	4	3	2	3
Face photo (front)	1	3	1	3
Face photo (side)	0	3	0	2

4.5.2. Pilot study: Phase 2

All sheep sniffed the stimulus during the test. Test sheep on average spent more than half their time standing within 1 m of the photo (Table 4.4). The two sheep which were slowest to sniff the photo (latencies of 60 and 63 s) spent the most time standing next to the photo (100 and 83% of time respectively). All other sheep sniffed the photo within 30 s of beginning the test. The mean proportion of time spent standing near the photo was 57% when the photo was located directly opposite the dog window (n=6) and 58% when the photo was moved to one of the other locations (n=4). The mean, minimum and maximum latencies, frequencies and durations of all sheep behavior in the test are given in Table 4.4.

Table 4.4. Mean, minimum and maximum values for behaviors of sheep during Phase 2 of the pilot study, during the attention bias test.

Statistic	Sniff photo Latency (s)	Sniff photo Frequency	Standing near Photo (%)	Looking at Photo (%)	Looking at Window (%)	Zones entered
Mean	18.9	3.4	57.4	21.3	15.8	5.0
Minimum	0	1	23	3	8	1
Maximum	63	7	100	41	30	9

4.5.3. Main trial

4.5.3.1. Attention behaviors

No differences were seen between treatment groups for time spent looking directly at the closed dog window or photo, however differences were seen between groups in time spent looking at the dog and photo walls (Table 4.5 and Figure 4.3). Specifically, Depressed sheep spent the most time looking towards the dog wall and least time looking towards the photo wall, while Anxious sheep spent the most time looking towards the photo wall and least time looking towards the dog wall (Figure 4.3). Both Anxious and Depressed sheep were slower to look at and sniff the photo than Control animals, although the difference between the Control and Anxious groups only tended towards significance (Table 4.6 and Figure 4.4). There were no differences between groups for duration of attention towards the door wall or back wall.

Table 4.5. Mean ± s.e.m. behavioral responses of sheep in each treatment group during the attention bias test.

Behavioral measure	Control	Anxious	Depressed	Test value	P value
Attention to dog window (s)	22.6 ± 2.4	20.7 ± 2.7	27.3 ± 3.0	$F_{(2,47)} = 2.44$	0.099
Attention to dog wall (s)	58.4 ± 3.8[a]	47.6 ± 5.3[b]	75.8 ± 5.3[c]	$F_{(2,47)} = 7.09$	<0.001
Attention to photo (s)	37.3 ± 4.4	36.7 ± 3.9	29.1 ± 4.6	$F_{(2,47)} = 1.57$	0.22
Attention to photo wall (s)	51.0 ± 5.2[ab]	57.1 ± 4.9[a]	39.2 ± 5.2[b]	$F_{(2,47)} = 4.96$	0.011
Attention to door wall (s)	45.5 ± 4.0	49.4 ± 3.8	41.4 ± 4.0	$F_{(2,47)} = 1.03$	0.37
Attention to back wall (s) [1]	3.1 ± 0.2 (22.3)	3.0 ± 0.2 (19.8)	3.0 ± 0.2 (19.3)	$F_{(2,47)} = 0.19$	0.83
Vigilance (mean rank duration) [2]	14.5 ± 2.3[a] (160)	36 ± 3.3[b] (171)	30.5 ± 3.7[b] (168)	$X^2_{(2)} = 12$	0.002
Standing near photo (s)	121 ± 9.9[a]	153 ± 9.3[b]	108 ± 9.9[a]	$F_{(2,47)} = 5.91$	0.005
Sniff photo (*n*) [1]	1.9 ± 0.1[a] (6.6)	1.4 ± 0.1[b] (1.4)	1.5 ± 0.1[b] (1.5)	$X^2_{(2)} = 11.4$	0.003
Sniff environment (*n*) [1]	1.9 ± 0.2 [a] (6.7)	0.8 ± 0.2[b] (2.2)	1.4 ± 0.2[ab] (4.1)	$X^2_{(2)} = 11.7$	0.003
Zones entered (*n*) [1]	1.74 ± 0.1[a] (5.7)	0.94 ± 0.2[b] (2.6)	1.68 ± 0.1[a] (5.4)	$X^2_{(2)} = 25.1$	<0.001

[a,b,c] Different superscripts within rows indicate a significant difference between treatments as determined using post-hoc analyses

[1] Least squares means are given on the log scale, back-transformed means are given in parentheses

[2] Mean ranks are given, raw means are given in parentheses

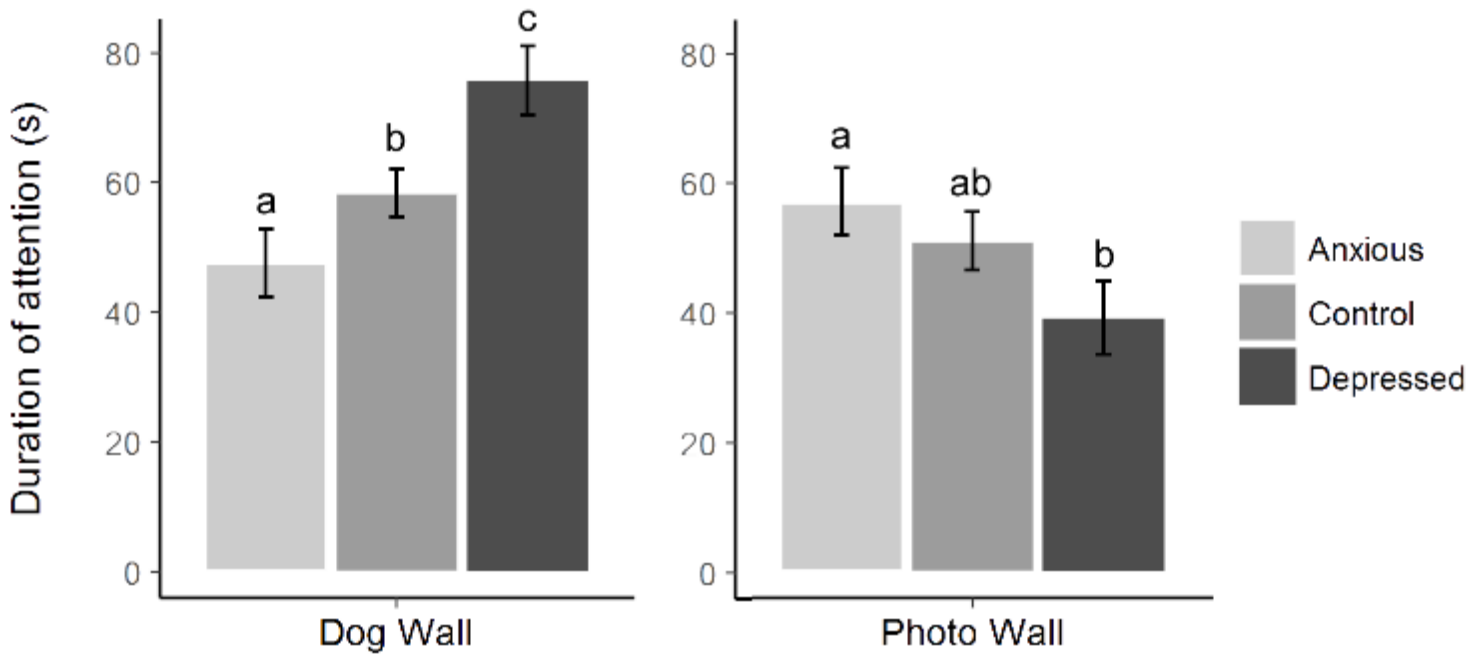

Figure 4.3. Mean ± s.e.m. time spent looking towards the dog wall versus the photo wall for each treatment group during attention bias testing.

Table 4.6. Hazard ratios for latency to look at the photo, sniff the photo, sniff the environment and enter the zone closest to the dog window as affected by treatment group.

Latency to	Group	Mean[1]	Coefficient[2]	SE (coeff)	Hazard ratio[3]	Wald (z)	P
Look at photo	**Control**	**2.7**	**Reference**				
	Anxious	5.6	-0.674	0.35	0.51 (0.25-1.02)	-1.91	0.057
	Depressed	8.0	-1.203	0.39	0.30 (0.14-0.65)	-3.08	0.002
	Anxious		**Reference**				
	Depressed		-0.529	0.37	0.59 (0.29-1.20)	-1.45	0.147
Sniff photo	**Control**	**5.6**	**Reference**				
	Anxious	18.4	-0.644	0.37	0.53 (0.26-1.08)	-1.75	0.08
	Depressed	13.3	-0.787	0.38	0.46 (0.22-0.95)	-2.09	0.037
	Anxious		**Reference**				
	Depressed		-0.143	0.35	0.87 (0.44-1.70)	-0.41	0.69
Sniff environment	**Control**	**38.1**	**Reference**				
	Anxious	123.3	-1.892	0.46	0.15 (0.06-0.37)	-4.12	<0.001
	Depressed	73.5	-0.894	0.40	0.41 (0.19-0.90)	-2.23	0.026
	Anxious		**Reference**				
	Depressed		0.998	0.44	2.7 (1.2-6.4)	2.29	0.022
Enter zone closest to dog	**Control**	**132.**	**Reference**				
	Anxious	179.4	-2.395	1.07	0.09 (0.01-0.74)	-2.24	0.025
	Depressed	163.2	-0.5684	0.59	0.57 (0.18-1.79)	-0.97	0.333
	Anxious		**Reference**				
	Depressed		1.83	1.10	6.20 (0.73-53.0)	1.67	0.095

[1] Raw mean latencies are given
[2] Regression coefficient from the Cox-proportional hazards model
[3] 95% confidence interval given in parentheses

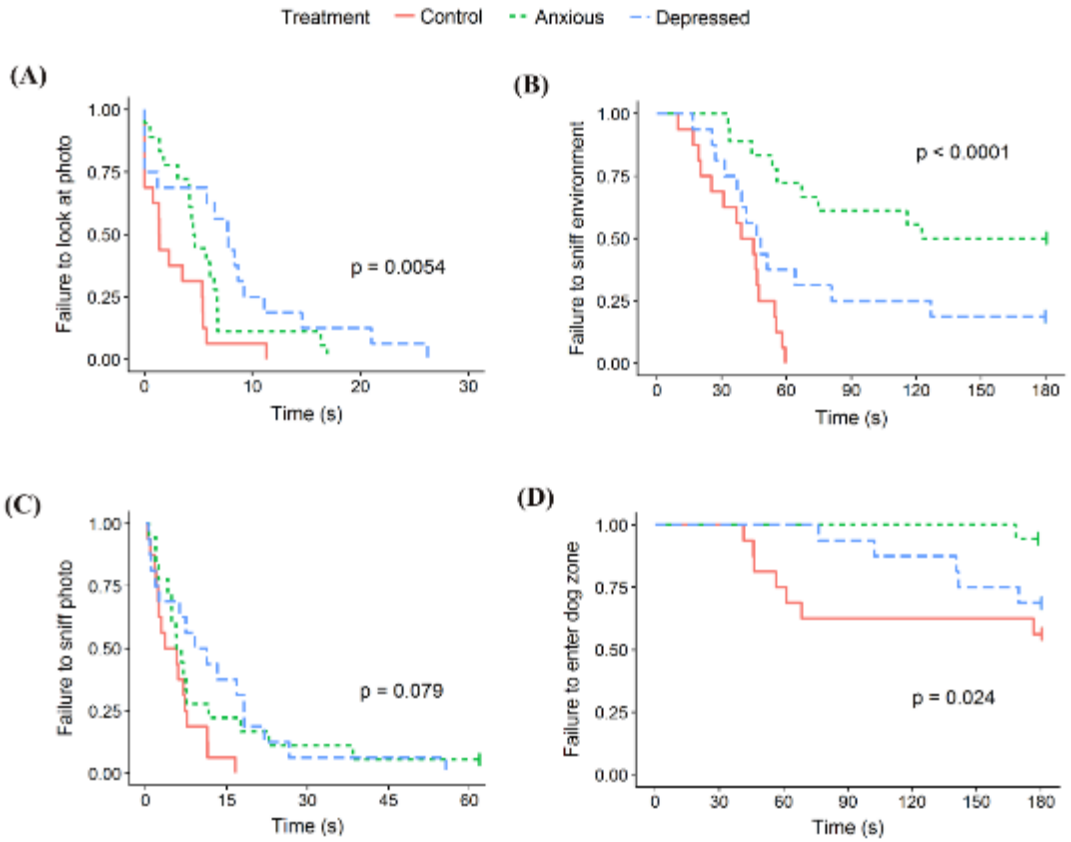

Figure 4.4. Kaplan-Meier curves for latency to look at the photo (A), sniff the photo (B), sniff the environment (C) and enter the zone closest to the dog (D).

Each time an animal exhibited the given behavior, the probability on the Y axis drops. Latencies to look at and sniff the photo are given for the first 60 s of testing only as no further events occurred after this time. The censored result in the Anxious group for latency to sniff the photo remained censored for the duration of the 180 s test.

4.5.3.2. Vigilance and other behaviors expressed during testing

Anxious and Depressed groups were more vigilant than Control animals during the attention bias test (Table 4.5). The Anxious and Depressed groups did not differ. Latency to become non-vigilant only tended to differ between groups (X^2 (2) = 5.39, p=0.068).

Sheep in the Anxious and Depressed groups were less likely to sniff the photo than Control animals, but did not differ from one another (Tables 4.5 and 4.6). Anxious sheep were less likely to sniff the environment, spent more time standing near the photo, entered fewer zones and were less likely to enter the zone closest to the dog than Control

animals, while the Depressed group did not differ from Control animals for these behaviors (Figure 4.4, Tables 4.5 and 4.6). The sniff environment frequency of the Depressed group was intermediate between the Control and Anxious groups, but did not significantly differ from either treatment, while latency to sniff the environment differed significantly between all groups (Figure 4.4, Tables 4.5 and 4.6). Number of steps taken did not differ between treatment groups (40.5, 30.1 and 37.4 steps for Control, Anxious and Depressed groups respectively, P=0.33).

More sheep in the Control group vocalized than sheep in the Anxious and Depressed groups. This was consistent for both open mouthed vocalizations (8, 2 and 0 sheep in the Control, Anxious and Depressed groups respectively, P<0.001) and close mouthed vocalizations (12, 6 and 7 sheep respectively, P=0.05). Five sheep urinated during the attention bias test, all of which were in the Anxious group. No animals defecated during testing. Only 2 sheep approached and sniffed the closed dog window, both of which were in the Control group.

4.5.4. Body temperature

In the repeated measures analysis on body temperature data, the Treatment x Time interaction was significant (X^2 (16) = 50.8, p<0.001). The main effects were also significant for both treatment (X^2 (2) = 9.8, p = 0.008) and time (X^2 (8) = 900.2, p<0.001). Contrasts indicated that body temperature did not differ between groups at times -40 and -30 (P>0.1). At 15 mins post attention bias testing, the Anxious group had a significantly higher body temperature than the Depressed group (t (45) = -2.1, P = 0.044), but only tended to be higher than the Control group (t (45) = -1.9, P = 0.069). The Anxious group had a higher body temperature than the Control and Depressed groups at all other time points (Figure 4.5). The Control and Depressed groups did not differ at any time point.

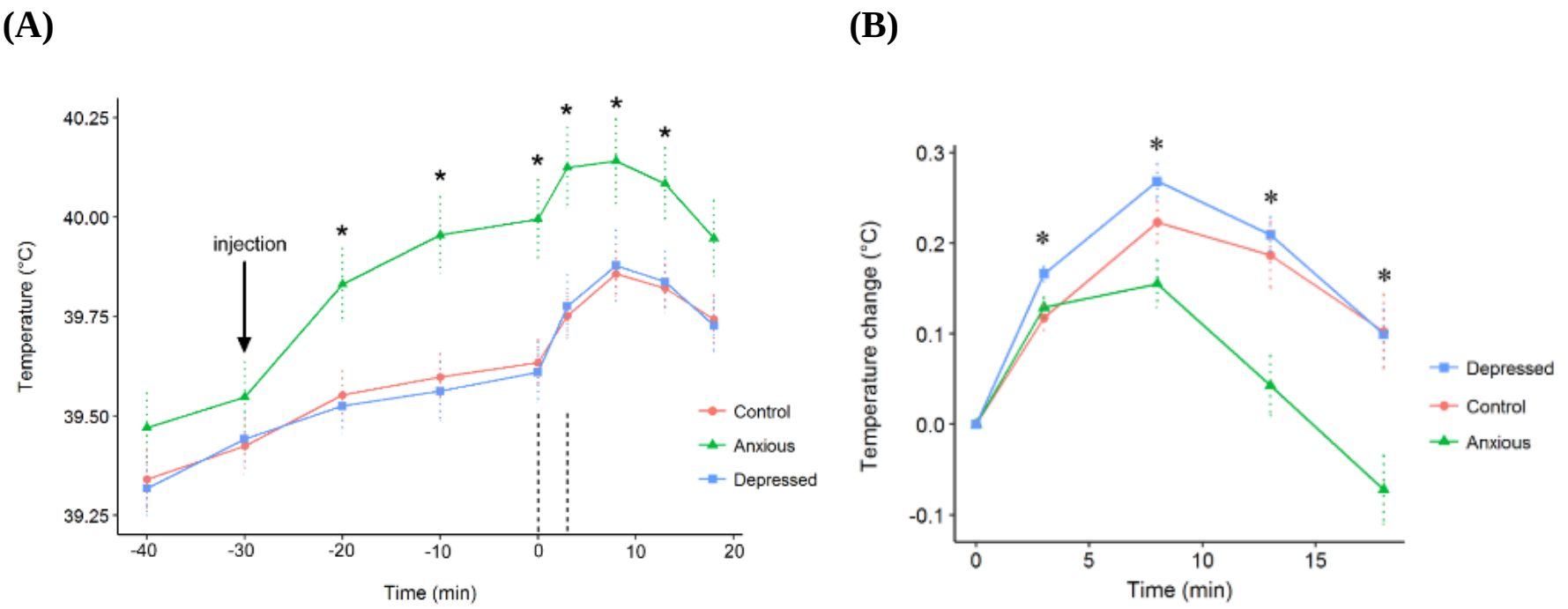

Figure 4.5. Mean (± se) body temperatures (A) and mean (± se) change in body temperatures (B) for the Control (●), Depressed (■) and Anxious (▲) groups.

The arrow denotes the time of injection prior to testing. The vertical dashed lines indicate the beginning and end of attention bias testing. (B) shows change in temperature from Time 0. The '*' symbol denotes a significant difference between the Anxious group mean and the other groups as determined using a repeated measures linear mixed models and post hoc tests.

The Treatment x Time interaction was significant for change in body temperature after attention bias testing (X^2 (8) = 57.9, p<0.001). The Depressed group showed a greater increase in body temperature than the other two groups immediately after attention bias testing (Time 3 min, LS mean 0.17°C), while the Control and Anxious groups did not differ (LS means 0.12 and 0.13°C respectively, P=0.008) (Figure 4.5). At all other time points, the Anxious group differed from the Control and Depressed groups (P<0.05) while the Control and Depressed groups did not differ (P>0.05).

4.5.5. Behavior in the first 60 s

No differences were found between the Control group and other treatments for any attention behaviors in the first 60 s of testing (P>0.05), however the Depressed group spent more time looking towards the dog wall than the Anxious group (Table 4.7). Results for latency to look at and sniff the photo did not differ from the full length test as no further events occurred after 60 s for these data. No differences were seen between groups for sniff photo frequency (Table 4.7).

Differences between treatment groups in the first 60 s of testing were consistent with the full length test for vigilance duration, time standing near the photo, sniff environment frequency and number of zones entered (Table 4.7). Latency to become non-vigilant significantly differed between treatment groups (likelihood ratio = 6.91, df=2, P=0.032), with Depressed sheep taking significantly longer to become non-vigilant than Control animals (mean latencies 48 and 35 s respectively, z=-2.51, P=0.012). The Anxious group did not differ from the Control or Depressed groups. Latency to sniff the environment differed significantly between all groups (likelihood ratio = 18.6, df=2, P<0.001).

Table 4.7. Mean ± s.e.m. behavioral responses of sheep during the first 60 s of the attention bias test.

Behavioral measure	Control	Anxious	Depressed	Test value	P value
Attention to dog window (s)	9.4 ± 1.0	9.1 ± 1.5	9.7 ± 1.1	$F_{(2,47)} = 0.6$	0.58
Attention to dog wall (s)	9.4 ± 1.3^{ab}	7.1 ± 1.3^{a}	13.0 ± 1.3^{b}	$F_{(2,47)} = 5.23$	0.008
Attention to photo (s)	15.3 ± 1.5	15.4 ± 1.4	11.3 ± 1.5	$F_{(2,47)} = 2.54$	0.089
Attention to photo wall (s)	19.4 ± 1.8	20.2 ± 1.7	15.7 ± 1.8	$F_{(2,47)} = 1.83$	0.17
Vigilance (mean rank duration) [1]	13.9 ± 2.4^{a} (54.9)	27.8 ± 3.3^{b} (57.8)	34.6 ± 2.8^{b} (58.9)	$X^2_{(2)} = 17$	<0.001
Standing near photo (s)	23.8 ± 3.6^{ab} (48.1)	34.7 ± 2.7^{a} (55.6)	16.8 ± 3.1^{b} (41.3)	$X^2_{(2)} = 13$	0.001
Sniff photo (*n*) [2]	1.3 ± 0.1 (3.8)	1.0 ± 0.1 (2.8)	1.0 ± 0.2 (2.8)	$X^2_{(2)} = 2.9$	0.23
Sniff environment (*n*) [2,3]	0.7 ± 0.2 (2.1)^{a}	-1.3 ± 0.5 (0.3)^{b}	-0.3 ± 0.3 (0.8)^{a}	$X^2_{(2)} = 28.1$	<0.001
Zones entered (*n*) [2]	1.25 ± 0.1^{a} (3.5)	0.61 ± 0.2^{b} (1.8)	1.32 ± 0.1^{a} (3.8)	$X^2_{(2)} = 13.5$	<0.001

[a,b] Different superscripts within rows indicate a significant difference between treatments as determined using post-hoc analyses
[1] Mean ranks are given, raw means are given in parentheses
[2] Least squares means are given on the log scale, back-transformed means are given in parentheses
[3] Generalized linear model fitting a Poisson distribution can generate negative least squares means on the log scale

4.6. Discussion

Sheep in an induced anxious state displayed an attention bias towards the positive stimulus and away from the threat compared to control animals, which contrasts with our hypothesis and previous studies (Lee et al., 2016; Monk et al., 2018). We suggest the differences in animal responses between the current and previous methods are likely due to the social aspect of the new positive stimulus. The threat provided by the test procedure is acutely stressful, as indicated by the stress-induced hyperthermia (SIH) seen in all treatment groups. Acute stress responses involve allocation of resources away from non-essential functions, such as feeding behavior, towards biological functions that aid survival or escape (Sherwood et al., 2005), which for a gregarious species with a strong flocking instinct can involve locating and staying with conspecifics (Lynch et al., 1992). Thus, attention towards conspecifics may be an appropriate response for sheep in a threatening situation, and be enhanced by anxiety. Modification of the test to replace feed with a photo changed the direction of the attention bias in anxious animals, indicating a context specific interpretation of responses is required for different attention bias test designs.

Depressed sheep showed greater attention towards the threat than Control animals as predicted, indicating the modified attention bias test may be used to assess states of depression in sheep. This result supports our hypothesis of an attention bias towards threat and/or away from positive stimuli in depressed individuals. Interestingly, the Depressed sheep showed an opposite bias in attention to that seen in Anxious sheep. If attention to conspecifics is an effective coping strategy when faced with a threat, as suggested above, then increased attention to threat in the Depressed group may represent an inappropriate coping response. This interpretation is supported by evidence that humans with depression are more likely to display maladaptive coping strategies to stressful situations than healthy individuals (Billings and Moos, 1984; Nolen-Hoeksema et al., 1999). On the other hand, enhanced attention to the threat may suggest the Depressed animals were less anxious during the test than Control animals. This interpretation is not supported by changes in body temperature (SIH) following testing, where the Depressed group exhibited a small, but significantly greater increase in body temperature, suggesting they were more stressed by the testing procedure than the Control sheep (Bouwknecht et al., 2007). Alternatively, or perhaps additionally, reduced attention towards the positive stimuli might reflect symptoms of anhedonia or

social withdrawal which are observed in humans with depression (Guze, 1995) and in depression-like animal models (Barr and Phillips, 1999; Moreau et al., 1992). In any case, sheep in both Anxious and Depressed states displayed attention biases which significantly differed from the Control group. The bidirectional nature of the attention bias between the different types of negative states could potentially make interpretation of animal responses difficult during future application of the test. Consideration of other behaviors during the test may help to differentiate the affective states of the animals.

Amongst other behaviors expressed during the test, both Anxious and Depressed sheep appeared to show signs of an increased fear response compared to the Control group. Vigilance behavior, typically associated with fearful and anxious states (Wemelsfelder and Farish, 2004), was higher in the Anxious and Depressed groups. Stress-induced hyperthermia and an increased urination frequency observed in the Anxious group are signs of autonomic hyperarousal, consistent with an anxious state and the findings of previous studies (Erhard et al., 2006; Lee et al., 2017; Pedernera-Romano et al., 2010; Sherwood et al., 2005). A greater increase in body temperature during testing in the Depressed group suggests an increased stress response to the test itself. Anxious and Depressed groups vocalized less than control animals, which may indicate fearfulness in contexts where a potential predator is present (Beausoleil et al., 2005; Romeyer and Bouissou, 1992). Anxious sheep were also least likely to sniff the environment and photograph, again suggesting that they were more fearful (Beausoleil et al., 2005, 2012; Romeyer and Bouissou, 1992). No differences were seen between any of the groups for number of steps taken, which is consistent with previous studies in sheep (Doyle et al., 2011; Lee et al., 2016; Monk et al., 2018). Locomotion behaviors can be context specific and difficult to interpret due to an inability to distinguish between conflicting motivations such as exploratory or fleeing behaviors (Romeyer and Bouissou, 1992). As such, the sniffing behaviors may better reflect exploration or interest in the stimuli and environment during the attention bias test. Overall, both Anxious and Depressed animals showed signs of increased fear during the test compared to control animals. In the context of the modified attention bias test, key fear related responses such as vigilance and exploratory behavior may be used to determine when an animal is in a more negative affective state, then the attention behaviors may be used to differentiate between states of depression and anxiety. Considering these behaviors together, as well as the context in which the test is applied, will allow for a greater understanding of the affective states of animals using the modified attention bias test.

The alternative positive stimulus appears to have been a successful replacement for food in the attention bias test, allowing for a more standardized application of the test and clearer interpretation of results when using pharmacological treatments. All but one animal had sniffed the photograph within the first 60 s of the attention bias test, indicating an absence of fear towards this stimulus (Beausoleil et al., 2005, 2012; Romeyer and Bouissou, 1992). Animals also spent a high proportion of their time in the test standing next to the photograph, suggesting the photograph was perceived as positive by the test animals and may have been recognized as a conspecific. The modified method removed variation from appetite and feeding motivation from the test, however it also potentially added variation from social motivation to the test. We suggest the former is likely to have a greater impact on results, with variation occurring not only between animals, but also within animals over time as they spend more time without feed prior to testing. As such, we suggest the modified method is an improvement over the original method, however further studies to directly compare the test designs would be useful to confirm this suggestion. Further, in the original method, sheep could position themselves in the arena so that they could look towards the dog window while eating food, making it difficult to differentiate attention to the positive versus negative stimuli. In the modified method, attention to the threat and attention to the positive stimulus are mutually exclusive, allowing for clearer interpretation of behavior. If future studies choose to use food as a positive stimulus, we suggest the food should be positioned in a way that sheep cannot easily look at the threat while eating, to more clearly differentiate their allocation of attention between these stimuli.

When interpreting the behavioral responses observed in the test, it is important to consider some of the other factors which may have impacted on animal behavior. The test procedure itself may induce some level of anxiety, however exposure to the test as an environmental source of anxiety was consistent across all treatment groups in the current study. For simplicity, we have assumed that an animal's displayed response is most strongly determined by the affective state it brings to the test arena, rather than by a new affective state arising from exposure to environmental stimuli during the course of the test. This assumption is supported by the treatment differences observed in both the current and previous studies, between groups with differing initial affective states (Lee et al., 2016; Monk et al., 2018). Whether the initial affective state of the animal further influences its behavioral response to anxiety-inducing components of the test procedure remains unknown.

Another factor which may have influenced behavioral responses during the test is the use of pharmacological treatments. Some of the animals in the Anxious group showed some form of abnormal behaviors in response to the drug treatment. Abnormal behaviors have been observed previously in young sheep administered mCPP at a dose rate of 2mg/kg (Doyle et al., 2015; Monk et al., 2018) but had not previously been observed in adult sheep using the same dose rate (Lee et al., 2009, 2016). In the current study, most of the behaviors observed were tail or head shakes, which we expect did not impact on other behaviors in the test. However a few sheep showed signs of ataxic gait, where it appeared they failed to lift their rear foot enough when stepping and therefore stumbled. If some of the sheep were having difficulty walking, this may have reduced behaviors related to locomotion such as number of steps and escape attempts. During visual inspection of the data, stumbling behaviors did not appear to have a pronounced impact on number of steps taken during the test, with no mCPP treated sheep appearing as outliers within the stepping data. Nevertheless, it cannot be ruled out that locomotion was impacted by the drug. Although the abnormal behaviors observed in this study were mild, it is suggested further studies using mCPP in sheep should use a lower dose rate of 1 to 1.5 mg/kg, as suggested by Doyle et al. (2015), regardless of animal age.

Further validation and refinement of the modified methodology will help to improve the application of the attention bias test in future studies and increase our understanding of animal responses during testing. The current study demonstrated that states of depression and anxiety can influence responses during the test, however the effects of other types of affective states on test performance need to be ascertained through further study. The influence of positive affective states could be of particular interest, as the presence of positive affective states make up an important component of animal well-being but have so far been relatively understudied when compared to negative states (de Vere and Kuczaj, 2016). A guiding principle for additional refinements to the test method should be to ensure its utility for application on farms. Monk et al. (2018) suggested that the original test duration could be shortened to enable its application to larger numbers of animals. However the results of the current study suggest this would not be appropriate for the modified attention bias test method, as attention behaviors did not differ between groups during the first 60 s of testing. Automation of behavioral measurement during the test may facilitate collation of behavioral data, which can be a lengthy and labor intensive process. Finally, any modifications of the test arena design

should ensure the test is suitable for use in existing handling facilities in on-farm settings.

4.7. Conclusion

The modified attention bias test presented in the current study potentially offers a method for assessing different types of negative affective states in sheep. Further, by assessing the direction in which an attention bias occurs, towards or away from the threatening stimulus, we may be able to differentiate between distinct anxiety and depression-like states. Modification of the method to replace the food with a photograph of a conspecific allows for more standardized application of the test and eliminates variation in results caused by changes in feeding motivation. Overall, these results support the use of the modified attention bias test for further research of affective states in sheep and potentially other livestock.

4.8. Conflicts 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.

4.9. Author Contributions

CL attained the funding for the project, JEM and CL contributed to the conception and design of the study, JEM and SB performed the experiments, JEM performed the statistical analysis, JEM wrote the first draft of the manuscript, CL, IC and SB assisted with interpretation of results and revision of the manuscript, all authors read and approved the submitted version of the manuscript.

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4.12. Data Availability Statement

The dataset generated for this study can be found in the CSIRO data access portal (DAP) [https://doi.org/10.25919/5b5016c4bca25].

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Chapter 5

The influence of pharmacologically-induced affective states on attention bias in sheep

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5.1. Abstract

When an individual attends to certain types of information more than others, the behavior is termed an attention bias. The occurrence of attention biases in humans and animals can depend on their affective states. Based on evidence from the human literature and prior studies in sheep, we hypothesized that an attention bias test could discriminate between pharmacologically-induced positive and negative affective states in sheep. The test measured allocation of attention between a threat and a positive stimulus using key measures of looking time and vigilance. Eighty 7-year-old Merino ewes were allocated to one of four treatment groups; Anxious (m-chlorophenylpiperazine), Calm (diazepam), Happy (morphine) and Control (saline). Drugs were administered 30 min prior to attention bias testing. The test was conducted in a 4 x 4.2 m arena with high opaque walls. An approximately life-size photograph of a sheep was positioned on one wall of the arena (positive stimulus). A small window with a retractable opaque cover was positioned on the opposite wall, behind which a dog was standing quietly (threat). The dog was visible for 3 s after a single sheep entered the arena, then the window was covered and the dog was removed. Sheep then remained in the arena for 3 min while behaviors were recorded. Key behaviors included time looking towards the dog wall or photo wall, duration of vigilance behavior and latency to become non-vigilant. In contrast with our hypothesis, no significant differences were found between treatment groups for duration of vigilance or looking behaviors, although Anxious sheep tended to be more vigilant than Control animals ($P<0.1$) and had a longer latency to become non-vigilant ($P<0.001$). Twenty-four of 80 animals were vigilant for the entire test duration. This censoring of data may explain why no differences were detected between groups for vigilance duration. Overall, a lack of difference between groups may suggest the test cannot discriminate positive and negative states in sheep. We suggest that the censoring of vigilance duration data, the use of insufficient drug doses, the potential influence of background noise and the age of the sheep may explain a lack of difference between groups. Due to these potential effects, it remains unclear whether the attention bias test can detect positive states in sheep.

5.2. Introduction

The assessment of attention biases in non-human animals may allow us to gain a better understanding of the underlying affective states that relate to their welfare. An attention bias occurs when an animal attends to certain types of information before or for longer than others. Attention biases are determined by the salience of the information, or its perceived importance to the individual, and can be influenced by the animal's transient emotional states and sustained by moods. For example, humans in anxious states pay more attention towards threatening information than non-anxious individuals (Bradley et al., 1995; Bradley, Mogg & Lee, 1997; Bar-Haim et al., 2007). Based on this principle, studies were conducted to determine whether an attention bias test developed for sheep was sensitive to changes in anxiety-like states (Lee et al., 2016; Monk et al., 2018b). The test measured time allocation of attention between a dog (threat) and a food reward (positive). The studies found that sheep in a pharmacologically-induced anxious state paid more attention towards the threat, evidenced by an increase in time spent looking towards the previous location of the dog and increased vigilance behavior compared to control animals. Further, sheep in an induced anxious state were less likely to feed than control animals. In comparison with controls, an induced calm state reduced time looking towards the threat, decreased vigilance and increased likeliness to feed. These results suggested the test could be used to assess anxious affective states in sheep. It remained unclear whether the pharmacological treatments modelled transient states or long-term moods, and whether the test could differentiate these types of states. Hereafter, we use the term affective states to encompass both transient emotions and moods.

The attention bias test for sheep was later modified by replacing the food with a photograph of a conspecific, to remove the potential influence of appetite on animal behavior (Monk et al., 2018a). In the modified method, sheep in pharmacologically-induced anxious and depressed states were more vigilant than control animals. However, the anxious group showed an attention bias towards the photograph rather than the threat. This unexpected result was attributed to the social aspect of the alternative positive stimulus and highlighted a need for context specific interpretations of behavioral responses. It appeared that within the context of the modified method, vigilance and exploratory behaviors, such as latency to sniff the environment, could be used to determine whether an animal was in a more negatively valenced affective state.

The direction of attention could be used to discriminate anxious and depressed states from a neutral state. Although the interpretation of responses changed between test methods, each study demonstrated that an attention bias test could be used to assess and differentiate contrasting affective states in sheep, thus providing a new approach for researchers to better understand the affective states of livestock.

While many animal studies have focused on reducing the occurrence of negative affective states, the presence of positive affective states also comprises an important, but relatively understudied component of animal well-being (de Vere & Kuczaj, 2016). A number of human studies have demonstrated attention biases towards rewarding stimuli in subjects experiencing positive moods (e.g. Tamir & Robinson, 2007; Grafton, Ang & MacLeod, 2012; Sanchez & Vazquez, 2014; Caudek, Ceccarini & Sica, 2017), although these results have not been consistent in all tested populations (Isaacowitz et al., 2008). In sheep, Lee et al. (2016) and Monk et al. (2018b) demonstrated that the anxiolytic drug diazepam induced an attention bias away from a threatening stimulus and towards a food reward compared to saline treated control animals. This suggests that the attention bias test may be used to assess positive, or at least less negative, affective states in sheep. However, it could not be confirmed whether increased attention towards the food was due to the induced affective state or an increase in appetite caused by the drug (Foltin, 2004; Gaskins, Massey & Ziccardi, 2008). The influence of a non-negative affective state on behavior in the modified attention test has not yet been established.

Pharmacological agents can be useful for modelling different types of affective states in animals, in a more standardized and repeatable manner than many environmental manipulations (Mendl et al., 2009; Doyle et al., 2015). Further, drugs can be used that remain active for the duration of testing, and provide information on the mechanisms and pathways underpinning animal behavior. Few studies have used pharmacological treatments in sheep to induce and assess positive affective states. The anxiolytic drug diazepam was used to reduce anxiety-like behaviors during development of the original attention bias test method (Lee et al., 2016; Monk et al., 2018b). Further, it has been used to reduce anxiety-like behaviors in a range of other contexts, such as during isolation and suddenness tests (e.g. Drake, 2006; Destrez et al., 2012). It has also been shown to attenuate stress-induced hyperthermia in other species, consistent with a less anxious state (Olivier et al., 2003; Bouwknecht, Olivier & Paylor, 2007; Lee et al., 2017). However, for each of these studies it is difficult to determine whether the state

induced by diazepam was truly positive as opposed to neutral or simply less negative than the other treatments. Verbeek et al. (2014) used the opioid agonist morphine to induce a positive affective state in sheep, due to its association with feelings of euphoria in humans (Riley et al., 2010). In sheep, the drug was found to enhance an optimistic judgement bias observed after feeding, consistent with having induced a more positive affective state (Verbeek et al., 2014). Therefore, morphine may be a useful pharmacological agent for confirming whether an attention bias test can discriminate euphoric-like positive affective states in sheep.

The aim of the current study was to determine whether the modified attention bias test could discriminate between positive and negative affective states in sheep. Specifically, we aimed to compare a high-anxiety state (Anxious), a low-anxiety state (Calm), a euphoric-like state (Happy) and controls, induced using the drugs m-chlorophenylpiperazine, diazepam, morphine and saline respectively. We hypothesized that the Anxious group would be more vigilant than control animals while the Calm and Happy groups would be less vigilant, in line with previous studies (Lee et al., 2016; Monk et al., 2018a,b). Further, we hypothesized that the Anxious group would show an increased body temperature response to the injection and would spend more time looking towards the positive stimulus during testing (photograph of a conspecific), as shown by Monk et al. (2018a). Given the differences in interpretation of behavioral responses between the original and modified test methods, it was difficult to predict the direction of attention for the Calm and Happy groups. However, our preliminary hypothesis was that they would both also pay more attention towards the positive stimulus (photo), in line with the human literature. We did not have an *a priori* hypotheses for how the Calm and Happy groups might differ from one another in vigilance or attention paid to the dog or photo. However, we did expect these groups would differ in behavioral measures of arousal such as activity and vocalizations, with the Happy group showing signs of a higher arousal state than the other groups, consistent with previous studies (Verbeek et al., 2012, 2014).

5.3. Materials & Methods

5.3.1. Animal Ethics

The protocol and conduct of the experiments were approved by the CSIRO F.D. McMaster Laboratory Animal Ethics Committee (AEC18-17), under the New South Wales Animal Research Act 1985.

5.3.2. Experimental design

To test whether the attention bias test could differentiate between different types of affective states, drugs were used to pharmacologically induce contrasting positive and negative affective states in ewes prior to testing. Specifically, drugs were used to induce high-anxiety states (Anxious), low-anxiety states (Calm), euphoric-like states (Happy) and control states (Control).

Eighty Merino ewes were weighed and randomly distributed between the treatment groups balancing for bodyweight. Sheep then had numbers painted on their wool for individual identification and were divided into two cohorts (n=40 per group) to be tested on separate days for logistical reasons. Treatment groups were evenly distributed between the two cohorts. All injections and tests for the 40 animals studied on a given day, occurred between 8:00 am and 1:30 pm. The experiment was conducted during October 2018.

5.3.3. Animal details

The sheep used in this experiment were non-lactating, non-pregnant Merino ewes, approximately 7 years old, with average bodyweight of 47.0 ± 5.4 kg. Sheep were managed extensively throughout their life and were kept at pasture prior to testing. Sheep had prior experience with dogs during routine on-farm management when being moved between paddocks and handling facilities, but had no experience with the attention bias test. All sheep were bred, raised and tested on the same farm in Armidale, Australia.

5.3.4. Drug details

All drugs were administered as a single intramuscular (i.m.) injection into the rump of the animal, 30 min prior to testing in the attention bias test. An anxiety-like state was induced in the Anxious group using the anxiogenic drug m-chlorophenylpiperazine (mCPP; Tocris, Bristol, UK). This drug has previously been shown to significantly impact on animal behavior 30 mins after i.m. administration (Lee et al., 2016; Monk et al., 2018a,b). The mCPP was administered at a dose rate of 1.5mg/kg. This is a reduced rate compared to previous studies, as recommended by Monk et al. (2018a), due to the observation of abnormal behaviors at the higher dose rate of 2mg/kg. Prior to treatment, mCPP was dissolved in BP Water for Injection (Baxter, Old Toongabbie, Australia) at a rate of 60 mg/ml. A calm-like state was induced in the Calm group using the anxiolytic

drug diazepam (Troy Laboratories, Sydney, Australia). The diazepam was administered at a dose rate of 0.1mg/kg as per previous studies (Lee et al., 2016; Monk et al., 2018b). The drug was administered i.m. rather than intra-venously (i.v.) for consistency across treatment groups. Studies in humans have shown complete bioavailability of diazepam after i.m. injection, with rapid absorption from muscle peaking at around 30 min (Divoll et al., 1983; Moore et al., 1991). A euphoric-like state was induced in the Happy group using the drug morphine (Hospira, Melbourne, Australia). The morphine was administered at a dose rate of 1mg/kg as per previous studies (Verbeek et al., 2012, 2014). Plasma morphine concentration is shown to peak within 20 min after i.m. administration in humans, with 100% systemic availability (Stanski, Greenblatt & Lowenstein, 1978). In sheep, Morphine is shown to have a half-life of approximately 119 min in blood plasma after i.v. administration (Bengtsson et al., 2009). The Control group were given 1ml of BP saline i.m. (Baxter, Old Toongabbie, Australia).

5.3.5. Attention bias test

The current study used the same attention bias test described by Monk et al. (2018a) (Figure 5.1). Briefly, the test arena consisted of a concrete yard surrounded by 1.8 m high opaque walls. There was a small window located on one wall of the arena, which could be completely covered with a retractable opaque cover. Directly opposite the window was an approximately life-size photograph of an unfamiliar female conspecific. Once a sheep entered the arena, an unfamiliar dog was visible to the sheep through the window for 3 s, then the window was covered and the dog was removed. The sheep then remained in the arena for 3 min, while behaviors were recorded using a Sony Handicam video camera (Sony, Australia, model number HDR-XR550) that was positioned above the test arena in an adjacent building. A line of metal panels were positioned inside the arena so that sheep could not move into the corners out of view of the camera (Figure 5.1). The total accessible area of the arena was 4 x 4.2 m.

Figure 5.1. Photograph of the attention bias test immediately after the test sheep entered the arena.

The dog was visible for 3 s, then a retractable opaque cover was lowered over the window and the dog was removed. Sheep remained in the test for 3 mins. The "#" symbol indicates the entrance of the arena, a camera was positioned above the arena to the right of the photograph (not visible in photograph). For a schematic diagram of the arena, see Monk et al. (2018a). Photo credit: Jessica Monk. Permission to publish has been given from the subject depicted in the photo.

5.3.6. Behavioral measures in the attention bias test

The behaviors recorded in the attention bias test are summarized in Table 5.1. Most behaviors were collated from video footage using The Observer XT 12.0 (Noldus Information Technology, Wageningen, The Netherlands). Behaviors were continuously recorded for the test duration. During the video analysis the test arena was divided into 9 grid sections (zones) which were overlaid on video footage, to calculate zones crossed, zones entered and zone durations (Table 5.1). Open- and close-mouthed vocalizations were scored on the day of testing by an experienced hidden observer, who was positioned behind the opaque matting out of view of the sheep. The same observer also scored the dog's behavior on a 3 point scale as: 1 – quietly stood still, 2 – lunged or crouched down or 3 – barked or growled at the sheep with any posture. A score of 3 was given on 6 occasions, for 1 animal in the Control group, 2 animals in the Anxious group and 3 animals in the Calm group.

Table 5.1. Ethogram of behaviours recorded during the attention bias test (Monk et al., 2018a)

Behavior	Definition
Attention	The direction in which the sheep is looking with binocular vision (Lee et al., 2016; Piggins and Phillips, 1996). The test arena was divided into 4 areas of attention: dog wall, photo wall, door wall and back wall. Total duration of attention was recorded for the dog and photo walls. Duration looking at the other walls were not analyzed as these areas were not central to our hypotheses.
Vigilance	Time spent with the head at or above shoulder height (Frid, 1997; Lee et al., 2016). Latency to become non-vigilant was also calculated.
Sniff photo	Number of times and latency to sniff the photo
Sniff environment	Number of times and latency to sniff the floor or walls of the test arena
Vocalizations	Number of open mouthed bleats and close mouthed bleats were recorded separately
Zones crossed	Number of zones crossed with both front feet placed into the new zone, or one front foot in the zone and the other on the line
Zones entered	Number of zones entered (1 to 9)
Zone duration	Total time spent in each of the 9 zones. Data for time spent in the zone closest to the photo were used for further analysis, as well as number of entries into the zone closest to the dog window.
Urinations	Number of urinations

Sheep treated with mCPP were also monitored for abnormal behaviors previously described by Doyle et al. (2015). These included ataxic gait, tail shaking, head shaking, body shaking and head rolling. Tail shaking was observed in 4 sheep during testing. No other abnormal behaviors were observed during testing.

5.3.7. Internal body temperature

Internal body temperature was recorded using Thermochron iButtons® (Model number DS1922L-F5, accuracy 0.5°C, resolution 0.063°C, weight 3.3g; Embedded Data Systems, Lawrenceburg, USA). The iButtons were attached to blank (progesterone-free) Controlled Internal Drug Release devices (CIDR®, Zoetis, Melbourne, Australia) using polyolefin heat-shrink tubing, as described by Lea et al. (2008). A CIDR was inserted into the vagina of each sheep one day prior to testing using an applicator lubricated with obstetrical lubricant. The iButtons were set to log at an interval of 20 s beginning 30 min prior to attention bias testing. Data were extracted using the program eTemperature version 8.32 (OnSolution, Castle Hill, Australia). Data from 4 temperature loggers were missing due to technical faults.

Body temperature data were extracted at times -30, -20, -10, -1, 6, 11, 15, 21 and 26 min relative to the beginning of attention bias testing. For each time point, the average

of 3 consecutive temperature recordings were used. Times -1 and 6 min were identified as the average baseline and peak temperatures before and after attention bias testing across treatment groups.

5.3.8. Statistical Methods

Data were analyzed using R version 3.5.1 (R Core Team, 2018). P values less than 0.05 were considered significant and $P<0.1$ were considered a tendency towards significance. All model residuals were checked for normality and homoscedasticity using Shapiro-Wilks test for normality and visual assessment of Q-Q and residuals vs fitted values plots. Treatment group, test order within each test day and dog behavior score were fitted as fixed effects in all linear models. Test order and dog behavior did not reach significance in any of the models and were subsequently removed using a backward elimination approach, considering both the Akaike and Bayesion information criterion. Cohort (test day) was fitted as a random effect in all mixed effects models. The package *nlme* was used to fit linear mixed effects models (Pinheiro et al., 2016). The package *lme4* was used to fit generalized linear mixed effects models (Bates et al., 2015). Post hoc multiple comparisons were conducted using a Tukey method for adjustment of P values. Where significant differences were found between groups, effect sizes were estimated using Pearson's correlation coefficient *r* (Field et al. 2012). Estimates of effect size were not made for count data.

Attention and vigilance data were analyzed using linear mixed effects models. Data for attention to photo wall and vigilance were log transformed to meet normality assumptions. A Kruskal-Wallis non-parametric ANOVA was also used to confirm the analysis of the vigilance data as the residuals were only marginally improved by transformation. Post hoc multiple comparison tests for the Kruskal-Wallis ANOVA were performed using the package pgirmess (Giraudoux, 2016).

Photo sniff frequency and number of zones entered were analyzed using generalized linear mixed effects models with a Poisson distribution for count data. Sniff environment frequency and zones crossed were analyzed using generalized linear mixed effects models with a negative binomial distribution due to evidence of over-dispersion. Vocalization data were analyzed using a negative binomial hurdle model using the package pscl, to account for the presence of excess zeros in the dataset (Zeileis, Kleiber & Jackman, 2008). Data for time spent in the zone closest to the photo were analyzed using a linear mixed effects model. The number of animals in each group that entered

the zone closest to the dog wall were analyzed using a Fisher's Exact Test. Post hoc multiple comparisons between groups were performed using the package *rcompanion* (Mangiafico, 2018). Urination data were also analyzed using a Fisher's Exact Test, examining the number of animals in each group that urinated.

All latency data were analyzed with Cox's proportional hazards model using survival analysis (Therneau & Grambsch, 2000; Therneau, 2015), as described by Monk et al. (2018b). These data included latencies to sniff the photo, sniff the environment and become non-vigilant. Animals that failed to perform each behavior within 180 s were deemed as censored results. Treatment and test day were fitted as fixed effects in all proportional hazards models.

All body temperature data were analyzed using a linear mixed effects model, fitting treatment, time and a treatment x time interaction as fixed effects. Sheep identity nested within test day was fitted as a random effect to account for the repeated measurements across time points. A subset of the body temperature data was taken from time -1 min onwards to better assess the influence of attention bias testing itself on body temperature responses. Change in body temperature from time -1 min was analyzed in the same way as the total temperature dataset, fitting a linear mixed effects model to account for repeated measures over time.

5.4. Results

5.4.1. Attention and vigilance

Raw attention and vigilance duration data are summarized in Figure 5.2. Linear mixed effects models showed that duration of attention towards the dog and photo walls did not differ significantly between treatment groups (Table 5.2 and Figure 5.3). However, the Anxious and Calm groups tended to spend less time looking towards the dog wall than the Control and Happy groups ($P<0.1$).

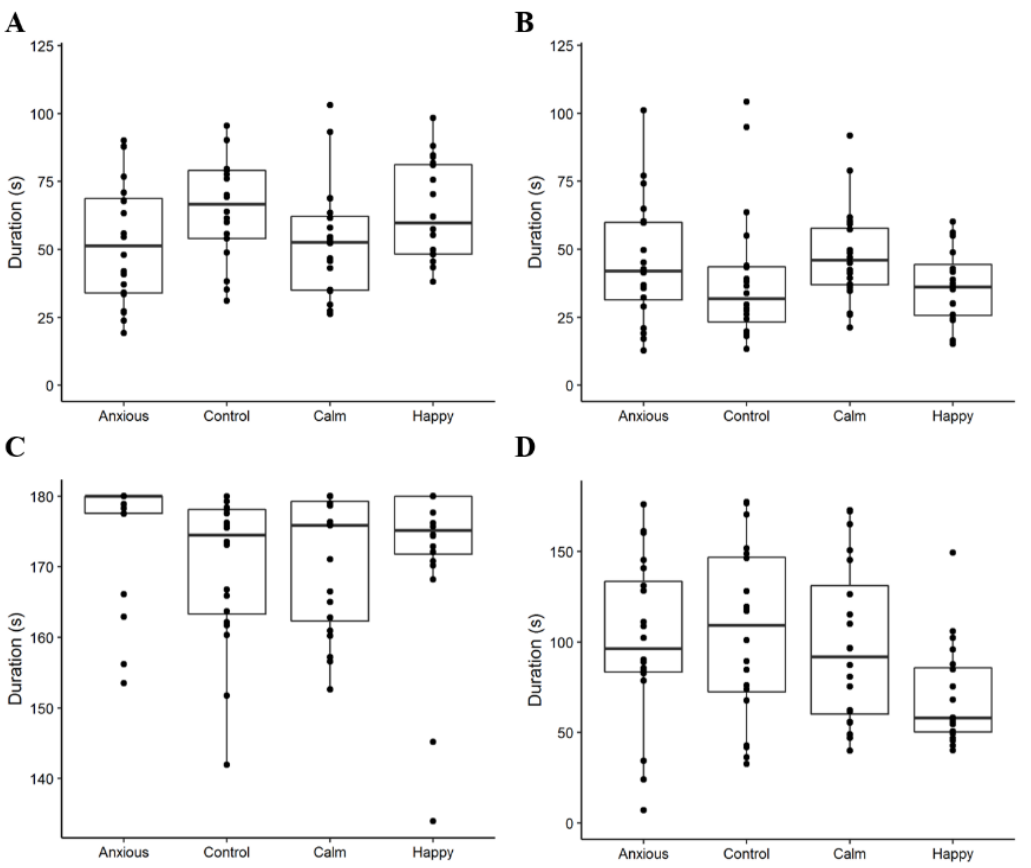

Figure 5.2. Boxplots displaying the distribution of observed duration data.

Boxplots show the median durations, the interquartile range (IQR) and the range of data within 1.5 x the IQR for duration of attention to the dog wall (A), attention to the photo wall (B), vigilance (C) and time spent standing in the zone closest to the photo (D). The dots represent raw duration data for each individual sheep within the treatment groups. We note that the plot axes are scaled differently to more clearly display the data within each observed variable.

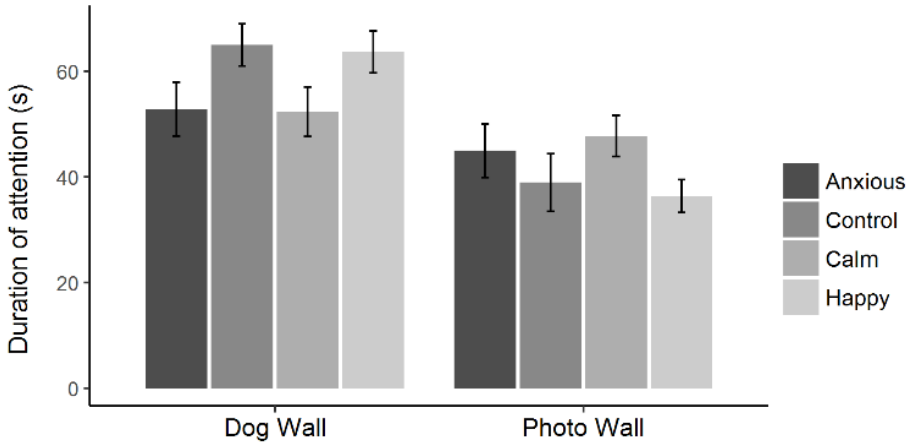

Figure 5.3. Mean ± s.e.m. time spent looking towards the dog wall and the photo wall for each treatment group during attention bias testing.

The times spent looking towards the dog and photo walls were analysed using linear mixed effects models, fitting treatment as a fixed effect and test day as a random effect.

Table 5.2. Mean ± s.e.m. behavioral responses of sheep in each treatment group during the attention bias test.

Behavioral measure	Anxious	Control	Calm	Happy	Test method	Test value (df)	P value
Attention to dog wall (s)	53.3 ± 6.7	64.9 ± 6.7	52.3 ± 6.7	63.6 ± 6.7	LME	F (3,76) = 2.4	0.075
Attention to photo wall (s) [1]	3.7 ± 0.2 (38.9)	3.5 ± 0.2 (33.4)	3.8 ± 0.2 (44.8)	3.5 ± 0.2 (33.6)	LME	F (3,76) = 2.0	0.123
Vigilance (s) [1, 2]	5.1 ± 0.1 (175.6)	5.1 ± 0.1 (169.7)	5.1 ± 0.1 (170.5)	5.1 ± 0.1 (171.6)	LME	F (3,76) = 0.6	0.30
Vigilance (mean rank)	52.5 ± 5.0	33.2 ± 4.5	36.5 ± 5.3	39.9 ± 4.9	KRUS	X2 (3) =8.1	**0.04**
Sniff photo (n) [1]	1.0 ± 0.1 (2.7)^{a}	1.4 ± 0.1 (4.0)^{ab}	1.6 ± 0.1 (4.7)^{b}	1.1 ± 0.1 (3.1)^{a}	GLME_P	X2 (3) = 14.2	**0.016**
Sniff environment (n) [1]	0.0 ± 0.4 (1.0)^{a}	1.1 ± 0.4 (2.9)^{ab}	1.3 ± 0.4 (3.6)^{b}	1.0 ± 0.5 (2.7)^{ab}	GLME_NB	X2 (3) = 9.9	**0.02**
Sniff closed window (n) [3]	1	1	1	4	FET	N/A	0.34
Zones crossed (n) [1]	2.7 ± 0.1 (14.1)^{a}	2.9 ± 0.1 (17.9)^{a}	2.8 ± 0.1 (16.5)^{a}	3.5 ± 0.1 (32.7)^{b}	GLME_NB	X2 (3) = 22	**<0.001**
Zones entered (n) [1]	1.5 ± 0.1 (4.5)^{a}	1.7 ± 0.1 (5.1)^{a}	1.7 ± 0.1 (5.5)^{ab}	2.0 ± 0.1 (7.5)^{b}	GLME_P	X2 (3) = 17.2	**<0.001**
Standing near photo (s)	102.8 ± 16.5^{ab}	105.0 ± 16.5^{a}	98.2 ± 16.5^{ab}	70.4 ± 16.5^{b}	LME	F (3,76) = 3.3	**0.025**
Enter zone close to dog (n) [3]	4^{a}	6^{ab}	7^{ab}	14^{b}	FET	N/A	**0.009**
Open-mouthed bleats (n)	0.7 ± 0.5^{a}	1.5 ± 0.6^{a}	3.3 ± 1.2^{a}	16.5 ± 2.6^{b}	GLME_NB_H	X2 (3) = 11.5	**0.009**
Close-mouthed bleats (n)	2.8 ± 0.6	4.1 ± 0.6	6.0 ± 0.9	7.4 ± 0.7	GLME_NB_H	X2 (3) = 4.0	0.26
Urinations (n) [3]	7	3	2	4	FET	N/A	0.31

[a,b,c] Different superscripts within rows indicate a significant difference between treatments as determined using post-hoc analyses, significant P values are emphasized with bold font

[1] Least squares means are given on the log scale, back-transformed means are given in parentheses

[2] Vigilance duration was censored at 180 s

[3] Raw number of animals of a total of 20 in each group are given

LME; linear mixed effects model fitting test day as a random effect, KRUS; Kruskal-Wallis one-way analysis of variance, GLME; generalized linear model with a Poisson (P) or Negative Binomial (NB) distribution, data including excess zeros used hurdle models (H), FET; Fisher's Exact Test was used to calculate probability, test statistic is not applicable (N/A), post hoc analyses were performed using the package *rcompanions*

Table 5.3. Hazard ratios for latency to sniff the photo, sniff the environment and become non-vigilant as affected by treatment group.

Latency to	Group	Mean (s)[1]	Censored (*n*)[2]	Coefficient[3]	SE (coeff)	Hazard ratio[4]	Wald (z)	P	Likelihood Ratio	df	P
Sniff photo	Control	19.1[a]	1	**Reference**					18.4	3	0.001
	Anxious	65.7[b]	4	-1.188	0.36	0.31 (0.15-0.62)	-3.3	0.001			
	Calm	16.4[a]	0	-0.095	0.33	0.91 (0.48-1.73)	-0.29	0.772			
	Happy	18.9[a]	0	-0.299	0.33	0.74 (0.39-1.43)	-0.89	0.372			
	Anxious			**Reference**							
	Calm			1.093	0.35	2.98 (1.49-5.97)	3.09	0.002			
	Happy			0.889	0.35	2.43 (1.23-4.81)	2.56	0.010			
	Calm			**Reference**							
	Happy			-0.204	0.32	0.816 (0.44-1.53)	-0.63	0.526			
Sniff environment	Control	102.4[a]	5	**Reference**					20.5	3	<0.001
	Anxious	147.3[b]	12	-1.189	0.44	0.3 (0.12-0.72)	-2.69	0.007			
	Calm	102.5[a]	8	-0.256	0.39	0.77 (0.36-1.65)	-0.66	0.510			
	Happy	91.2[a]	4	0.128	0.36	1.13 (0.56-2.3)	0.35	0.723			
	Anxious			**Reference**							
	Calm			0.934	0.46	2.54 (1.03-6.27)	2.03	0.043			
	Happy			1.317	0.44	3.73 (1.58-8.82)	3	0.003			
	Calm			**Reference**							
	Happy			0.384	0.38	1.46 (0.69-3.11)	1	0.317			
Non-vigilance	Control	65.5[a]	2	**Reference**					19.3	3	<0.001
	Anxious	132.9[b]	11	-1.522	0.42	0.21 (0.09-0.49)	-3.65	0.000			
	Calm	99.0[a]	5	-0.671	0.35	0.51 (0.25-1.02)	-1.9	0.058			
	Happy	100.2[a]	6	-0.674	0.36	0.51 (0.25-1.02)	-1.88	0.060			
	Anxious			**Reference**							
	Calm			0.851	0.42	2.34 (1.02-5.37)	2.01	0.044			
	Happy			0.848	0.43	2.33 (1-5.43)	1.97	0.049			
	Calm			**Reference**							
	Happy			-0.003	0.37	0.99 (0.48-2.06)	-0.01	0.993			

[1] Raw mean latencies are given, superscripts indicate significant differences between groups for each behavior
[2] Number of animals which failed to exhibit the given behavior within 180 s were deemed as censored results
[3] Regression coefficient from the Cox-proportional hazards model
[4] 95% confidence interval given in parentheses

The linear mixed effects model on vigilance duration data showed no significant differences between treatment groups (Table 5.2). The Kruskal-Wallis ANOVA on vigilance duration data showed an overall treatment effect; however, post-hoc multiple comparisons showed no differences between the groups (Table 5.2). Survival analyses showed the Anxious group had a significantly higher latency to become non-vigilant than the other groups, while the Calm and Happy groups tended to have a higher latency to become non-vigilant than the Control group (Table 5.3 and Figure 5.4).

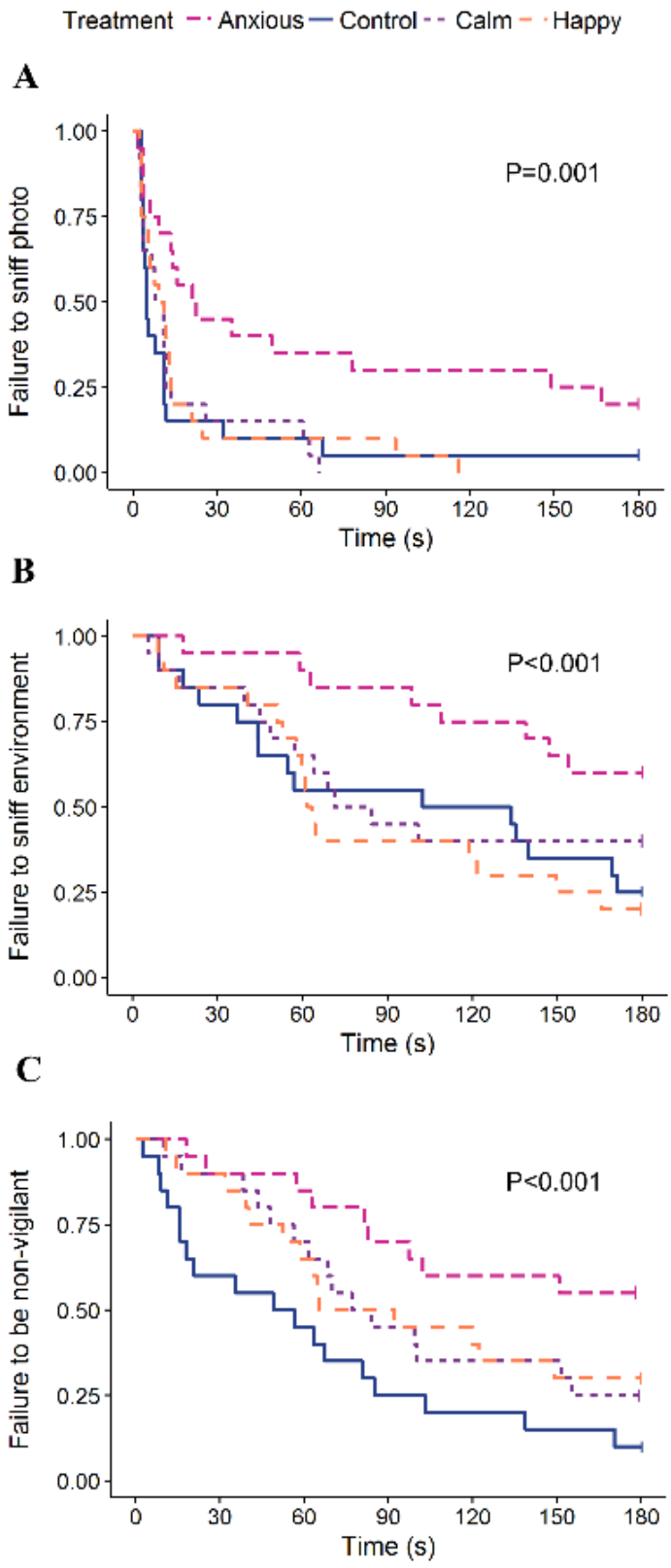

Figure 5.4. Kaplan-Meier curves for latency to sniff the photo (A), sniff the environment (B) and become non-vigilant (C).

Each time an animal exhibited the given behavior, the probability on the Y axis drops.

5.4.2. Other behaviors

The Calm group had the highest frequencies of sniffing the photo and environment while the Anxious group sniffed the photo and environment the least; however, neither group differed significantly from the Controls (Table 5.2). Anxious sheep had a longer latency to sniff the photo and environment than all other treatment groups (Table 5.3 and Figure 5.4).

The Happy group crossed and entered more zones than the other groups, while the other groups did not differ (Table 5.2). The Happy sheep also spent the least amount of time standing near the photo (r=0.26-0.30) and performed more open-mouthed vocalizations than the other groups (Table 5.2 and Figure 5.2). More sheep in the Happy group entered the zone closest to the dog wall compared to the Control, Calm and Anxious groups (Table 5.2). No statistical differences were found between groups for the number of animals that urinated (Table 5.2).

5.4.3. Body temperature

The repeated measures analysis on body temperature data from the baseline at -30 min showed a significant Treatment x Time interaction ($F_{(24, 576)} = 4.5$, $P<0.001$). Contrasts from the model summary indicated that body temperature did not differ between groups at times -30 min or -20 min ($P>0.1$) (Figure 5.5). At time -10 min, the Anxious group had a significantly higher body temperature than the Calm and Happy groups, but only tended to be higher than the Control group (t (71) = -1.8, P=0.069, r=0.21). The Anxious group had a higher body temperature than the other groups at all other time points (Figure 5.5, r=0.26-0.34). The Control, Calm and Happy groups did not differ at any time point.

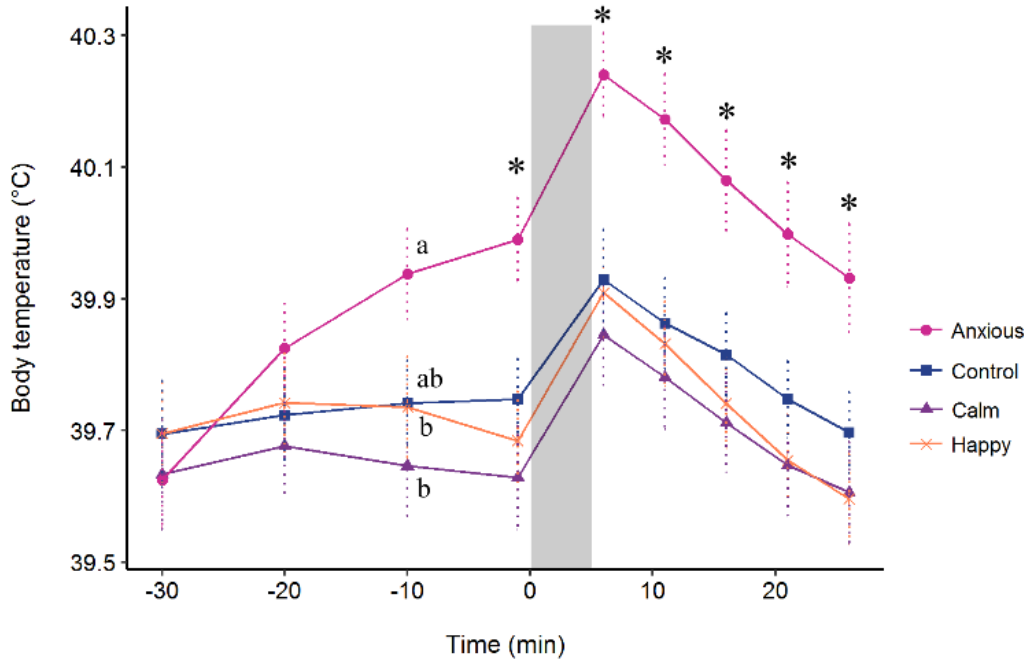

Figure 5.5. Mean ± s.e.m. body temperatures for the Anxious (●), Control (■), Happy (x) and Calm (▲) groups.

All injections were administered at time -30. The grey bar denotes the time of attention bias testing. The letters (a,b) indicate significant differences between groups at time -10. The '*' symbol denotes a significant difference between the Anxious group mean and all other groups as determined using a repeated measures linear mixed model.

For change in body temperature after time -1 min, the Time x Treatment interaction was not significant ($F_{(15, 360)}$ = 0.70, P=0.78). The model fitting fixed effects only without the interaction showed no significant effect of treatment on change in body temperature ($F_{(3, 72)}$ = 0.24, P=0.865).

5.5. Discussion

The pharmacological treatments induced a number of significant effects on behaviors and body temperature; however, in contrast with our hypothesis, the key behaviors of vigilance and looking time did not differ between treatment groups in the current study. There are a number of possible explanations for this, which will be discussed in greater detail throughout the following paragraphs. Firstly, these results could suggest that the modified attention bias test cannot provide a reliable measure of affective states in sheep. Treatment differences between the Control and Anxious groups observed previously during a modified attention bias test were not replicated in the current study (Monk et al., 2018a). This could mean that the results observed in the previous study were an anomaly. It is worth noting, however, that the Anxious group tended to spend

less time looking towards the dog wall and displayed a higher level of vigilance than the Control animals, albeit not strongly supported statistically, which is consistent with our expectations and the previous study (Monk et al., 2018a). Further, the Anxious group did have a significantly longer latency to become non-vigilant than the other groups. Finally, the Calm group tended to spend less time looking towards the dog wall than the Control and Happy groups. Thus, we would suggest the current study does not indicate the attention bias test is not useful as a measure of affective states in sheep, but rather that it may not be as sensitive to these changes as previously shown, or that additional factors impacted on animal behavior during this study, as discussed below.

One external factor that may have influenced animal behavior in the current study was the presence of background noise during testing. Throughout both test days, there was unexpected work being conducted in a nearby sheep handling facility. This meant there was some distant background noise consisting of conspecific vocalizations and vehicle movement. At the time of testing, the researchers had not expected this level of noise to impact on the behavior of the sheep being tested. Further, the noise was consistent and repetitive, spanning across the test period so that all treatment groups were exposed to a similar level of noise. However, the noise may have caused the test animals to reach a maximum level of vigilance. The overall time spent vigilant was higher in the current study relative to previous studies (Lee et al., 2016; Monk et al., 2018a,b). Further, there was a high number of censored data points, with 24 of 80 animals showing vigilance behavior for the entire test duration. The prevalence of censored data was much higher than in the previous study, during which only 4 of 50 animals were vigilant for the entire duration of the test (raw data generated by Monk et al., 2018a). If the duration of vigilance reached a behavioral and temporal maximum within the given test duration, this would have reduced the amount of variation in the data and power of the test to discriminate between treatment groups. Notably, more sheep in the Anxious group were vigilant for the entire test duration. Thus, for vigilance duration, the Anxious group mean was impacted to a greater extent than other groups, and may have disproportionately disguised the extent of heightened vigilance in this group. This is reflected by the survival analysis on latency to become non-vigilant, which accounted for the censoring of vigilance data and showed a significant treatment effect. The background noise may have also impacted on looking time measures, as the noise was coming from the direction of the dog wall. However, the mean times spent looking towards the dog wall for all animals were 59 and 61 s for the current study and Monk

et al. (2018a), respectively, suggesting the influence on direction of attention was minimal. Nevertheless, it cannot be ruled out that the presence of background noise impacted on vigilance and looking behaviors in the test. Consideration of the potential influence of background noise on animal responses will be of particular importance if applying the test in a commercial, on-farm setting, in which affective states are induced by the animals' environments.

An alternative explanation for a lack of response could be that the drugs or doses were inappropriate for modifying affective states or did not induce the expected affective states. During the current study, the Calm animals tended to spend less time looking towards the dog wall as predicted. However differences between the Calm and Control groups were not significant for any observed behavior, contrasting with previous studies using the same dose rate of diazepam (Lee et al., 2016; Monk et al., 2018b). During the original attention bias test method, vigilance and eating behaviors were mutually exclusive. This means that increased time spent eating would have automatically caused a reduction in vigilance behavior. This is important to note as diazepam has been shown to increase appetite in other animal species (Foltin, 2004; Gaskins, Massey & Ziccardi, 2008). Thus, the decrease in vigilance shown previously may have been due to a drug effect on appetite rather than the influence of a calm state on attention biases in sheep. Another factor worth noting is that the drug had previously been administered intra-venously rather than intra-muscularly. In humans, complete bioavailability of diazepam has been demonstrated after intra-muscular injection, with peak levels occurring around 30 min (Divoll et al., 1983; Moore et al., 1991). However, no studies in sheep have administered diazepam intra-muscularly at the same dose rate used in the current study. As diazepam or its metabolites were not directly measured in the current study, the efficacy of the drug cannot be confirmed. Further, injection of diazepam did not attenuate the stress-induced hyperthermia caused by attention bias testing. This contrasts with previous studies in cattle (Lee et al., 2017) and rodents and suggests the drug may not have had a strong anxiolytic effect in the current study (Olivier et al., 2003; Bouwknecht, Olivier & Paylor, 2007). It is also worth noting that a number of studies using diazepam in livestock and humans have found inconclusive or inconsistent effects (Clarke et al., 2014; Doyle et al., 2015; Lee et al., 2017; Mandelli et al., 1978). Further studies including pharmacokinetic assessment are required to validate the use of diazepam as an anxiolytic treatment in sheep and other livestock species.

Evidence for the use of morphine to manipulate affective states in sheep is currently limited, with few studies using the drug with this intended effect. In the current study, morphine had no effect on key measures of attention bias, but did seem to increase arousal as predicted. Happy sheep crossed and entered more zones and were more vocal than Control animals, which is consistent with previous studies using the same dose of morphine (Verbeek et al., 2012, 2014). While it appears morphine has an effect on arousal, its influence on the valence component of affect is less clear. Happy sheep did not show reduced vigilance or increased exploratory behavior during the test, previously thought to indicate a less-negative state (Monk et al., 2018a). Instead, increased activity during isolation can be considered a fearful response for sheep (Romeyer & Bouissou, 1992; Forkman et al., 2007). Previously, Verbeek et al. (2012) demonstrated changes in ear posture after morphine treatment which were suggestive of decreased fearfulness (Reefmann et al., 2009). Verbeek et al. (2014) demonstrated an enhanced optimistic judgement bias after consumption of a food reward, suggestive of a more positive mood after treatment (Paul, Harding & Mendl, 2005). Therefore, it is possible that increased activity during the current study did not reflect increased fearfulness, but rather supports the suggestion by Verbeek et al. (2012) that the opioid system may be involved in the arousal component of affective state. The potential influence of morphine on the valence component of affect in sheep remains inconclusive. Further studies validating the use of this drug, or exploring alternative methods for inducing positive affective states, would be useful for understanding affective states and attention biases in sheep.

The drug mCPP has been used previously to induce anxious states in sheep, including studies of attention bias. However the current study used a reduced rate of 1.5mg/kg compared to the rate of 2mg/kg used previously (Lee et al., 2016; Monk et al., 2018a,b). It could therefore be possible the reduced dose-rate was insufficient to cause an equivalent anxiety-like response. However, the mCPP treated sheep showed a significant increase in body temperature after injection compared to the other treatment groups. Although mCPP did not potentiate the stress-induced hyperthermia caused by attention bias testing, the drug itself caused a significant increase in body temperature compared to all other treatment groups, which is consistent with an anxiety-like state and previous studies (Sherwood, Klandorf & Yancey, 2005; Bouwknecht, Olivier & Paylor, 2007; Pedernera-Romano et al., 2010; Lee et al., 2017; Monk et al., 2018a). The Anxious group also showed behavioral signs of increased anxiety during testing, displaying a higher latency to become non-vigilant and a higher latency to sniff the

photo and environment compared to Control animals (Romeyer & Bouissou, 1992; Beausoleil, Stafford & Mellor, 2005; Beausoleil et al., 2012). Doyle et al. (2015) had also found that a lower dose of 1mg/kg induced anxiety-like behaviors in young sheep during runway, startle and isolation tests. Overall, it appears that the dose of 1.5mg/kg did cause an anxious-like response prior to and during testing. It is recommended that further studies continue to use the reduced dose rate of 1.5mg/kg in adult sheep to lessen the presence of unwanted side-effects. We suggest the key behaviors of vigilance duration and looking time did not differ between the Anxious and Control groups due to the censoring of vigilance data or the influence of other confounding factors.

Additional factors such as animal age, sex, parity and experience with dogs may have also influenced animal responses in the test. Previously, attention biases have been demonstrated in both male and female sheep ranging from 5 months old to 2 years old, while the current study assessed 7-year-old ewes. In humans, age has been found to alter the direction of attention biases (Isaacowitz et al., 2008). In sheep, previous experience with humans, age and parity have been shown to alter fearfulness (Viérin & Bouissou, 2002; Dodd et al., 2012). Notably, routine handling procedures on-farm, such as shearing and mustering to handling facilities with the use of dogs, are associated with indicators of behavioral and physiological stress in sheep and are shown to influence animal behavior during future procedures (Dwyer, 2009). Given that these older animals predominantly had negative experiences with humans and dogs throughout their lives, they may have developed a negative association with handling, which may have influenced their responses during testing. Additionally, animal age may have impacted the effect and metabolism of the drugs used in the study. For example, in humans, age is shown to impact the volume distribution and clearance rate of diazepam, as well as the metabolite concentration and peak time (Klotz et al., 1975; Divoll et al., 1983). In rats, morphine is shown to have a variable effect on behavior and nociception in 8-week-old versus 24-week-old animals (Paul, Gueven & Dietis, 2018). Understanding the potential influence of these factors on animal responses will allow for a clearer interpretation of behavior during attention bias studies.

Another factor that should be noted is the choice of positive and negative stimuli in the current test methodology. The negative stimulus was a live dog, which was presented for only 3 s of the test. The positive stimulus was a photograph of a conspecific, which was present for the entire test duration. As such, the positive and negative stimuli used in the current study were not balanced in intensity and presentation time. Within the

context of the current study, attention biases are being assessed within treatment groups relative to other treatment groups. This means the key question is not whether an individual pays more attention towards the dog window relative to the photograph, but rather whether a treatment group pays more attention towards the dog window relative to the other treatment groups being tested. Consequently, discrepancies between the positive and negative stimulus intensities and stimulus presentation times should not have impacted on our results, as these factors were consistent across all tested animals. However, it cannot be ruled out that the drugs may have impacted an animal's perception of the stimuli and therefore their responses. For example, if the Anxious sheep perceived the photograph as a conspecific to a greater degree than the other groups, this could explain an additional attraction to the stimulus. Further studies should be conducted to better understand how animals perceive and respond to the stimuli presented during attention bias tests. Such studies should not only consider the salience of the stimuli, but should also consider the type of stimuli used with regards to the primary sensory systems of the animals being tested (Raoult & Gygax, 2018, Winters et al., 2015).

Additionally, it is important to consider that the stimuli used in the current study may not be generalizable to other studies and populations. We used a single dog for all tests to control for the potential effects of dog disposition or temperament on sheep responses. However, different dogs may evoke different behavioral responses in sheep during future studies. We note here that the photograph used in the current study has been made publicly available by Monk et al. (2018a) for use in further research; however, this photo may be less suitable for studies in different sheep breeds. Sheep have been shown to discriminate valenced photographs of conspecifics, and to generalize this discrimination of valenced faces to photos of different individuals (Bellegarde et al., 2017). As such, we do not expect that minor changes to conspecific photographs would have a great impact on animal responses. Nevertheless, future studies aiming to better understand how animals perceive and appraise different types of stimuli should take this into consideration.

The current study highlights a number of key areas for further research. Studies understanding the potential influence of factors such as age, experience and background noise on animal responses may allow the method to be adjusted or interpreted accordingly for different populations of animals or for different testing environments. It is suggested the test might be better suited to younger groups of animals that have

had less experience with dogs and with routine handling practices that could influence their responses in the test. Inclusion of a habituation period could reduce the overall vigilance levels by reducing the fear-eliciting elements of the test itself, allowing clearer separation of vigilance between animals (Erhard, Elston & Davidson, 2006). However, this would limit the test's application to a larger population of animals in an on-farm setting. Understanding the influence of background noise will be of particular importance if applying the test in a commercial, on-farm setting. It would be useful to examine the impact of other environmental and pharmacological manipulations on responses in the modified attention bias test. Studies examining environmental manipulations should utilize experiences and environments that are relevant to livestock production systems to facilitate the future application of the test in an on-farm setting. Studies examining pharmacological models should aim to better understand the way in which the models impact on the valence of affective state. This will best be done by utilizing a variety of methods, such as place preference tests and operant conditioning tasks. Finally, further studies should be conducted to examine the influence of different types of positive and negative stimuli on animal responses.

5.6. Conclusions

It remains unclear whether the attention bias test can be used to detect positive affective states in sheep. Further, the current study was not able to replicate previous findings that negative affective states influenced responses in the modified attention bias test. It is suggested the current study should be repeated on a population of younger animals, making sure to reduce or eliminate background noise during testing, which may have confounded results. Further studies should be conducted to confirm the effects of the given pharmacological agents and to ensure the doses and administration routes are appropriate to induce specific affective states. It may be useful to explore alternative pharmacological agents for inducing affective states in sheep, and to examine the impact of environmental manipulations on attention biases.

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Chapter 6

Repeatability of an attention bias test for sheep

Paper formatted to *Animals*

6.1. Abstract

This study examined the repeatability of behavioural responses in an attention bias test developed for sheep as a measure of affective states. Repeatability was assessed to better understand the influence of consistent underlying traits versus transient affective states on animal responses during testing. Sheep were assessed in the attention bias test 3 times. During testing, individual sheep were exposed to a dog for 3 s in a 4 x 4 m arena, then the dog was removed and their behaviours were recorded for the following 3 min. We hypothesized that duration of vigilance behavior, defined as having the head at or above shoulder height, would have moderate-high repeatability across tests. We hypothesized that time spent looking towards the previous location of the dog and latency to feed would have low repeatability. Data were modelled using restricted maximum likelihood linear mixed-effects models, fitting animal ID as a random effect. Vigilance behavior was moderately repeatable as predicted (r= 0.58 ± 0.06 s.e.). Latency to eat (r= 0.20 ± 0.07 s.e.) and attention to the dog (r= 0.08 ± 0.06 s.e.) had low repeatability as predicted. Overall, these results suggest that vigilance behavior during attention bias testing is more heavily influenced by underlying temperament or personality traits, while attention and feeding behaviours are influenced by transient affective states or external factors.

6.2. Introduction

With growing recognition of animal sentience and emotions, the emotional or affective states of animals are increasingly being considered as a vital component of animal welfare. However, potential measures of affective states in non-human animals have only recently been explored and are currently not well-understood. One method which has shown promise for the assessment of affective states in livestock is an attention bias test. An attention bias is where an individual alters their allocation of attention towards certain types of information depending on their affective states (Bar-Haim et al., 2007; Bradley et al., 1995, 1997). An attention bias test has recently been developed for sheep to assess the variability between animals in allocation of attention towards a predator threat (Lee et al., 2016). Various versions of the test have been shown to be influenced by short-term pharmacological manipulations to induce anxiety-like (Lee et al., 2016; Monk et al., 2018b) and depression-like affective states (Monk et al., 2018a). However,

as the test has only recently been developed, many questions remain as to what information the test can provide on the affective state of an animal.

There are various frameworks for conceptualising and studying affective states in non-human animals; however, for the purpose of this study we will adopt the framework described by Mendl et al. (2010). Affect can be conceptualised as a location within a 2-dimensional space delineated by the dimensions of valence and arousal; where valence indicates the positivity or negativity of a state and arousal indicates the intensity or level of activation (Mendl et al., 2010). Affective states include short-term, discrete emotions, which are triggered by specific events, as well as longer-term moods, which can be thought of as the running mean of an animal's position within the affective space (Mendl et al., 2010). An animal's propensity to be in a particular affective state may be influenced by personality or temperament. The concepts of personality, temperament, behavioural syndromes and coping styles refer to the consistency of an animal's behavioural responses across time and situations or contexts (Finkemeier et al., 2018). Together, the personality of an animal and its affective state interact to determine the way in which the animal responds to environmental stimuli (Finkemeier et al., 2018).

Studies using the attention bias test developed by Lee et al. (2016) have shown that transient emotional states or moods have an influence on sheep behaviour during attention bias testing. However, it remains unclear which of these aspects primarily drive behavioural responses during testing. Further, the potential influence of stable, underlying personality or temperament traits on animal responses remains unclear, particularly in a context where no emotion-modifying treatments are applied. Studies examining repeatability of animal responses during consecutive attention bias tests, without manipulation of affect, may begin to provide information on which aspects of an animal's affective state or personality most strongly influence animal behaviour in the test. This information will allow for a clearer interpretation of an animal's responses during future use of the test, as an assessment of animal welfare.

Understanding the repeatability of behavioural tests is also important for the interpretation of animal responses during repeated measurements. Taking repeated measures on the same animals before and after an experimental manipulation can help to remove between-animal variation and thus increase the power of an experiment to detect an effect. In a practical setting, repeated measurements can allow for the tracking of an individual's responses to monitor their well-being over time. However, animals

can become habituated or sensitised to tests upon repeated exposure (Doyle et al., 2010; Erhard et al., 2006). This is particularly important for tests which rely on novelty as part of the procedure (Erhard et al., 2006). A change in mean reactivity over consecutive tests may not be of concern if the purpose of the test is to compare relative responses within a given population of animals. However, if the rates at which animals habituate or become sensitized to a particular test situation differ, then it may not be appropriate to repeat the test with the same animals. It is therefore important to understand the effect of repeated testing on animal responses for a clearer interpretation of behaviour in this context.

The current study aimed to examine the effect of repeated testing on sheep responses in an attention bias test. As measures of attention bias are thought to reflect shorter-term, transient affective states, we hypothesised that key measures of looking time or latency to feed would have low repeatability over time. While vigilance has also been considered as a key measure of attention bias (Lee et al., 2016; Monk et al., 2018b), it has been suggested that it may primarily relate to fearfulness and would therefore be expected to have moderate-high repeatability (Beauchamp, 2017; Monk et al., 2018a). We expected rankings of zones crossed would have moderate-high repeatability, as locomotive behaviours are found to be repeatable within similar contexts such as the arena test (Kilgour, 1998; Kilgour and Szantar-Coddington, 1995; Wolf et al., 2008). Behaviours such as time standing in the zone closest to the door and pawing at the door were expected to have low repeatability, as animals learn to wait at the exit door at different rates. Finally, we hypothesised that mean behavioural responses during testing would change between trials as animals became habituated to the novel test arena (Doyle et al., 2010; Erhard et al., 2006). To test these hypotheses, we used data collected as part of a broader experiment which assessed attention bias in adult ewes on 3 occasions, using the test described by Monk et al. (2018b).

6.3. Methods

6.3.1. Animal Ethics

The protocol and conduct of the study were approved by the University of New England Animal Ethics Committee, under the New South Wales Animal Research Act 1985 (Animal Research Authority numbers 16-003 and 17-015).

6.3.2. Animal details

One hundred adult ewes were used in this study. The sheep belonged to the Sheep CRC Information Nucleus Flock in Armidale, NSW, Australia. The flock included Merino sheep (*n*=73) and Merino x Border Leicester (or similar) sheep (*n*=27). Sheep were approximately 4 years old and weighed an average of 52.5 ± 6.4 kg at the beginning of the experiment. The average weight was 57.1 ± 6.6 kg at the time of testing one year later. All sheep were raised together under extensive farming conditions and were housed at pasture for the duration of the study. The sheep had regular contact with humans throughout their lives and had previous exposure to behavioural tests such as the isolation box test described below.

6.3.3. Experimental Design

This experiment was conducted over 2 years from March 2016 to April 2017. The current study used data generated as part of a larger experiment, which aimed to examine the relationships between a suite of behavioural tests and physiological measures in sheep. The ewes used in this experiment had been selected from a larger population of 340 ewes, based on their immune competence phenotype, as assessed by Hine et al. (2017). The population was ranked on immune competence, then the top 50 (High) and bottom 50 (Low) ranked ewes were selected for the experiment.

At the beginning of the experiment, all sheep individually underwent the following consecutive procedures, in the listed order, over a period of approximately 14 min: blood sampling, attention bias testing, arena testing, isolation box testing, eye temperature measurement, flight speed assessment and a second blood sampling. The details for each procedure are given below. Sheep were then returned to the farm and were managed under typical Australian extensive farming conditions for approximately one year. After that time, the circuit of testing procedures was repeated on 81 of the same animals. Two weeks after completing the second testing circuit, all sheep were tested in the attention bias test a third time. No additional procedures occurred at that time.

6.3.4. Attention Bias test

The current study used the same attention bias test arena and testing procedure as described by Monk et al. (2018b). The test comprised a 4 x 4.2 m arena with 1.8 m high opaque walls. Approximately 1.5 kg of lucerne hay was positioned in the middle of the arena. A small window was positioned on one side of the arena, which could be completely obscured by a retractable opaque cover. An unfamiliar kelpie-x-border-collie dog stood quietly behind the open window at the beginning of the test as the sheep entered the arena. The dog was visible for 3 s, then the window was covered and the dog was removed. A timer began once the window was fully covered and sheep remained in the test for a further 3 min while behaviours were recorded. The behavioural responses recorded during testing are summarised in Table 6.1. Behaviours were collated from video footage using The Observer XT 12.0 (Noldus Information Technology, Wageningen, The Netherlands). A 3 x 4 grid was overlaid onto the video footage for calculation of zones crossed and time spent in the zone closest to the exit door (Figure 6.1). Prior to testing, sheep were held in a paddock with limited feed available overnight, but were given *ad lib* access to water.

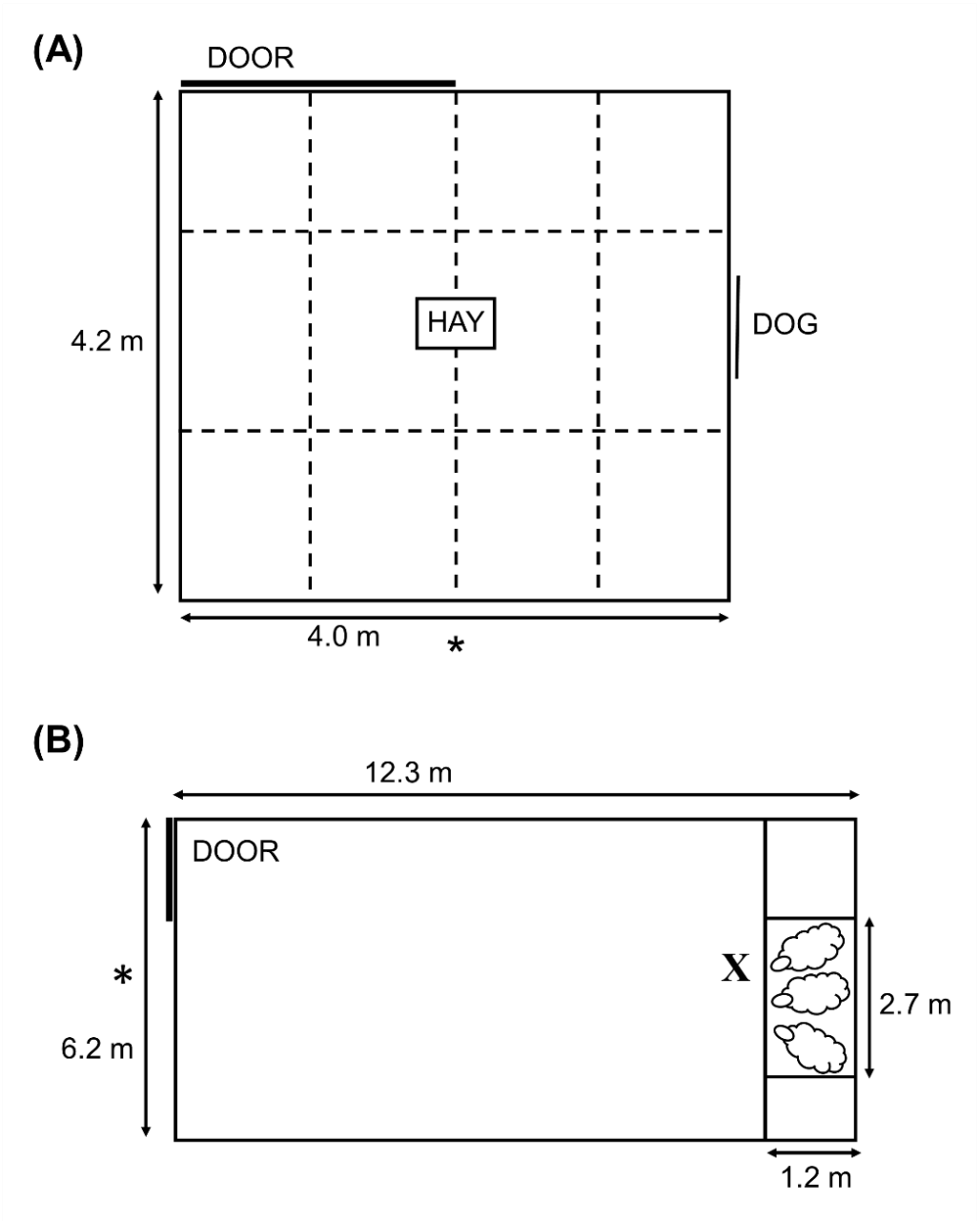

Figure 6.1. Schematic diagrams of the attention bias test (A) and the arena test (B).

The symbol "*" denotes the positions of two cameras. The "X" denotes the position of a stationary human standing in front of a pen containing 3 sheep. The walls of the attention bias test were 1.8 m high and covered in opaque matting. The walls of the arena were 1 m high and covered in shade-cloth.

Table 6.1. Ethogram of behaviours recorded during the attention bias tests (Monk et al., 2018b).

Behaviour	Definition
Vigilance	Time spent with the head at or above shoulder height.
Attention to dog wall	Time spent looking towards the closed dog window with binocular vision, for the first 60 s of testing.
Eating	An eating bout began when a sheep took a bite of hay and ended when the sheep became vigilant or moved more than 30 cm from the hay. Number of eating bouts (eating frequency) and latency to eat were recorded.
Zones crossed	Number of zones crossed with both front feet in a new zone, or with one front foot in the zone and the other on the line.
Standing near door	Duration standing in the zone closest to the door.
Pawing	Lifting a front foot and making contact with the door in a pawing motion or digging at the ground in front of the door.

6.3.5. Additional behavioural tests and measures

The current study focused on the attention bias test and did not examine any of the additional behavioural tests and measures. As such, these additional methods will only be described briefly. Each additional test was selected to measure a different aspect of animal behaviour or to assess physiological stress responses.

Prior to testing, all sheep were weighed and had numbers painted on their rumps for individual identification. Internal body temperature was recorded throughout testing using Thermochron iButtons® (Embedded Data Systems, Lawrenceburg, United States), which were attached to blank, progesterone-free Controlled Internal Drug Release devices (CIDR®, Zoetis, Melbourne, Australia) as described by Lea et al. (2008). An iButton was inserted into the vagina of each sheep one day prior to testing and was removed after the testing circuit had been completed.

At the beginning of the 14 min testing circuit, baseline blood samples were collected via jugular venepuncture by an experienced handler, immediately prior to entering the attention bias test. To assess the stress response to testing, blood samples were taken again at the end of the circuit, immediately after flight speed assessment.

Immediately after attention bias testing, sheep were moved into an arena test similar to that described by Murphy et al. (1994). The sheep entered the arena depicted in Figure 6.1 for a total of 3 min. At one end of the arena, a small pen held 3 spare conspecifics which were not otherwise used for testing. A stationary human was positioned in front

of the conspecifics, standing quietly. After 3 min, the sheep was walked from the arena into an isolation box test.

The isolation box was a 1.5 x 0.5 x 2 m wooden box with an open roof covered by shade cloth (Bickell et al., 2009; Murphy et al., 1994). The number of movements and vocalisations within the box were recorded using an electronic agitation meter (School of Animal Biology, University of Western Australia). Sheep remained in the isolation box for 30 s.

After the isolation box test, sheep were restrained in a rollover sheep handling cradle, which was kept upright, for approximately 5 min to allow excess heat to dissipate from their wool. The temperature of the sheep's eye was then measured using an infra-red thermography camera (ThermaCam T640, FLIR Systems AB, Danderyd, Sweden).

Sheep were then moved into a weigh crate for assessment of flight time. Sheep were held for 5 s, then the weigh crate was opened. The time taken for the sheep to traverse approximately 2 m after exit from the crate into a holding yard was electronically recorded using infrared sensors (Ruddweigh Australia Pty Ltd., Guyra, Australia).

6.3.6. Statistical analysis

Data were analysed using R version 3.5.1 (R Core Team, 2018). Data were modelled with restricted maximum likelihood (REML) mixed-effects models using the package *lme4* (Bates et al., 2015). Eating frequency data were modelled fitting a Poisson distribution for count data, all other models were fitted with a Gaussian distribution. All model residuals were checked using visual assessment of residuals vs fitted values plots and histograms. A square root transformation was applied to time standing-near-door data. Sheep ID was fitted as a random effect in all models. Breed and immune grouping were fitted as fixed effects in all models and weight at the beginning of the experiment was fitted as a covariate. Immune grouping and weight were subsequently removed from all models using a backward stepwise reduction, considering the Akaike Information Criterion and Bayesian Information Criterion. Breed was retained for latency to eat and eating frequency data, but was removed from all other models.

Repeatability (r) was calculated from the between-animal (σ_B^2) and within-animal (σ_W^2) components of variance for each behaviour as $r = \sigma_B^2/(\sigma_B^2 + \sigma_W^2)$ (Bell et al., 2009; Dingemanse et al., 2010). Repeatability estimates were confirmed and uncertainty in the estimates was quantified using the *rptR* package (Stoffel et al., 2017). This package

relies on the mixed-effects models fitted using the *lme4* package and uses parametric bootstrapping for estimation of confidence intervals and standard errors. Uncertainty estimates were based on 1000 bootstrapping runs. Repeatability estimates were also calculated in the same way for subsets of the dataset, that examined data from trials 1 and 2 only, then trials 2 and 3 only. Repeatability estimates of <0.4 were considered to be low, 0.4-0.7 to be moderate and >0.7 to be high, as suggested by Harper (1994).

To compare mean behavioural responses across the trials, data were modelled as above, using maximum likelihood mixed-effects models instead of REML, and fitting trial number as an additional fixed effect. Post hoc multiple comparisons were conducted using a Tukey method for adjustment of P-values. The number of animals which pawed at the exit door were analysed using a Fisher's Exact Test, post hoc multiple comparisons were performed using the package *rcompanion* (Mangiafico, 2018). Repeatability estimates were not made for pawing behaviour due to a low occurrence of this behaviour.

6.4. Results

Raw behavioural data are summarised in Figure 6.2. Vigilance was the most repeatable behaviour (Table 6.2). Attention to the dog was least repeatable across all trials (Table 6.2). Vigilance, zones crossed and attention to dog data were more repeatable between trials 2 and 3 than between trials 1 and 2 (Table 6.2). The feeding behaviours and time standing near the door were more repeatable between trials 1 and 2 than between trials 2 and 3 (Table 6.2). Duration of time spent displaying vigilance remained consistent across all trials (Table 6.3). Duration of attention towards the dog wall decreased over the trials, while time standing near the door, pawing at the door and zones crossed increased over the trials (Table 6.3).

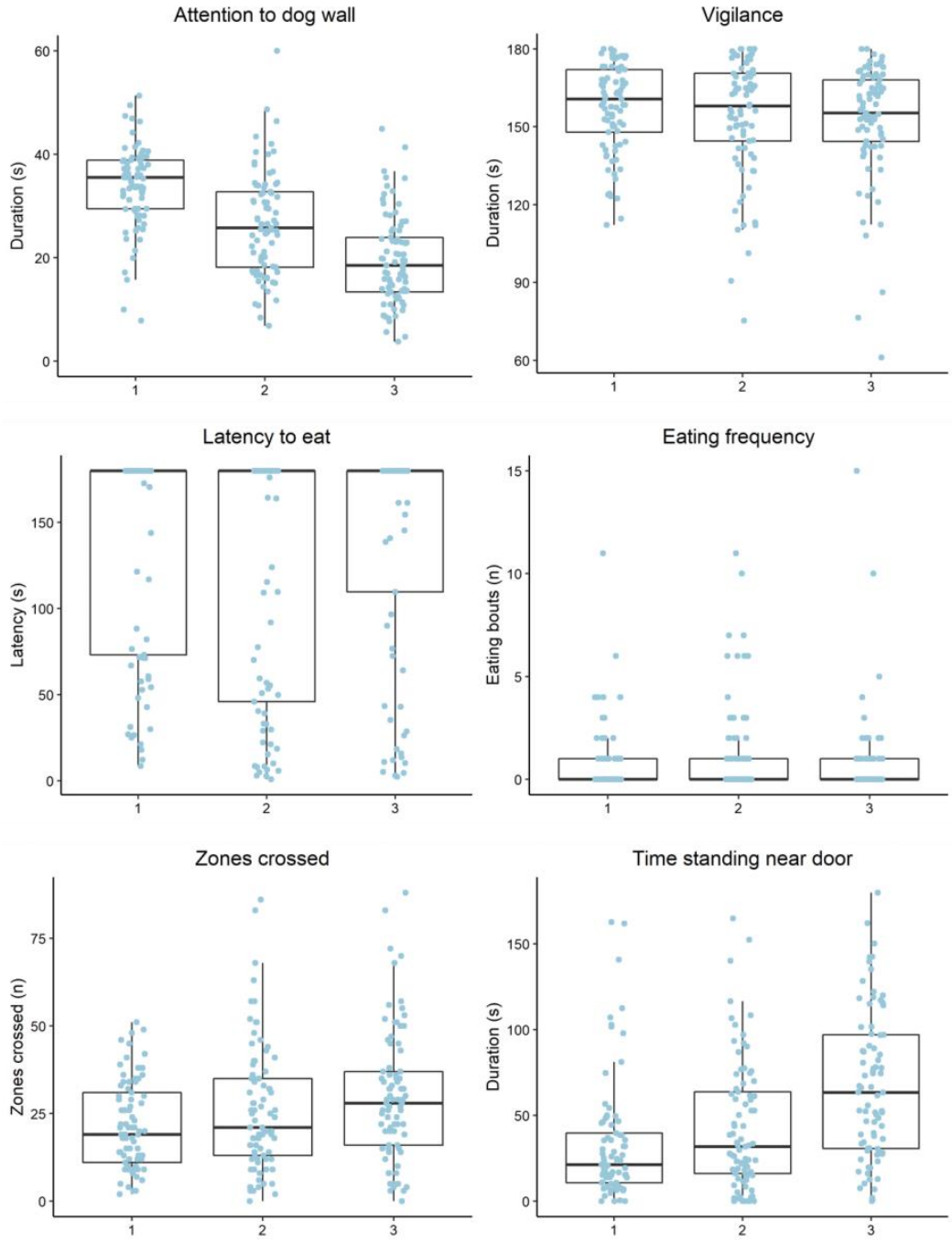

Figure 6.2. Boxplots displaying the distribution of observed behavioural data during attention bias testing over three repeated tests.

Boxplots display the median values, the interquartile range (IQR) and range of data within 1.5 x the IQR. The dots represent raw data for individual sheep within each trial. We note that the plot axes are scaled differently to more clearly display the data within each observed variable.

Table 6.2. Repeatability estimates for behavioural responses in the attention bias tests.

Repeatability was estimated using the package *rptR.* Maximum models included fixed effects of breed, immune competence and weight, fixed effects were removed based on AIC.

Behaviour	All trials			Trials 1 and 2 only			Trials 2 and 3 only			Fixed effects retained
	r	s.e.	CI	r	s.e.	CI	r	s.e.	CI	
Vigilance (s)	0.58	0.06	0.44, 0.68	0.55	0.08	0.38, 0.68	0.70	0.06	0.57, 0.79	none
Zones crossed (n)	0.47	0.07	0.34, 0.59	0.43	0.09	0.23, 0.59	0.59	0.07	0.44, 0.72	none
Eating frequency (n)[1]	0.36	0.12	0.11, 0.56	0.50	0.17	0.13, 0.76	0.21	0.13	0.00, 0.48	breed
Time at door (s)	0.29	0.07	0.14, 0.43	0.46	0.09	0.27, 0.61	0.35	0.09	0.15, 0.52	none
Latency to eat (s)	0.20	0.07	0.07, 0.34	0.31	0.10	0.10, 0.50	0.12	0.10	0.00, 0.34	breed
Attention to dog (s)	0.08	0.06	0.00, 0.22	0.05	0.08	0.00, 0.27	0.28	0.10	0.10, 0.46	none

[1] Original scale approximations for repeatability estimates are presented

Table 6.3. Mean ± s.e.m. data for behavioural responses in the attention bias tests across 3 trials.

Behaviour	Mean ± s.e.m.			Model	Test value (df)	P
	Trial 1	Trial 2	Trial 3			
Vigilance (s)	157.2 ± 1.8	154 ± 2.5	152.5 ± 2.4	*lmer*	X^2 (2) = 5.27	0.072
Zones crossed (n)	21.5 ± 1.3[a]	26.1 ± 2[b]	30 ± 2[b]	*lmer*	X^2 (2) = 21.3	<0.001
Eating frequency (n)[1]	0.7 ± 0.1 (29)[a]	1.3 ± 0.2 (37)[b]	0.8 ± 0.2 (27)[a]	*glmer*	X^2 (2) = 15.6	<0.001
Time at door (s)	33.4 ± 3.9[a]	42.7 ± 4.1[a]	67 ± 4.7[b]	*lmer*	X^2 (2) = 54.6	<0.001
Latency to eat (s)	139 ± 6.7	121.3 ± 8	140.7 ± 7.1	*lmer*	X^2 (2) = 5.72	0.057
Attention to dog (s)	33.8 ± 0.8[a]	26.7 ± 1.1[b]	19.5 ± 1.0[c]	*lmer*	X^2 (2) = 148	<0.001
Pawing at door (n)[1]	0.1 ± 0.0 (3)[a]	0.6 ± 0.2 (11)[ab]	0.9 ± 0.3 (13)[b]	FET	N/A	0.02

[1] Raw numbers of animals which exhibited the behavior are given in parentheses

[a,b,c] Different superscripts within rows indicate significant differences between trials as determined using post hoc analyses

lmer: linear mixed-effects model, *glmer*: generalised linear mixed effects model with Poisson distribution, FET; fisher's exact test

6.5. Discussion

Repeatability estimates for vigilance duration and zones crossed were moderate to high, which was consistent with our hypotheses. The zones crossed repeatability estimates were comparable to those found for measures of activity during arena tests in sheep (Kilgour, 1998; Kilgour and Szantar-Coddington, 1995; Wolf et al., 2008). The observed consistency of animal responses over consecutive attention bias tests suggests that vigilance and measures of activity were strongly influenced by underlying temperament or personality traits. This suggestion is supported by previous studies using the arena test, which found consistency in activity not only over time, but also across testing contexts (Beausoleil et al., 2012). Further, this is also consistent with suggestions that vigilance may be used as a measure of trait fearfulness in sheep and other grazing ruminants across a range of different contexts (Beauchamp, 2017).

An alternative interpretation is that vigilance and zones crossed were influenced by shorter-term emotional states, but that the emotional states induced by isolation in the testing environment remained consistent over the trials. Previous studies have shown vigilance behaviour in the attention bias test is altered by pharmacological models that temporarily induce affective states (Lee et al., 2016; Monk et al., 2018a, 2018b). This suggests short-term emotional states have some influence on this behaviour. Importantly though, the attention bias test arena was novel during the first exposure but was no longer novel thereafter, changing the context of the test between trials. Further, the attention bias test was the first of many stress-inducing testing procedures conducted during the testing circuit. While this would not have impacted on behaviours during the first trial, sheep may have perceived the attention bias test differently during the second and third trials, in anticipation of the entire testing circuit. Thus, we suggest the animals' affective states may not have been consistent between all trials, particularly between trials 2 and 3, and that the interpretation that vigilance and zones crossed were influenced by short-term emotional states is therefore less likely to be correct.

The duration of attention to the dog wall and feeding behaviours had low repeatability, which was also consistent with our hypotheses. These findings suggest that attention and feeding behaviours may be heavily influenced by discrete emotional states, moods or unidentified, temporary environmental effects, rather than personality or temperament traits. Alternatively, low repeatability of attention to dog data may represent individual variation in learning or acclimation to the testing environment. A

general decrease in attention to the dog window over consecutive trials may indicate that sheep learnt the threatening cue was not further reinforced and that a high level of attention towards this potential threat was not required, in the context of the attention bias test. Considering this interpretation, it could be valuable for additional studies to examine the animals' rates of change in attention towards the dog over consecutive tests as a new variable. The rate at which animals acclimate to novel environments may relate to other aspects of an animal's personality or learning abilities, and can itself have welfare implications within livestock production systems (Monk et al., 2018c; Wechsler and Lea, 2007).

Many of the mean behavioural responses of sheep changed across consecutive trials as predicted. This effect was particularly strong for attention to the dog and time standing near the door, which showed an inverse relationship. Attention to the dog likely decreased over time as sheep learnt the threat was not further reinforced after the window was closed. A shift in attention towards the door may therefore indicate that sheep reallocated their attentional resources away from the unreinforced predator threat and towards escaping the arena, to reunite with their flock-mates. An increased number of sheep actively pawing at the door further supports this suggestion. Such a shift in motivations would also explain an increase in zones crossed over consecutive trials, as sheep spent more time exploring, to find a way out of the arena. In contrast, mean duration of vigilance behaviour did not differ between trials. Vigilance is an important and innate behavioural response of sheep to both isolation and the threat of predation (Beauchamp, 2017; Frid, 1997; Kendrick, 2008). Thus, it is not surprising that vigilance remained consistent and quite high across all of the trials. Given that an animal's responses change with repeated attention bias testing, it is important to ensure that all sheep being tested at a given time have had the same level of experience with the test, so that the influence of habituation or extinction does not confound interpretation of animal responses.

The results of this study provide useful information on the degree to which the attention bias test developed for sheep measures discrete emotions, moods, or traits. However, there are still a number of key questions remaining. Our results suggest that behavioural responses such as vigilance and zones crossed may be useful as measures of temperament. However, further studies examining consistency of vigilance and activity across a variety of contexts, including the attention bias test, will help to further confirm these behavioural responses as measures of temperament or personality. Additionally,

repeatability estimates can give some indication of the heritability of a trait (Dohm, 2002). Measures of activity in sheep, within the context of the arena, are shown to be heritable (Wolf et al., 2008). Given the moderate to high repeatability of vigilance behaviour, it may be possible that this behavioural response has a genetic basis as well, which can be incorporated into selection programs. However, further work is required to better understand how vigilance alone might relate to animal welfare or production before determining whether this would be a trait of interest for which to select.

The key measures of attention bias; duration looking towards the dog and latency to feed, had low repeatability, suggesting they were readily influenced by external environmental factors, emotions or moods. Moving forward with the attention bias test, it will be important to tease apart these potential effects. If these behavioural responses are readily influenced by unrelated external factors, the test may have little use for the assessment of animal welfare. If the test only measures transient labile emotional states, caused by short-term events which occurred immediately prior to testing, the test may have some limited applications, but again may not be a useful measure of the welfare state of an animal. If the test measures longer term moods, this would be ideal for the assessment of welfare states by giving information on the cumulative effect of recent events which have impacted on the animal. Previous studies have demonstrated an influence of affective state on these key measures of attention bias (Lee et al., 2016; Monk et al., 2018a, 2018b). Thus, we suggest that these responses are likely to be most influenced by emotions or moods, rather than unrelated external factors.

In conclusion, some aspects of behaviour in the attention bias test were more repeatable than others, which largely aligned with our initial hypotheses. Further studies aiming to more clearly tease apart the influence of emotion and mood are essential for better understanding key measures of attention bias in the test. The differentiation of emotions and moods may be done using environmental manipulations which vary in duration and intensity. Alternatively, or additionally, the careful selection of pharmacological agents and treatment regimens may also allow differentiation of moods and emotions. Studies which overlay models of short term positive and negative emotions onto models of positive or negative mood may help to understand how emotions and moods interact in the generation of an attention bias. Of course, it is important to keep in mind that in practice, the behavioural responses of an animal will be influenced by an interaction between temperament, mood, emotions and other external factors. Further, the degree

to which each of these aspects influences an individual's responses may vary between situations and over time.

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Chapter 7

General Discussion: Interpretation and application of the attention bias test for sheep

The primary aim of this thesis was to further develop and validate a novel method for the assessment of attention biases in sheep, as a measure of animal welfare. The thesis began with the following set of hypotheses:

1) An attention bias test can be used to assess and differentiate positive and negative affective states in sheep
2) The attention bias test developed by Lee et al. (2016) can be refined to improve its practical application and to better facilitate interpretation of animal responses
3) Behaviour during the attention bias test is primarily influenced by affect, but some aspects of behaviour are also influenced by consistent temperament or personality traits

This discussion will address each of these hypotheses individually before discussing the implications of our findings with regards to animal welfare more broadly.

Chapters 2 to 5 examined the effect of several types of positive and negative affective states on the attention bias test for sheep. The findings of these studies are summarised in Table 7.1. Previously, Verbeek et al. (2019) had demonstrated that an attention bias test was influenced by environmentally induced chronic stress, however the results of chapter 2 suggest this potential bias cannot be explained by pharmacological induction of physiological stress alone. This may mean the attention bias test was unable to detect a negative state induced by chronic stress, in contrast with the hypothesis. However, it could also mean that the pharmacological model of stress did not impact on affective state in the study. Chapters 3 and 4 demonstrated that attention bias tests may be useful as a measure of anxious states, which is consistent with the hypothesis, studies in humans and the findings of Lee et al. (2016). Further, chapter 4 demonstrated that the modified test is also influenced by depressed states and that the method can differentiate these two negative states. The potential effect of positive affect on animal responses during testing remains inconclusive.

Table 7.1. Validation studies using variations of the attention bias test described by Lee et al. (2016). The table summarizes observed differences for key behavioural responses in each test. All studies used Merino sheep.

Study	Positive stimulus	Test duration (s)		Treatment	*n*	Findings relative to controls				Conclusions
		Dog	No-dog			Look at dog	Look at positive	Vigilant	Eat/sniff latency	
(Verbeek et al., 2019)	Pelleted ration in familiar bowl	30	180	Lying deprivation (endogenous chronic stress)	15	-	-	↓#	↓#	Chronic stress through lying deprivation caused reduced vigilance or increased attention to the food reward
Chapter 2	Pelleted ration in familiar bowl	30	180	22 days of ACTH (exogenous chronic stress)	14	-	na	-	-	Cortisol response alone may not explain previously observed changes in attention after chronic stress
(Lee et al., 2016)	Pelleted ration in familiar bowl	10	180	mCPP (anxiogenic)	20	↑*	na	-	-	The test method was sensitive to changes in anxious states, consistent with human literature
				Diazepam (anxiolytic)	20	↓*	na	-	↓*	
Chapter 3 (Monk et al., 2018b)	Lucerne hay	3	180	mCPP (anxiogenic)	20	-	na	↑*	↑*	The test could be shortened to 45 s and the habituation period to the feed bowl removed
				Diazepam (anxiolytic)	20	↓*	na	-	-	
	Lucerne hay	3	180	Presentation of dog versus empty window	20	-	na	↑*	↑*	The dog was perceived as a threat, however window movement alone also captured attention
Chapter 4 (Monk et al., 2018a)	Photograph of a conspecific	3	180	mCPP (anxiogenic)	16	↓*	↑^	↑*	↑^	The modified test was sensitive to different types of negative states and could differentiate them, a different interpretation of behavior was required for the new method
				pCPA (depressant)	16	↑*	↓^	↑*	↑*	
Chapter 5	Photograph of a conspecific	3	180	mCPP (anxiogenic)	20	↓^	-	-	↑*	The test was not sensitive to changes in positive states, however this may have been due to confounding factors
				Diazepam (anxiolytic)	20	↓^	-	-	-	
				Morphine (euphoric)	20	-	-	-	-	

Arrows (↑,↓) indicate the direction in which a treatment group was different from the control animals, where the P value was reported as <0.05 (*) or 0.05<0.1 (^), or where models fitting treatment performed better than a model fitting the intercept only (#). The symbol "-" denotes no difference between the treatment and control groups, "na" indicates the behaviour was not measured. *Look at dog* or *look at positive*: duration looking towards the dog window or positive stimulus, *vigilant:* duration vigilant with the head at or above shoulder height, *eat/sniff latency:* latency to eat the food or sniff the photo

There were some inconsistencies in the effect of pharmacological models between Lee et al. (2016) and the chapters of this thesis (Table 7.1). For example, Lee et al. (2016) found mCPP treated animals spent significantly longer looking towards the dog compared to the control group, however this finding was not replicated in chapter 3. On the other hand, chapter 3 showed a significant effect of mCPP on vigilance compared to control animals, which was not shown by Lee et al. (2016). However, in each case, mCPP treated animals differed significantly from the diazepam treated animals and mean durations for each group moved in the expected directions. These differences in the significance of treatment effects between studies may be due to high variation between individuals within the treatment groups. Such variation is often observed during behavioural tests (e.g. Wolf et al., 2008; Gygax & Vögeli, 2016; Bushby et al., 2018) and could be due to the variable influence of drugs on individuals, the animal's affective states prior to testing or other aspects of animal temperament or personality. Overall, it is clear that affective states have some impact on the attention bias test developed throughout this thesis. However, these studies only partially supported the hypothesis that the attention bias test can be used to assess and differentiate a range of positive and negative affective states in sheep.

This thesis presented a range of methodologies for the assessment of attention biases in sheep. Each of the methodologies presented from chapters 3 onwards required no prior habituation period, thus improving the practical application of the test as hypothesised. Further, chapter 3 showed the test duration can potentially be shortened to further improve its practical application. Although, the results from chapter 4 suggested that shortening the modified test duration may not be appropriate.

The influence of pharmacological models on appetite was identified as a potentially confounding factor in chapters 2 and 3. Due to this, the method was modified to replace the food reward with an alternative positive stimulus, to aid interpretation of responses during testing. However, this modification caused a reversal in the direction of the attention bias observed in sheep treated with the drug mCPP. This finding very clearly demonstrates that a context specific interpretation of responses is required for attention bias tests, particularly where the stimuli are modified. This observation is consistent with human studies which show the observation of an attention bias depends on the salience of the stimuli, and potentially also the intensity or the type of salient stimuli (Zvielli, Bernstein & Koster, 2014; Pergamin-Hight et al., 2015).

The difference in interpretation between test methods therefore raises the question of which approach is better. The modified method removed the potential influence of feeding motivation and appetite during testing. However, there is a new key issue raised by this modification with regards to the interpretation of animal behaviour. When an animal is faced with a predator threat, the primary motivation underlying behavioural responses would be to avoid being predated. For a sheep, this may mean looking towards the threat with binocular vision to gain information on the relative distance of the threat to the animal (Kendrick, 2008). However, it may also drive flocking behaviour and attention towards the social stimulus (Dwyer, 2004; Wemelsfelder & Farish, 2004). Additionally, an animal which is less threatened by the predator may also exhibit flocking behaviour while isolated and display attention towards the social stimulus (Dwyer, 2004). Thus, there are potentially multiple contrasting motivations driving the same behavioural response during attention bias testing. This may make interpretation of behavioural responses difficult during future application of the modified test. On the other hand, an animal which is more threatened by a predator is unlikely to show interest in feed. Thus, interest in food more clearly represents a shift in underlying motivations, and importantly attention, away from the threat of predation. I therefore suggest that in the absence of factors that have a large influence on appetite, the original attention bias test method may be preferred.

Chapter 6 examined the repeatability of the attention bias test over 3 consecutive trials, as a first step to understanding whether behaviour in the test is most influenced by affective states or temperament. The findings of this study suggested that duration looking towards the dog and latency to feed were more transient and are likely more influenced by emotions or moods, while vigilance behaviour and zones crossed are more heavily influenced by underlying temperament traits. Importantly though, chapters 3 and 4 demonstrated that vigilance was significantly altered by transient affective states, indicating that the responses of an animal during testing are likely to be driven by a combination of temperament, emotion and mood. Overall, these findings supported the hypothesis that responses in the attention bias test are primarily influenced by affect, but that some aspects of behaviour are also influenced by consistent temperament or personality traits.

An important aim of this thesis was to develop a method that can be used as a measure of animal welfare. It is therefore important to discuss the potential role of attention bias tests in animal welfare assessment. Overall, the attention bias test developed throughout

this thesis shows promise as a measure of negative affective states. Given this, I currently see two key areas in which an attention bias test may be applied for the improvement of animal welfare. The first application is as a tool for further research. Measures of affective state can be used in research to better understand the ways in which our production systems and environments impact on the mental well-being of livestock. This will then allow for the improvement of management practices and facilities to enhance animal welfare. The second application is for welfare assessment or assurance schemes on-farm. This potential application would largely rely on refining the test to be more practical. It would also rely on the ability of the attention bias test to assess mood, rather than transient, short term emotional states. Importantly though, this novel cognitive approach for the assessment of affect in livestock is still new and under development. More research is therefore required before the test can be applied in these ways, as a robust measure of affect.

There are a number of key areas identified for further research on the attention bias test developed throughout this thesis. It remains unclear whether responses in the test are more heavily influenced by emotions or moods. Studies aiming to more clearly tease apart these aspects of affective state are essential for understanding animal responses during testing, and to determine the potential role of the test as a measure of animal welfare. Additional factors such as previous experiences, breed, animal age and background noise may have an influence on animal responses during testing. Further studies should be conducted to better understand the influence of such factors. This will be essential if the test is to be applied across populations of animals with different prior experiences or in environments where background noise may be unavoidable. The method developed throughout this thesis is quite rapid to apply, however, analysis of video footage remains a time consuming and labour-intensive process. Further, the test requires a specialised testing arena to conduct. Automation of behavioural measurement and adaptation of the test to existing sheep handling facilities will therefore improve its practical application in future. Modifications to the method that allow comparisons of attention towards a range of different positive and negative stimuli may give more insight into the affective state of an animal.

It should be noted that a number of other research groups have also been developing measures of attention bias for livestock and other animal species within the last few years. A recent review by Crump et al. (2018) summarises many of the affect-driven attention bias tests developed for use in animals. One interesting method developed by

Raoult and Gygax (2018) also used a looking time task for the assessment of attention in sheep. This study used a dual-presentation test paradigm to examine the influence of valenced video stimuli on attention in sheep. Video stimuli also varied in intensity, using videos of conspecifics and dogs filmed at different distances from the camera. Measures of attention included orienting of the head and ears and neural activity monitored using a functional near-infrared spectroscopy (fNIRS) sensor mounted to the heads of the animals. However the study did not find any differences in attention paid between the positive and negative stimuli and it was suggested video footage may not be appropriate stimuli for such a test paradigm.

Another study by Bellegarde et al. (2017) trained Scottish Mule sheep to discriminate between images of the faces of conspecifics photographed in a neutral or negative situation. Sheep which had been trained to associate a reward with the negative photograph learned the association at a faster rate than those assigned the neutral photograph. This was interpreted as an attention bias towards the negative cue, which facilitated engagement with the photo and learning of the association. While this study was not designed to assess attention biases, it presents another potential approach for the assessment of attention biases in livestock. Together, these studies demonstrate a growing interest within the research community in the use of cognitive methods for assessment of affective states in livestock. They also demonstrate some alternative approaches to assessing attention biases in livestock.

This cognitive approach is very new and the method still requires further refinement and validation before it can be considered as a robust measure of affect. However, the attention bias test developed throughout this thesis shows promise as a measure of affective state in sheep, particularly negative or anxiety-like states. Key measures of attention during testing include looking behaviours and other demonstrations of interest in food or a positive stimulus. These measures are likely to be most informative when assessing affective states. Vigilance behaviour may be better thought of as an animal's propensity to be in a negative affective state, within the context of the attention bias test. Consideration of all of these behaviours together will allow for a clearer interpretation of animal responses during testing.

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