Analysis of the Judgement Bias Test for Welfare Assessment in Rats

A Thesis by Publication Submitted in
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Degree of Doctor of Philosophy (PhD)

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Contents

Abstract

The identification of cognitive biases has become an important measure of animal affective (emotional) state, and therefore, animal welfare. Negative cognitive biases can be evidenced by animals preferentially processing novel information pessimistically and judgement-bias testing is the commonplace methodology to detect such biases. As the use of judgement-biases has increased in the scientific community, concerns regarding the efficacy and repeatability of these methods has been questioned. This body of work began with the aim of identifying the effects that a common housing procedure would have on judgement bias expression in rats. However, after analysis of data multiple confounding factors were identified that were argued to have seriously impacted on validity of methods employed. Therefore, investigations in this thesis focussed on a commonly employed, active choice judgement bias test as used in rats. Confounding factors that have received limited attention in the literature have been applied to these studies. These factors include phase of oestrous, social status, housing density and space allocation. It was aimed to investigate if the judgement bias test employed can find practical utility in welfare assessment of the laboratory rat.

In addition to investigating the effects of external factors on animal expression to the judgement bias test, investigations into the mechanistic nature of the test were also undertaken. Extinction of learning, which results in the animals failing to perform the test parameters, is a commonly reported limitation of these methods, as are significantly long training times often associated with their use. These two factors were also identified and studied in this thesis, with the aim of improving practicality of the judgement bias discrimination task (JBT) to allow its employment in applied animal husbandry situations. This thesis has identified many potentially confounding concerns of judgement bias testing in rats. Whilst evidence suggests that the JBT may never find practical use as a welfare assessment technique, it remains an extremely important indicator of animal affect. Improved understanding of these confounding factors will increase validity of the JBT as a tool for detection of affective state.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future be used in a submission in my name for any other degree or diploma in any university of other tertiary institution without the prior approval of the University of Adelaide and where applicable any partner institutions responsible for the joint award of this degree.

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Signed Date

Timothy Hugh Barker

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~~ Do it for her ~~

List of Publications by Candidate

Published Journal Papers

1. **Barker T.H.,** Howarth G.S. & Whittaker A.L. (2016) The effects of metabolic cage housing and sex on cognitive bias expression in rats. *Applied Animal Behaviour Science*, *177*, 70-76.
2. **Barker T.H.**, Bobrovskaya L., Howarth G.S. & Whittaker A.L. (2017) Female rats display fewer optimistic responses in a judgment bias test in the absence of a physiological stress response. *Physiology & Behavior*, *173,* 124-131.
3. **Barker T.H.**, George R.P., Howarth G.S. & Whittaker A.L. (2017) Assessment of housing density, space allocation and social hierarchy of laboratory rats on behavioural measures of welfare. *PLoS ONE* 12(9): e0185135.
4. **Barker T.H.,** Howarth G.S. & Whittaker A.L. (2018) Increased latencies to respond in a judgment bias test are not associated with pessimistic biases in rats. *Behavioural Processes, 146,* 64-66.
5. **Barker T.H.,** Kind, K.L., Groves, P.D., Howarth, G.S. & Whittaker A.L. (2018) Oestrous phase cyclicity influences judgment biasing in rats. *Behavioural Processes, In Press, Accepted Proof, DOI*:10.1016/j.beproc.2017.11.016

Journal Papers Under Review

1. **Barker T.H.,** Howarth G.S. & Whittaker A.L. Imposed subordination in rats impedes learning as determined by the judgment bias test, resubmitted after peer review to *Journal of Veterinary Behavior: Clinical Application and Research.*

Published Supporting Journal Papers

1. George R.P., **Barker T.H.,** Lymn K.A., Bigatton D., Howarth G.S., & Whittaker A.L. Judgement Bias Test to Assess Affective State and Potential Therapeutics in a Rat Model of Chemotherapy-Induced Mucositis. *Scientific Reports*, DOI:10.1038/s41598-018-26403-7

Peer Reviewed Conference Papers

1. **Barker T.H.**, Howarth G.S. & Whittaker A. L. (2015) The effects of housing in metabolic cages on cognitive bias expression in rats. Presented at *Australian and New Zealand Laboratory Animal Association Conference*, Adelaide, Australia, August-September 2015.
2. **Barker T.H.**, Howarth G.S., & Whittaker A.L. (2016) Judgment Biases: Metabolic cage housing and sex effects. Poster Presented at *Australian and New Zealand Laboratory Animal Association Conference,* Auckland, New Zealand, August-September 2016.
3. **Barker T.H.**, Howarth G.S., & Whittaker A.L. (2016) Cognitive biases: Metabolic cage housing; sex and comparative measures. Presented at *International Society for Applied Ethology Congress*, Edinburgh, Scotland, July 2016.
4. **Barker T.H.**, George R.P., Howarth G.S. & Whittaker A.L. (2017) The Assessment of Housing Density and Social Hierarchy of Laboratory Rats on Behavioural Measures to Assess Welfare. Presented at *2017 Australian & New Zealand Council for the Care of Animals in Research and Teaching Conference, 'Maintaining Social License in a Changing World',* Queenstown, New Zealand, September 2017.

Thesis Structure

This thesis is presented as a ‘Thesis by Publication’ and consists of published journal articles and manuscripts currently under review. The manuscripts are formatted according to journal requirements. Consequently, reference format and English spelling differ throughout this thesis. A consolidated list of references is provided at the end of the main body of this thesis.

Additional publications not directly related to this program of study, in which the candidate was involved, are included as appendices.

CHAPTER 1.

Introduction

1.1. Review of Literature and Background to Project

1.1.1. Animal Welfare and Welfare Assessment

Welfare is a measurable construct that refers to the state of a non-human animal (henceforth, referred to as animal) in relation to its environment (Broom, 1991). Poor welfare has often been associated with animal suffering (Dawkins, 1990), and whilst suffering and poor welfare occur in tandem, poor welfare does not occur solely due to, or is always an implication of, animal suffering (Broom, 1991). Animal welfare has been best defined according to three main schools of thought. Whilst some theories of what constitutes ‘good welfare’ once comprised only one of these schools (Dawkins, 1990), good welfare is now understood to be a factor of all three (Fraser et al., 1997; Boissy et al., 2007). The first school is physical status and fitness - the attempts of the animal to cope with its environment (Fraser and Broom, 1990). The second is mental status - the subjective experience of what animals ‘feel’ (Duncan, 1993). The final school is naturalness or telos - promoting the performance of natural behaviours and leading natural lives (Rollin, 1993).

It has been suggested that two types of distortions arise when assessing animal welfare (Fraser et al., 1997). The first distortion is assessing welfare purely empirically. Fraser et al. (1997) argued that animal welfare, as a concept, should be based on values as it pertains to what is inherently *better* or *worse* for animals, and these values are innately subjective to the assessor. However, traditionally animal welfare was considered as a ‘scientific concept’ that could be measured objectively, for example stress could be measured through cortisol levels and animal health through incidence of disease (Stafleu et al., 1996). This distortion is discussed in greater detail in section 1.1.2. The second distortion is the definition of animal welfare used by a scientist according to the three main schools discussed above. Often, individuals have considered one of these schools as being ‘more important’ than the others, leading to skewed conceptions of what constitutes good welfare. The presupposition of considering one school as more important than another has created an environment where welfare assessment has been inherently difficult to standardise (Fraser et al., 1997).

As such, methods for welfare assessment have tended to focus on minimisation of the negative experience, whilst maintaining internal homeostasis (Yeates and Main, 2008; Mellor, 2012). An example of this distinction can be made when assessing the ‘Five Freedoms’ afforded to animals (Webster, 1994) which include (1) Freedom from hunger and thirst; (2) Freedom from discomfort; (3) Freedom from pain, injury or disease; (4) Freedom to express normal behaviour; and (5) Freedom from fear and distress. Of these freedoms, the first school (physical status) is featured more prominently than either the second (mental state), or third (telos) schools. In addition, four of these five freedoms explicitly value minimisation of the negative experience (Yeates and Main, 2008). Conversely, freedom five promotes animals being able to express normal behaviours, however freedom four, the only representative of the second school of welfare, does not promote prevalence of a positive subjective experience. This has created a paradigm whereby the majority of welfare assessment techniques only value minimisation of these negative experiences without encouraging a complementary positive experience (Mellor, 2015).

1.1.2. Animal Affect

With this understanding, there has been a recent shift in attitude to assess welfare state with greater emphasis on subjective experience, also described as affective state (Yeates and Main, 2008; Mellor, 2012), to encourage positive experiences. The affective state of an animal is a psychophysiological construct (Harmon-Jones et al., 2013a) composed of three underlying components. The first of these components is valence - the intrinsic attractiveness or aversiveness of a stimulus to provoke positive or negative valence, respectively (Frijda, 1986; Harmon-Jones et al., 2011). The second component is motivational intensity - the strength of desire for the animal to approach or avoid the stimulus (Harmon-Jones et al., 2013b). The final component is arousal - the strength of activation of the sympathetic nervous system in response to the stimulus (Gable and Harmon-Jones, 2013). Affective states have also been used as a term to interchangeably refer to both mood and emotions (Paul et al., 2005), with emotions being defined as acute and attached to stimuli (e.g. fear), and mood being extended and not necessarily derivative of immediate stimuli (e.g. depression). For the consideration of this thesis and accompanying publications, the affective state of an animal is defined as per the works of Mendl et al. (2010a) which considers an animal's long term mood states, combined with their reactions to current emotion-inducing stimuli. Figure 1.1 is an adaption of the model presented by Mendl et al. (2010a) and details a two-dimensional framework of core affective experiences and associated degree of emotional arousal and valence consideration.

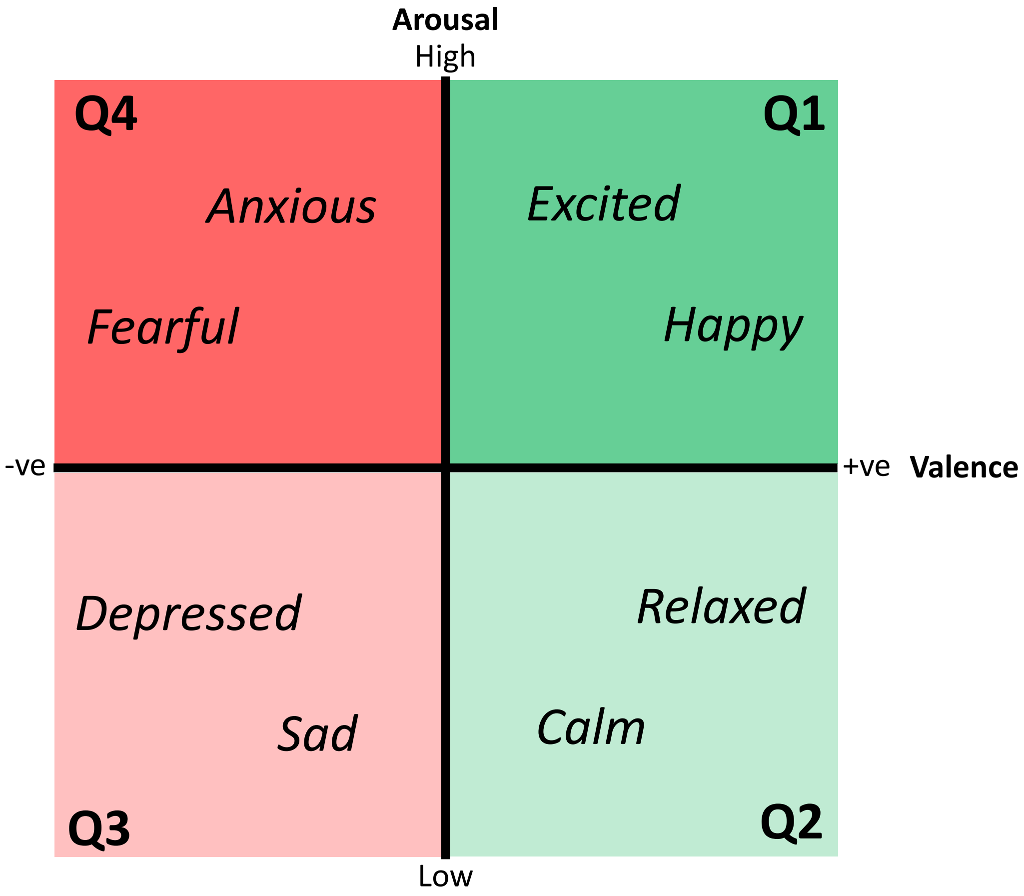

Figure 1 – Example of core affective states and their associated degree of emotional arousal and valence consideration.

Words in italics represent possible locations of core, commonly-reported affective states and discrete emotional responses. Quadrants 1 (Q1) and 2 (Q2) are associated with positive valence, and therefore positive affective states. Q3 and Q4 are associated with negative valence and therefore negative affective states. Q1 and Q4 are associated with high arousal, these affective states follow a large emotional response. Q2 and Q3 are associated with low arousal, these affective states follow a small emotional response. Adapted from Russell and Barrett (1999), Burgdorf and Panksepp (2006) and Mendl et al. (2010).

Using the framework shown in Figure 1, and the three components of an affective state construct, it is possible to conceptualise how an animal's affective state might shift. Mendl et al. (2010a) used the example of feeding motivation, as this motivation to eat causes an animal to seek sustenance. If the animal is successful it may lead to a highly aroused, positive state of excitement (Q1). After consumption of the food, arousal intensity may fall, but the positive experience of having eaten remains (Q2). If the animal fails to find food, this may lead to frustration or anxiety, highly aroused, negative states (Q4). If this failure persists over time it may culminate in low-arousal states of sadness or depression (Q3) (Mendl et al., 2010a).

Being able to accurately identify and quantify an affective state has become crucial in assessing and improving animal welfare (Balcombe, 2006; Brydges and Hall, 2017). However, identifying an affective state cannot be achieved directly (Mendl et al., 2010a; Mellor, 2012). Moreover, affective states, such as anxiety or depression (negative) or excitement and contentment (positive), are closely related and need to be identified and considered accordingly. Traditional methods used to assess welfare typically fail at truly identifying animal affect, instead being reflective of the components of animal affect, that is, emotional arousal or valence. These methods rely on associating easily identified phenotypic changes to imply a change in animal affect, and therefore, welfare state. The most widely-investigated phenotypic changes are neurophysiological and behavioural parameters (Mendl et al., 2009; Novak et al., 2015). These approaches are inherently flawed at detecting animal affect. For example, increases in faecal-corticosterone concentration have been observed in rats housed in metabolic cages (Kalliokoski et al., 2013). This housing type has been identified as a significant stressor for mice and rats (Whittaker et al., 2012; Whittaker et al., 2016a). However, male rats experienced an almost 2-fold increase in plasma corticosterone concentrations immediately after engaging in sexual activity (Bonilla-Jaimem et al., 2006). Physiological measures indicate emotional arousal and are unable to discern how the emotional response is considered by the animal, that is, whether the response is positively or negatively valenced. The above mentioned studies indicate that the animals experienced strong emotional arousal to stimuli, however one stimulus was negative (Kalliokoski et al., 2013) and the other positive (Bonilla-Jaimem et al., 2006).

Behavioural indices of animal affect are typically able to identify valence through the type and frequency of behaviours elicited in the context of the animal’s environment. For example, play behaviour is largely associated with animal contentment, a positive affective state. Increased periods of immobility or reduced motivation to move are conversely indicative of animals feeling anxious or depressed (Sams-Dodd, 1995). However, there are often difficulties in interpretation of changes in behaviours in relation to the confines of a research question. As previously discussed, behaviours occur because of the animal’s motivational state (Temple et al., 2011; Seehuus et al., 2013), which therefore infers that observable behavioural patterns are a measure of motivation. The motivational state of an animal is also not consistent between animals (Oswald et al., 2011), therefore welfare measures that solely study behavioural indices may be confounded by differing motivational values that individual animals hold. Rats that had a high motivation to eat palatable food (binge-eating prone, BEP) tolerated higher levels of foot-shock for a food reward than rats with a low motivation for palatable food (binge-eating resistant, BER). Despite the aversive consequence for eating palatable food, BEP rats would continue to do so, simply due to being genetically pre-disposed to having a higher motivation for that reward (Oswald et al., 2011). Animals may display certain behaviours not due to the research parameters in question, but due to innate differences in their motivational state.

There remains an important niche in welfare assessment to accurately identify and quantify animal affect, whilst being free from the restrictive properties of physiological and behavioural indices (Mellor, 2015). One of the fastest-growing concepts with the potential to capitalise on this niche involves detection of cognitive bias through a judgement bias paradigm (Mendl et al., 2009).

1.1.3. Judgement Bias Discrimination Tasks

Theories of cognition propose clear and measurable relations between the affective state and cognitive functions, such as decision making and information processing (Harding et al., 2004; Paul et al., 2005). This phenomenon is known to exist in humans (Mineka et al., 1998; Mogg and Bradley, 1998; Amir et al., 2005; Standage et al., 2014) and animals (Bateson et al., 2011; Brydges et al., 2011; Burman et al., 2011; Salmeto et al., 2011; Briefer and McElligott, 2013; Destrez et al., 2014) and observable patterns between studies have remained largely consistent. People that self-identified as being anxious or depressed (Amir et al., 2005), and animals subjected to conditions known to induce anxiety or depression (Mendl et al., 2009), promote the pessimistic judgement of ambiguous stimuli.

Pessimism and pessimistic-like tendencies lead to behavioural adaptions that have evolutionary advantages. The negatively valenced states of anxiety and depression produce short-term value for an animal's immediate survival (Bethell, 2015a). Anxiety is associated with an increase in epinephrine production, which facilitates an animal's fight or flight response (Mogg et al., 1995), whereas depression is linked to inactivity, social isolation and reduced food consumption (Willner et al., 1998; Nettle and Bateson, 2012; Gordon and Rogers, 2015), behaviours which promote energy conservation and a reduction in risk-taking actions (Bethell, 2015a). In contrast, optimism is associated with animals in positive affective states, which is inferred to induce opposite effects.

Categorising behavioural output as optimistic or pessimistic presents a measurable endo-phenotype that can objectively identify animal affect. This categorisation can be achieved through application of a cognitive bias test, most commonly performed using a judgement bias discrimination task (JBT). First proposed by Harding et al. (2004), the JBT involves teaching animals to respond with two different behaviours to two different stimulants. A positive reward is associated with a unique stimulus. Upon exposure to the stimulus the animal will learn to display a certain behaviour to obtain the positive reward. The animal is then taught that an aversive reward is associated with a second, different stimulus. Upon exposure to this second stimulus the animal will learn to display a second behaviour to avoid the aversive reward. When the animal is introduced to a stimulant intermediate between the two learned stimuli, judgement to this ambiguity can be observed through

behaviours the animal exhibits (Figure 2). If the animal exhibits behaviours consistent with the positive reward, that animal has displayed an optimistic cognitive bias. If the animal displays behaviours associated with the aversive reward, it has displayed a pessimistic cognitive bias.

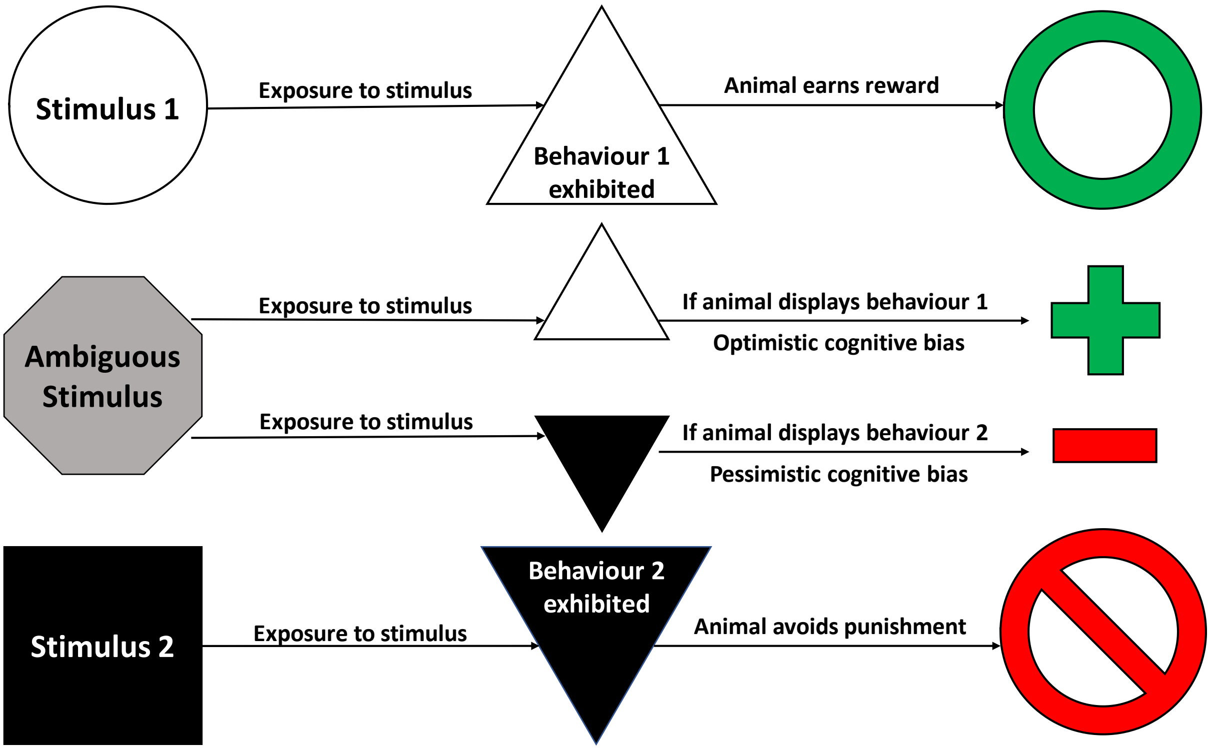

Figure 2 – Flowchart of the judgment bias paradigm learning procedure.

Positive reward is always associated with stimulus 1. Exposure to stimulus 1 results in expression of behaviour 1 to obtain the reward. The negative reward is always associated with stimulus 2. Exposure to stimulus 2 results in expression of behaviour 2 to avoid punishment. A stimulus directly intermediate between the two previously learned stimuli is introduced. Depending on the behaviours expressed to the ambiguity, a cognitive bias can be discerned.

The cognitive processing of a stimulus influences emotional response, whilst the affective state of an animal will bias this cognitive processing to produce variable emotional responses. This is the basis for the use of judgement biases to assess affective state of animals as it allows establishment of clearly defined, objective measures of cognitive performance (judgement to ambiguity) as an indicator of emotional state (Mendl et al., 2009). If an animal responds to a JBT displaying an optimistic bias, we can assume that the animal is in a positive affective state. The ability to classify judgement as being optimistic or pessimistic allows for research parameters to be established that can quantify how particular conditions or stimulants influence emotional response valence. This can then inform best practices to encourage prevalence of optimistic cognitive biases, hence achieving the goal of assessing welfare using an indicator of animal affect to encourage positive experience.

JBTs can be designed in multiple ways in accordance with learning capabilities of the animal and parameters of interest (Mendl et al., 2009; Bethell, 2015a). The most common categorisations of these tests are a go/no-go test or an active response test (Mendl et al., 2009; Bethell, 2015a; Roelofs et al., 2016). A go/no-go test involves presenting two stimuli to an animal, one stimulus encourages the animal to make an action (go response) regarded as optimistic, whilst the second stimulus discourages the animal to perform the action (no-go response) regarded as pessimistic. This procedure type has been utilised in multiple studies to identify cognitive bias in many animal species (Harding et al., 2004; Asher et al., 2009; Doyle et al., 2010a; Anderson et al., 2012; Bethell et al., 2012a; Douglas et al., 2012; Destrez et al., 2013; Neave et al., 2013; Daros et al., 2014; Destrez et al., 2014; Starling et al., 2014; Verbeek et al., 2014b; Horváth et al., 2016; d'Ettorre et al., 2017; Lalot et al., 2017; Le Ray et al., 2017; McGuire et al., 2017). The active response test differs in that both positive and negative stimulants require the animal to make an active response, where an active response is defined as being a deliberate, quantifiable action by the subject animal (Burman et al., 2008). Whilst training animals to perform a go/no-go JBT has resulted in greater success compared to active choice JBT (Hintze et al., 2017), there are well documented concerns associated with the go/no-go methodology. The main concerns being the length of training times, continued exposure to negative events and the inability to associate a lack of response with negative cognitive biases (Brydges et al. 2011; Brydges and Hall 2017; Barker et al. 2018). Therefore, the following discussion is largely limited to JBTs utilising active response methods.

1.1.4. Confounding Design and Under-Studied Anomalies of Active Choice JBTs

1.1.4.1. Stimulant and Reward Pairings

An important consideration in design of an active choice JBT is the requirement for active behaviour to be identical in response to both stimulants. Discussion of the JBT has so far focused on test design that utilised a positive reward associated with stimulus 1, and an aversive punishment associated with stimulus 2 (Figure 2). Active response JBTs have successfully been developed using this stimulant/reward typing (Rygula et al., 2012; Papciak et al., 2013; Rygula et al., 2014a; Rygula et al., 2015a; Rygula et al., 2015b; Saito et al., 2016; Drozd et al., 2017; Golebiowska and Rygula, 2017a; b; Curzytek et al., 2018). Mice and rats have been trained to activate one lever in response to stimulus 1 (2000Hz tone at 75dB) and were rewarded with the positive reward (sucrose solution). Upon experiencing stimulus 2 (9000Hz tone at 75dB) the animals activated a second lever to avoid punishment (mild foot-shock and/or white noise).

Whilst this stimulant/reward pairing has produced successful results, its continued use has been discouraged. Repeated exposure of an animal to a punishment is detrimental to animal welfare and has the potential to influence affective state of the animal to reflect aversive testing conditions (Brydges et al., 2011). The learning capabilities of animals subjected to intensive stressful regimes have also been subject to criticism (Vögeli et al., 2014). To best observe a positive affect with a JBT, the stimulant/reward pairing must not be aversive to the animal, and a positive versus less-positive stimulant/reward pairing has been suggested (Brydges et al., 2011). This pairing type has shown repeated success in active choice JBTs and is most commonly associated with a large food reward as the positive reward and a small food reward as the less-positive reward (Brilot et al., 2010; Murphy et al., 2013; Keen et al., 2014; Hernandez et al., 2015; Murphy et al., 2015; Brydges and Hall, 2017; Clegg et al., 2017). Different pairing methods, such as an accessible food reward versus an inaccessible food reward (Burman et al., 2011; Carreras et al., 2015; Carreras et al., 2016; Potes et al., 2017), or an instant reward versus delayed reward (Matheson et al., 2008), have also shown success in an active choice JBT. However, the JBT model utilised in this study used a favoured food-item to act as the positive reward versus a non-favored food-item to act as the less-positive reward. Favoured food-items were selected through preference testing prior to the animal being trained on the JBT (Brydges et al., 2011), and this stimulant/reward typing has been used to repeated success (Brydges et al., 2011; Brydges et al., 2012; Pomerantz et al., 2012; Chaby et al., 2013).

1.1.4.2. The Active Response

As discussed in section 1.1.3. there has been a push, to utilise JBT design that requires the animals to make a deliberate, quantifiable action for both the go and no-go response (Burman et al. 2008). These tests are typically referred as either active response or go-go tests. The first JBT designed that attempted to utilize an active response for both the positive and negative responses was achieved in a study on rats. The animals were trained to associate a spatial location with an obtainable food reward (positive reward) and another spatial location with an unobtainable food reward (negative reward) (Burman et al., 2008). The latency to approach both spatial locations was used as the testing parameter as the animals were required to elicit an action (approach the ‘rewarded’ location) before being removed from testing scenario. Latency to approach novelty has been evidenced to identify preference in animals, therefore decreased latency to approach an object can correctly identify an increased desire to that object (Bateson and Kacelnik, 1995). This JBT design, using spatial location and ‘latency’ as the active response, has been reported consistently within the JBT literature (Burman et al., 2008; Burman et al., 2009; Boleij et al., 2012; Richter et al., 2012; Kloke et al., 2014; Baciadonna et al., 2016). However, it is contested that latency is not an appropriate measure to identify an active response as latency to approach a reward can be affected by decreased motivation or inactivity (Brilot et al., 2010; Hernandez et al., 2015). A decrease in approach latency could perhaps not be due to negative cognitive bias, but due to decreased desire to obtain the reward (Karagiannis et al., 2015), or decrease in general activity levels (Salmeto et al., 2011; Hymel and

Sufka, 2012). This anomaly was the foundation for the study presented in chapter 7, which concludes that increased latency is not associated with pessimistic biases (Barker et al., 2018).

It is therefore suggested that active response tests require animals to make a deliberate active response to the rewarded stimulus (Hernandez et al., 2015; Barker et al., 2018). Active responses that have been documented include foraging behaviours (Brydges et al., 2011; Chaby et al., 2013; Barker et al., 2016), removal of lids from a rewarded receptacle (Brilot et al., 2010; Pomerantz et al., 2012; Keen et al., 2014; Gordon and Rogers, 2015) or attempting to consume the reward item that would normally be present during a training trial (Boleij et al., 2012; Seehuus et al., 2013; Titulaer et al., 2013; Kis et al., 2015).

1.1.4.3. Extinction of Learning

One of the most commonly reported problems of the JBT arises from animal extinction of learning. This phenomenon is experienced by animals that learn outcomes of the JBT and, therefore, do not perform the required behaviours necessary for cognitive bias to be observed (Brilot et al., 2010; Doyle et al., 2010b; Jamieson et al., 2012; Briefer and McElligott, 2013; Murphy et al., 2013; Freymond et al., 2014; Karagiannis et al., 2015; Barker et al., 2016; Barker et al., 2018). Many JBTs employed do not associate a reward item with the intermediate, ambiguous stimulus (Roelofs et al., 2016), and utilise repeated testing of the animal to the intermediate probe to increase statistical power (Barker et al., 2018). Therefore, a requirement remains to balance the number of exposures each animal receives to the intermediate probe to achieve statistical power without encouraging onset of learning extinction. Techniques that have achieved this balance include preventing animals from acting as their own controls (Barker et al., 2016) and introduction of training trials between ambiguous stimulus exposure (Burman et al., 2009; Doyle et al., 2010b; Brydges and Hall, 2017).

Extinction of learning is further discussed by Chaby et al. (2013) who note that during first exposure to the ambiguous probe animals interpret the intermediate ambiguous stimulus based only on their own subjective experiences. However, each subsequent exposure is further influenced by that animal’s previous interactions with the ambiguous stimulus (Doyle et al., 2010a). Chaby et al. (2013) identified that significant differences in cognitive expression between treatment groups were only observed for the initial ambiguous trial. The authors suggested that analysis of initial ambiguity trials should be conducted separately from any subsequent trials using the ambiguous stimulus to account for this learning discrepancy in the discussion of results (Chaby et al., 2013).

Despite the conclusions of Chaby et al. (2013), extinction was not observed in the Brydges et al. (2011) study, that utilised the same methods. Neither was it observed in subsequent studies also utilising this methodology (Brydges et al., 2012; Barker et al., 2016; Barker et al., 2017a; Barker et al., 2017b; Barker et al., 2018). This may be due to the training paradigm used by Chaby et al. (2013). In the original (Brydges et al., 2011) study, a training phase immediately prior to the testing phase was recorded. This particular phase involved removing the reward (positive or low-positive) from a normally rewarded training trial. The animals still behaved as if the stimulant was rewarded (by foraging in the normally rewarded bowl), but a reward would not be present, therefore the animals learnt to expect that not every trial would be rewarded, potentially reducing chance that learning extinction would occur (Barker et al., 2016). It is also important to note that unrewarded training trials occurred for a limited time period (five days) and took place after the animals had already demonstrated the ability to perform the judgement bias test to the researcher’s established criterion. This is important as unrewarded trials could possibly hamper the ability of animals to learn and subsequently perform the JBT. This particular training phase was not documented to have occurred in the Chaby et al. (2013) study, which could provide reason as to why this particular study reported that learning extinction in the animals had occurred, whereas other studies utilising the same methodology did not witness this effect. Some studies have attempted to introduce partial reinforcement of ambiguous locations (Bateson and Matheson, 2007; Matheson et al., 2008). However, this strategy is argued to permanently alter reward status of the intermediate stimulus for the animal, so that its presence is no longer ‘truly intermediate’ because the animal has learned that presence of the intermediate stimulus results in higher chance of a reward being offered than presence of the negative stimulus, and judgement to this ambiguity may change accordingly.

1.1.4.4. Sex and Oestrous Cyclicity

Brown et al. (2016) discussed that previous JBT study has focused on either a male or female subject with only a handful to have controlled for both sexes in experimental design and statistical analysis (Briefer and McElligott, 2013; Asher et al., 2016; Barker et al., 2016; Brown et al., 2016; Carreras et al., 2016; Takeshita and Sato, 2016; Barker et al., 2017a; Roelofs et al., 2017). Few of these studies report no difference between male and female judgement (Asher et al., 2016; Carreras et al., 2016; Roelofs et al., 2017). However, the remaining majority present evidence of such difference (Briefer and McElligott, 2013; Barker et al., 2016; Brown et al., 2016; Takeshita and Sato, 2016; Barker et al., 2017a). From these studies, female animals responded with greater optimistic biases after experiencing a stressful treatment compared to males (Briefer and McElligott, 2013). This finding was later reproduced for this thesis in a study of Sprague-Dawley rats and is presented in chapter 2 (Barker et al., 2016). However, in a later study with increased statistical power also reproduced in this thesis (Chapter 3), female Sprague-Dawley rats responded similarly to males after experiencing metabolic cage housing (stressful treatment) whilst responding with fewer optimistic biases in control housing (Barker et al., 2017a). Female Lister-hooded rats have displayed decreased latencies to respond to ambiguous locations in a spatial judgement bias test compared to rewarded locations, whilst males recorded no difference, indicating optimism in the female cohort (Brown et al., 2016). In studies of Japanese pygmy squid (*Idiosepius paradoxus* females are more likely to display extended pessimism compared to males (Takeshita and Sato, 2016). Whilst consensus as to the difference between male and female performance in the JBT has yet to be elucidated, it is apparent that male and female performance is not always equivalent, with female performance being recorded as seemingly more variable than that of males (Barker et al., 2017a). As discussed in a review by

Girbovan and Plamondon (2013), there is a need, in at least rodent studies to include sex and its interactions as an explanatory variable in analysis of data collected.

Variability in the female cohort could perhaps be explained by the female-dependant factor of oestrous. This question forms the foundation for the study presented as Chapter 6. The oestrous cycle of rats lasts approximately 5 days and is comprised of four unique phases (proestrus, oestrus, metoestrus and dioestrus), that can be categorised by ovarian hormone concentrations and presence of varying cell types (Goldman et al., 2007; Paccola et al., 2013; Levine, 2015). The influence of ovarian hormones has been strongly correlated with variations in rodent behaviour (Kastenberger et al., 2012) and logical progression suggests that oestrous cyclicity may be a significant explanatory factor behind variability of female response to the JBT. Whilst the scope of this research and discussion has focused on the rodent, the factor of oestrous cyclicity is suggested to be an important predictor variable that should be accounted for when designing JBTs that make use of a female cohort.

1.1.4.5. Removal of/from the Stressor

Reports on the JBT often describe the hypothesised negatively valenced animals responding with clearly optimistic biases. This is described in the work of Doyle et al. (2010a) who first reported that removal of a stressor or from a stressful environment can promote animals to respond with an optimistic bias, when it was hypothesised that a pessimistic bias would be observed. The majority of studies that report a disputed hypothesis follow this pattern where removal from an imposed stressor has triggered an optimistic response (Doyle et al., 2010a; Sanger et al., 2011; Briefer and McElligott, 2013; Düpjan et al., 2013; Keen et al., 2014; Verbeek et al., 2014a; Hernandez et al., 2015; Barker et al., 2016).

The phenomenon could be argued to have been attributed to the imposed stressor not being strong enough. However, as discussed by Doyle et al. (2010a), animals facing the stressor (sheep being physically restrained) exhibited increased serum cortisol concentrations, identifying the restraint to be a highly stressful procedure. Meanwhile, bears (*Ursus arctos horribilis)* displaying stereotypic behaviours (pacing) were also shown to exhibit an optimistic bias (Keen et al., 2014). Rats displaying stereotypic behaviours also spent more time in ‘positively rewarded locations’ (Novak et al., 2015). Doyle et al. (2010a) discussed the findings of the sheep study and suggested that restrained sheep exposed to an extremely aversive stimulus altered their ‘risk-taking threshold’ (Doyle et al., 2010a). This suggested that the aversive presence of the dog was less aversive than the restraint treatment. Therefore, restrained sheep were more likely to ‘risk’ approaching ambiguous locations compared to control sheep. Meanwhile, Keen et al. (2014) identified that in the study involving bears, the familiar testing area, interaction with humans and food reward all contributed to make the testing procedure an optimistic event in and of itself. Therefore, despite the bears displaying stereotypic behaviours, movement into the testing chambers overrode any differences in affect associated with observed stereotypic behaviours. There is further discussion to this effect, namely that there is evidence to suggest elevated glucocorticoids (corticosterone), released during a stress response, motivate animals to consume food (Willner et al., 1998; Dallman, 2010). As discussed by Hernandez et al. (2015), this factor increases the incentive value of food rewards used, therefore animals in the negative state respond with seemingly optimistic biases. This prompts development of JBTs that do not utilise food as the rewarding element.

A disputed hypothesis was also observed in studies involving goats (Briefer and McElligott, 2013) and rats (Barker et al., 2016). Both studies utilised a sample including males and females, and interestingly observation of optimistic decisions after experiencing an imposed stressor was only observed in the female cohort adding to the discussion of section 1.1.4.4 that male and female response to a JBT are not equivalent.

1.2. Research Aims and Questions

The main aim of this body of research was to improve practicality and reliability of the judgement bias discrimination task as a tool for assessment of animal welfare in the rat. By investigating under-studied, external factors such as oestrous cyclicity (Chapter 6), or the as yet un-discussed factor of social hierarchy and subordination stress (Chapters 4 and 5), this study aimed to refine the process and considerations that need to be accounted when designing and employing future JBT methods in all species. Investigations of latency (Chapter 7) and learning rates (Chapter 5) elucidate the methods by which the JBT can best be employed and results analysed. In summary, the aims of this project were to:

1. Determine the effects that metabolic cage housing had on male and female rat judgement when exposed to the JBT, and then to correlate biases with known physiological and behavioural measures indicative of a stress response.

2. Identify the effects of social subordination stress on the response of male rats exposed to the JBT. The effects of subordination stress were considered in high stocking density cages versus low-density cages, and large space allocation cages versus small space allocation cages.

3. Examine the relationship between oestrous phase cyclicity and judgement bias expression of female rats. Social status of rats was again controlled for to identify if any significant interactions existed between social status and oestrous phase on judgement bias.

4. Understand learning rates of female rats subjected to the JBT and evaluate the efficacy of training paradigms. Social status of rats was controlled for to identify effects of subordination stress on learning rate.

5. Evaluate the effect of learning extinction in male rats after consecutive exposures to the intermediate, ambiguous probe of the JBT.

1.3. Research Methodology

Specific details of experimental design, housing conditions and animal status are presented in the following chapters. However, the judgement bias discrimination task reported in each chapter is consistent, with modifications discussed where relevant. The JBT employed is described next.

1.3.1. Apparatus and set-up

The judgement bias test followed the methods of Brydges et al. (2011). Two Perspex boxes (610mm x 435mm x 500mm) (henceforth referred as the 'start box' and the 'goal box') were connected via a PVC pipe (800mm with 100mm diameter). Inside the goal box, two reward bowls were positioned in each corner opposite the pipe. The right-hand bowl was filled with coriander scented sand, while the left-hand bowl was filled with cinnamon scented sand (1% by weight of spice to sifted sand) (Figure 3). All testing was performed under red-light conditions in a temperature-maintained facility.

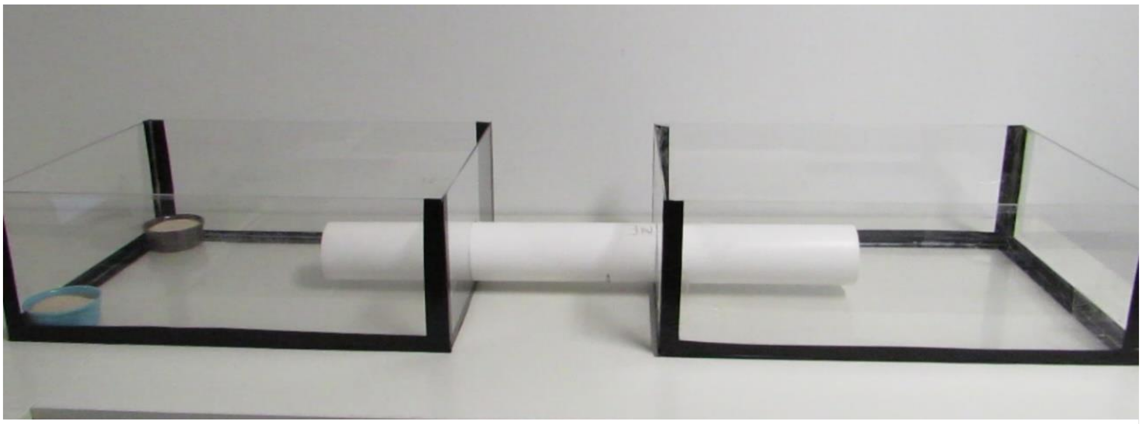

Figure 3 – Judgement Bias Discrimination Task – Testing Apparatus

Originally designed and developed by Brydges et al. (2011)

The positive reward items used were milk chocolate baking chips (Cadbury, London, England) and the less-positive reward items used were Cheerios (Uncle Toby's, Victoria, Australia). For every rat, each reward item was paired specifically with a bowl location and scented sand. Reward association remained consistent for each rat throughout the experiment but was counter balanced between associations. The training stimuli were sandpapers of different grades that lined the entire inside of the pipe. The first stimulus was coarse sandpaper (P80 grade). The second stimulus was fine sandpaper (P1200 grade). An intermediate grade of sandpaper (P180) was used as the ambiguous probe during testing. Each stimulus (coarse and fine sandpaper) was associated with a particular reward item (e.g. chocolate reward always paired with coarse sandpaper and located in the coriander scented bowl).

Based on experimental conditions of interest, animals were sorted into testing groups prior to training. If for any reason this was not inductive of animal learning (e.g. housing in metabolic cages, as seen in Chapters 2 and 3), animals were trained in control conditions before being moved to testing conditions.

1.3.2. Phase A – Local Habituation

Prior to training on the JBT, each rat was handled for two 10-minute periods. The first period between 0900 and 1200 hours, the second period between 1400 and 1700 hours. Phase A lasted for five days.

1.3.3. Phase B - Apparatus Habituation

Rats were placed into the testing apparatus, four times a day for 5-minute intervals. Food bowls contained the reward items appropriate to the individual rat, these rewards were placed on the surface of the sand in the reward bowls. No sandpaper was present within the PVC pipe. Phase B lasted for five days.

1.3.4. Phase C – First training

Phase C marks the beginning of animal training. The testing apparatus now contained the appropriate sandpaper stimuli. Each rat was subjected to four training trials per day. Two of these trials occurred between 0900-1200 hours and two between 1300-1700 hours. These times correspond with the dark phase of the animal’s circadian cycle. For each time period, one trial contained the positive reward item and corresponding sandpaper, and the second trial contained the less-positive reward item and corresponding sandpaper. These occurred randomly. The reward items were placed on the surface of the scented sand in the appropriately corresponding bowl.

Rats were placed in the start box, upon which a timer is started. Recordings were made for latency of the rat to enter the pipe after placement into the start-box, to first exit the pipe and enter the goal-box, to approach any reward bowl and approach the correct (reward containing) bowl. Timing was stopped once the animal had begun to consume the reward item. Once the rat had consumed the reward it was removed from the apparatus, which was then cleaned with 70% ethanol solution. If the rats failed to consume the reward within ten minutes the trial was considered a failure and the animal was removed from the apparatus. Rats were eligible to be promoted to Phase D after they had completed five days of performing three of the four trials correctly per day. At this point, it was assumed that the animals had learned this discrimination. A table of commonly used definitions is included as Table 1.

Term	Definition
Approach	When the rat actively and intentionally placed its forelimbs and face into a reward bowl to extract the reward.
Forage	When the rat continuously and deliberately displaced the sand in the food bowl to obtain the reward.
Consumption	When the rat actively and intentionally interacted with the food by bringing it to its mouth.
Success	Successful trial was determined after the animal had approached and foraged in the correct (reward containing) food bowl before approaching or foraging in the incorrect food bowl.
Promotion	Animals were promoted to the succeeding trial (where appropriate) after achieving ¾ successful trials per day, for five consecutive days.
Failure	If the rat failed to consume the reward within 10 minutes of being placed into the testing chamber.

Table 1– Definitions of commonly used terminology for the Judgment Bias Paradigm

1.3.5. Phase D – Second training

This phase was similar to phase C; however, the reward item was no longer placed on top of the sand in the appropriate reward bowl but was buried into the reward bowl. Each successful extraction of the reward increased depth of reward burial for the next trial. These burial levels are defined as: (1) Level. Where the reward item is buried into the top layer of the sand, so that it is still visible to the rat, but needed extraction to obtain. (2) Light Cover. The reward is now completely buried in the sand yet remains close to the surface and is only lightly covered. (3) Quarter. The reward is now completely buried in the sand, approximately one quarter deep from the bottom of the reward bowl and the surface of the sand. (4) Half. The final burial stage, the reward is now buried directly in the middle and centre of the reward bowl, completely covered by sand. As with phase C, criteria for promotion to the next phase remained consistent.

1.3.6. Phase E – Third training

This phase was similar to Phase D; however, the reward item was always buried directly in the middle and centre of the reward bowl, completely covered by sand. One trial of the four per day was randomly chosen to be unrewarded. The animals undertook the test as per normal, however upon foraging in the correct bowl, no reward item was present. This teaches the animal that not every trial is rewarded, which combats the onset of learning extinction during the testing phase. As with phase D, criteria for promotion to the next phase remained consistent.

1.3.7. Phase F – Final Training

This phase was similar to Phase E; however, the unrewarded trial was now paired with the intermediate grade of sandpaper. Animals were trained on Phase F for five days. It is important to note that this phase was omitted entirely in adaptations of this method, as discussed in the relevant chapters.

1.3.8. Phase G – Testing

Phase G marked the start of the testing period. Rats were moved into testing groups according to experimental parameters and given appropriate time to re-habituate to the new conditions. However, this step may be omitted based on the experimental conditions of interest. The rats received four trials per day, with one test being randomly selected to be an ambiguous test trial per day. For the ambiguous trial, the reward bowls were both unrewarded and the intermediate grade of sandpaper was present in the pipe. The animal was removed immediately after foraging in any reward bowl. If the animal foraged in the reward bowl that it had learned would normally contain the chocolate reward, then the animal displayed an optimistic cognitive bias. If the animal foraged in the reward bowl that it had learned would normally contain the Cheerio reward, then the animal displayed a pessimistic cognitive bias.

1.4. Discussion of Scientific Papers

This thesis is presented as a series of published journal articles, or manuscripts that have been submitted for publication. Figure 4 is an illustrative representation of study development and scientific papers produced.

Chapter 2 (*The effects of metabolic cage housing and sex on cognitive bias expression in rats)* presents a publication of work performed prior to candidature commencement, but which was written and submitted for publication after candidature had been accepted. This study was conducted to identify the effects of metabolic cage housing on male and female rats, on their judgements to the JBT. As hypothesised, metabolic cage housing caused significant reduction in the number of optimistic interpretations made to the ambiguous probe. This study was the first to suggest a discrepancy existed between male and female rats based on their JBT performance. Whilst these findings were novel, post-study power calculations revealed that the number of females utilised in the study (n=12) was under-powered based on a standard 80% assignment of power. This led to the design of the published study presented in Chapter 3 (Female rats display fewer optimistic responses in a judgment bias test in the absence of a physiological stress response). By increasing statistical power and including physiological correlates of stress it was identified that, after being moved to metabolic cages, both males and females responded with decreased optimistic biases. However, discrepancy between the sexes remained. In the control groups, females responded with significantly fewer optimistic biases than males. Two factors were suggested as to why this discrepancy existed. The first was the female dependant factor of oestrous (Frye, 1995; Frye et al., 2000; Frye and Walf, 2002; Walf et al., 2006; Paris and Frye, 2008; Walf et al., 2008) and the second was that group-housed rodents are suggested to constantly alter their social status in order to gain higher social standing (Van Loo et al., 2001). Females have been evidenced to be more sensitive to social status changes than males (Hurst et al., 1996; Hurst et al., 1999). These assumptions led to the design of published studies in Chapters 4 (*Assessment of housing density, space allocation and social hierarchy of laboratory rats on behavioural measures of welfare*) and 6 (*Oestrous phase cyclicity influences judgment biasing in rats*).

To assess if social status was a factor in judgement bias testing, this study utilised a male-only sample. It was discussed that until the effects of oestrous had been explored, using males was the more pertinent option to establish foundational results. This study investigated the effects of social status, housing density and space allocation on rat performance in a series of behavioural tests, namely the open-field, social-interaction and novel-object recognition tests, in addition to the JBT. It was identified that subordinate rats responded with significantly fewer optimistic biases than their dominant counter-parts, suggesting that subordination stress, as imposed through group-housing, could be a significant modifier of judgement biases. During this study, it was noted that subordinate animals seemingly took longer to learn parameters of the JBT than did their dominant cage-mates. This led to the development of the study presented in Chapter 5 (*Imposed subordination in rats impedes learning on a judgment bias test*) which investigated the effects of subordination stress on a female cohort, whilst investigating learning aptitude. The study identified that dominant rats took significantly fewer days to learn the JBT than their subordinate cage-mates. Imposed subordination was concluded to be a significant detriment of learning aptitude and a hindrance to the practicality of JBT design.

Chapter 6 presents published work on oestrous cyclicity. This study assessed the factors of social status and oestrous to investigate whether oestrous cycle rotation, or an interaction of this with social status, discouraged prevalence of optimistic biases. Rats in dioestrous phases and those considered to be subordinate demonstrated a decreased percentage of optimistic responses. However, no interaction between oestrous phase or social status was observed.

Chapter 7 (*Increased latencies to respond in a judgment bias test are not associated with pessimistic biases in rats*), while disconnected from the previous, was designed to assess the effects of learning extinction, in order to better understand the practicality of this JBT design. Whilst no learning extinction was observed, there was a significant increase in latency to respond to the ambiguous probe following eight days of continuous probe exposure. Following day 8, there was a significant increase in latency to make both optimistic and pessimistic responses to the ambiguous probe. This implied that use of response latency, after 8 consecutive days of training, as a measure in judgement bias testing can falsely identify pessimism. This suggested that future JBT design needs to include an active response indicator to avoid this confounding variable.

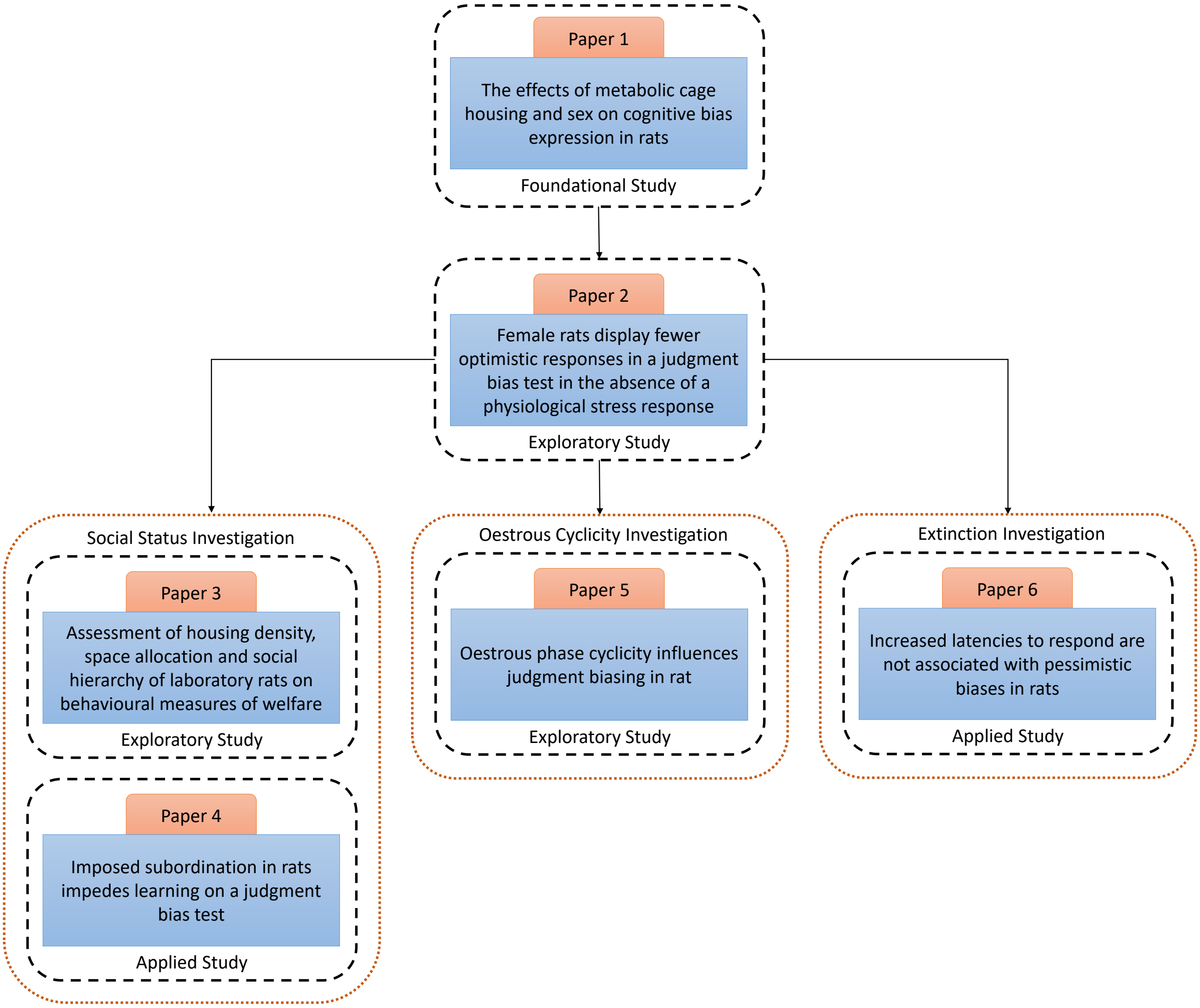

Figure 4 –Experimental design process and development of scientific papers.

CHAPTER 2.

The effects of metabolic cage housing and sex on cognitive bias expression in rats

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2.1. Statement of Authorship

Title of Paper	**The effects of metabolic cage housing and sex on cognitive bias expression in rats.**
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Applied Animal Behaviour Science Jan 17th, 2016 Volume 177, pages 70-76

Principal Author

Name of Principal Author (Candidate)	**Timothy Hugh Barker**		
Contribution to the Paper	Experimental design and procedure. Statistical analysis. Wrote manuscript. Acted as corresponding author.		
Overall percentage (%)	80		
Certification:	This paper reports on original research I conducted and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	27/03/2018

Co-Author Contributions

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

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	**Gordon Stanley Howarth**		
Contribution to the Paper	Principal supervisor Provided guidance on content and structure of manuscript Editing of manuscript		
Signature		Date	27/03/2018

Name of Co-Author	**Alexandra Louise Whittaker**		
Contribution to the Paper	Principal supervisor. Assisted with experimental design and procedures. Provided guidance on content and structure of manuscript. Editing of manuscript.		
Signature		Date	27/03/2018

2.2. Statement of Context

This chapter presents the foundational study on which this body of work is based. Metabolic cage housing had been associated with significant increases in physiological indicators of stress in both rats and mice (Gomez-Sanchez and Gomez-Sanchez, 1991; Gil et al., 1999; Eriksson et al., 2004; Kalliokoski et al., 2013; Whittaker et al., 2016a). However, previous study to identify the effects this housing had on animal affect was limited to behavioural and physiological measures (Kalliokoski et al., 2013; Whittaker et al., 2016a). Therefore, this research project presented a unique opportunity to apply an established JBT for use in rats to identify the effects metabolic cage housing has on affective state using a novel method of affective state detection. Whilst metabolic cages significantly reduced optimistic decisions made by males, this effect was not observed in females. This study was the first to detail that, in rats, male and female cognitive bias expression is not equivalent. This study guided later experimental design to probe this knowledge gap forming the basis of experimental work described in Chapter 3.

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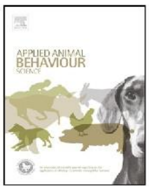

The effects of metabolic cage housing and sex on cognitive bias expression in rats

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ABSTRACT

The use of metabolic cages is established housing protocol in small-animal, gastrointestinal research. Physiological data, as primarily observed through secretions of the hypothalamic–pituitary–adrenal axis has shown that rodents housed in this manner experience an increased stress response. Few studies have observed behavioural measures of stress, or the emotional impact, in response to metabolic cage housing in rodents. This study investigated the impact of moving rats from standard group housing to individual metabolic cage housing on the affective states of rats as measured through a judgement bias paradigm. It was assumed that a change from standard housing to metabolic cages would impact on the rats' affective state. It was therefore hypothesised that rats moved to metabolic cages would show fewer optimistic responses to an ambiguous stimulus compared to rats remaining in standard housing. Rats (*Rattus norvegicus*) ($n = 24$) were trained to learn the correct response needed to obtain a reward, given the type of stimulus present (rough versus smooth sandpaper). One stimulus was associated with a high-positive reward (chocolate), whilst the other was associated with a low-positive reward (cereal). Upon learning the discrimination, the rats were introduced to a stimulus intermediate between their learned stimuli (intermediate sandpaper) creating an ambiguous probe. Responses to the probe were regarded as optimistic if the rat responded to an ambiguous cue as if it were a positively rewarded stimulus, or pessimistic, if response to an ambiguous cue mimicked that of a negatively rewarded stimulus. Male rats moved to metabolic cages ($n = 6$) showed significantly fewer optimistic responses to the probes (0.16 ± 0.16), than control males that remained in open-top cages (4.5 ± 0.34) ($p = <0.001$). No differences in optimistic decisions were observed in females moved to metabolic cages ($n = 6$) compared to standard housing ($n = 6$) ($p = 0.0524$). This demonstrated, that upon being moved to metabolic cages, male rats responded with increased negative behavioural judgements. The data also demonstrated that gender can alter rodent judgement in cognitive experimentation. This has implications for all cognitive bias studies, and for the continued use of metabolic cages in small-animal research.

1. Introduction

Metabolic cages are an established housing practice in research studies that involve evaluation of drug pharmacology or gastrointestinal research in rodents (Hwang et al., 2013). The system allows for the accurate assessment of the output and input measures of the housed rodents. Metabolic cages designed for rodents are constructed to maintain the integrity of the data being measured. However, in doing so, the physical constraints of the cage and their subsequent limitations create a housing environment that does not provide for all aspects of good welfare (Greco et al., 1989; Manser et al., 1995; van Praag et al., 2000; Nagy et al., 2002; Eriksson et al., 2004; Whittaker et al., 2012; Ravenelle et al., 2014).

When evaluating metabolic cages as a complete unit, the majority of research conducted on rodent welfare in these cages has primarily focused on physiological responses. For example, it was first reported by Gomez-Sanchez and Gomez-Sanchez (1991) that metabolic cage housing led to increased corticosterone excretions in rats. This was confirmed by Kalliokoski et al. (2013) who reported that mice housed in this type of cage had ten times greater corticosterone output, measured in their faeces then control mice. Metabolic cages have been associated with increased weight loss and reduced immunoglobulin A (IgA) secretions in rats (Eriksson et al., 2004), both of which are indicative of a stress response (Guhad and Hau, 1996; Royo et al., 2004). Metabolic cages have also been associated with increases in catecholamine levels and endogenous monoamine-oxidase activity (Gil et al., 1999).

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Physiological measures have had wide application in the assessment of rodent affective state in response to metabolic cage housing. However such techniques have associative flaws as they tend to be a measure of emotional arousal rather than emotional valence (Yeates and Main, 2008; Mendl et al., 2009). As reviewed by Yeates and Main (2008), emotional arousal is a measure of how strongly an emotional response is elicited by the animal, whilst emotional valence is a measure of whether the emotional response elicited by the animal is positive or negative in regards to the subjective experience of that animal. Therefore, without proper application of methods of evaluation that specifically measure emotional valence, there is no way to determine how an emotional response is perceived by an animal (Yeates and Main, 2008; Mendl et al., 2009). Behavioural observations, coupled with evaluation of physiological parameters provide some insight into the nature of the stress response in animals (Amir et al., 2005; Yeates and Main, 2008; Mendl et al., 2009; Salmeto et al., 2011). Despite this new understanding in the assessment of the stress response, few studies have been conducted into metabolic cage housing and the associated effects on rodent behaviour. This is largely due to the physical and social constraints that the cage imposes, making many behavioural tests inapplicable.

Research into human psychology has provided evidence that alterations in cognitive processing, so-called ‘cognitive biases’, are a reliable indicator of affective state and emotional valence (Wells and Matthews, 1996; Amir et al., 2005; Mendl et al., 2009). For example, subjects in a negative affective state (e.g. anxiety, depression) made negative judgements regarding ambiguous stimuli more often than subjects in a positive affective state (Amir et al., 2005).

This principle was adapted to animals in the rodent study by Harding et al. (2004). This work demonstrated that cognitive biasing as a result of affective state manipulation existed in multiple animal species. Therefore the measurement of cognitive biases presents a unique, under-studied protocol in the assessment of rodent affective state that is not limited by the physical and social constraints of the metabolic cage. As operationally defined by Douglas et al. (2012) and throughout the current study, optimism is defined as responding to an ambiguous cue as if it were a positively rewarded stimulus, whilst pessimism is defined as responding to an ambiguous cue as if it were a negatively rewarded stimulus.

Environmental enrichment has been demonstrated to induce optimism in animals such as rats, pigs and macaques (Bateson and Matheson, 2007; Matheson et al., 2008; Brydges et al., 2011; Bethell et al., 2012; Douglas et al., 2012). The removal of this enrichment has also been associated with pessimism in starlings and rats (Bateson and Matheson, 2007; Burman et al., 2008). Pessimistic judgement biases have also been associated with restraint in sheep (Doyle et al., 2010a,b; Bethell et al., 2012), pain in calves (Neave et al., 2013) and predatory threat in chickens (Salmeto et al., 2011), all three of which, are negatively valanced emotional states (Yeates and Main, 2008).

Previous studies have determined that environmental conditions can bias the cognitive processing of animals. (Bateson and Matheson, 2007; Matheson et al., 2008; Brydges et al., 2011; Bethell et al., 2012; Douglas et al., 2012; Wichman et al., 2012). Therefore, in the current study, the judgement-bias test for rodents as established by Brydges et al. (2011), was adapted with the primary aim of investigating the impact of re-housing in a metabolic cage on the affective state of rats using this judgement-bias paradigm. It was assumed that moving group-housed rats into metabolic cages would negatively impact their affective state. Therefore it was hypothesised that rats moved to a metabolic cage would exhibit a decreased number of optimistic responses to an ambiguous probe compared to rats that remained in standard open-top cages. Male and female rats were included in the study, and it was hypothesised that there would be no difference in cognitive bias expression between sexes, a theory that has never been tested, representing an under-studied area within the field of cognitive biases. Rats were housed in the metabolic cages for 7 nights (8 days). This period in the metabolic cage best represents the conditions in which these animals are housed when following typical laboratory work that involves the use of these cages (Mashtoub et al., 2013; Wang et al., 2013; Whittaker et al., 2015a).

The detection of cognitive biasing resulting from metabolic cage housing has profound implications for the use of metabolic cages in future studies involving rats. These biases would provide some of the first indications as to the valence of the emotional state of rats when moved to this housing system.

2. Materials and methods

Animal housing and experimental protocols were approved by the Animal Ethics Committee of the University of Adelaide and conducted in accordance with the provisions of the Australian Code for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2013).

2.1. Subjects and housing

Male ($n = 12$) and female ($n = 12$) Sprague Dawley rats were sourced from a barrier-maintained, specific-pathogen-free (SPF) facility (University of Adelaide, Laboratory Animal Services, Adelaide, Australia). This sample size ($n = 24$) was utilised based on previous literature using similar methodology (Brydges et al., 2011). At 3 weeks of age rats were housed in same sex groups of 3 in standard polycarbonate open-top rat cages (415 mm × 260 mm × 145 mm, Tecniplast, NSW, Australia) lined with paper based bedding (Animal Bedding, Fibrecycle Pty Ltd., Qld, Australia) and furnished with a chewing object (Nylabone Products, NJ, USA). Standard rat chow (Rat and Mouse Cubes, Specialty Feeds, WA, Australia) and potable reverse osmosis (RO) water were provided ad libitum. All animals were identified by marking the base of the tail with a non-toxic marker pen. During the testing phases, twelve rats (6 males, 6 females) were housed in metabolic cages (220 mm in diameter × 120 mm tall, Tecniplast, NSW, Australia), with a metal grid floor and no shelter. Room temperature remained at 21–23 °C. The photoperiod was set on a 12 h light/dark cycle.

2.2. Apparatus

The testing chamber was similar to that utilised by Brydges et al. (2011), and comprised two transparent perspex boxes (610 mm × 435 mm × 215 mm). The “start box” was connected by a PVC pipe 90 mm in diameter and 800 mm long, to the “goal box”. During the experiment this pipe was lined with either coarse (P80), or fine (P1200) sandpaper (Flexovit, NY, USA) according to the learned association of the rat (see below). The goal box contained one blue and one brown bowl (each 90 mm diameter, 20 mm deep), in either corner (Fig. 1), these bowls were present and their positions fixed throughout the testing. Milk chocolate baking chips (Cadbury, London, England) and cheerios (UncleToby’s, Victoria, Australia) were used for the high-positive and low-positive reward items respectively. Previous cognitive bias work see Harding et al. (2004) utilise a positive versus negative reward scheme, in that the negative reward is mildly aversive to the animal. However, this experiment follows from the work of Brydges et al. (2011) in using a high-positive versus low-positive reward item. The decision to use this reward scheme was two-fold. Firstly, it was discussed that attempted detection of a positive effect works most optimally when a positive or neutral reinforcer is utilised (Mendl et al., 2009). Secondly, as discussed by Brydges et al. (2011), repeated exposure to

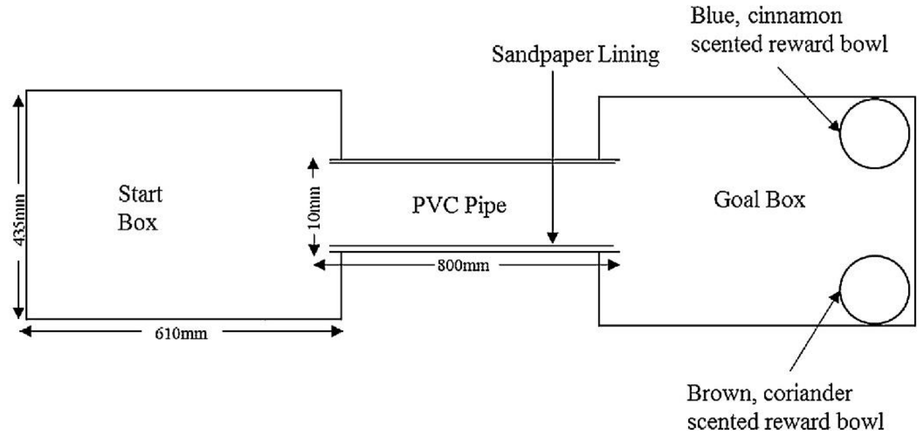

Fig. 1. Schematic of testing apparatus used during the training and testing procedures.

an aversive stimulus during repeated cognitive bias testing (animals receive 2 tests per day per reward) may impact negatively on the state of the animals.

Sandpaper of two different grades, P80 and P1200, were utilised as the rough and fine stimuli respectively, as it has been previously demonstrated that rats are able to effectively discriminate between sandpapers of different grain sizes, through the tactile feedback provided by their whiskers (Morita et al., 2011). The sandpaper was placed in the PVC pipe because that is where there is maximal contact between the rat's whiskers and the sandpaper surface.

2.3. Experimental design

Rats were randomly allocated to either remain in open-top, group housing ($n = 12$) or to be moved to a metabolic cage ($n = 12$), counter-balanced between sexes. The rats would enter these housing modes after the training period which took 162 days. During the training procedures animals were open-top/group housed, and were approximately 23–25 weeks of age.

Each rat was assigned an association to the reward items, such that equal numbers of rats associated chocolate with fine sandpaper, and cheerio with coarse sandpaper (vice-versa for remaining animals). The rats were then assigned an association for bowl colour to reward item, half associated chocolate with the brown bowl and cheerio with the blue bowl (vice versa for remaining animals). These associations were randomly assigned and were counter- balanced between sexes and between treatment groups. The reward bowls are distinct for each rat, in that the bowl colour, scent of sand within the bowl and the location of the bowl all remain consistent, and as such work in unison to signal reward content to the rat. All tests were performed during the light phase of the photoperiod.

2.4. Habituation phase (phases A–B)

The experimental phase is summarised in Fig. 2. To acclimatise the rats to the experimental conditions, each rat was handled for 20 min each day for 5 days (phase A). This involved 10 min handling between 0900 and 1200, and 10 min handling between 1400 and 1700. During this period animals had access to both food rewards.

Phase B then commenced. Rats were placed into the testing chambers for 20 min per day with the food bowls filled with either cinnamon (blue bowl) or coriander (brown bowl) scented sand (1% by weight of spice to sifted sand). Both bowls contained the reward items that were appropriate for the individual rat's assigned associations. During phase B no sandpaper lined the PVC pipe. This phase continued for 5 days.

2.5. Training phase (phase C–F)

In phase C rats undertook four training trials a day, two occurred between 0900 and 1200 h and two from 1400 to 1700 h. For each trial, a single reward was placed on the surface of the appropriate corresponding sand. Rats were then placed (individually) into the start box, and a timer was started. During these trials, the appropriate corresponding sandpaper lined the PVC pipe according to which reward was being utilised in the trial. Each rat had two trials for the chocolate and the cheerio per day, the order of which was randomly determined. Time was recorded for the rat to first leave the start box, enter the goal box, approach any reward bowl, approach the correct reward bowl and start consumption. Approach behaviours were defined when the rat actively and intentionally placed its forelimbs and face into a reward bowl in an attempt to extract the reward. Consumption of the reward was defined as when the animal actively and intentionally interacted with the food by bringing it to its mouth. If the rat failed to consume the reward within 10 min of being placed into the testing chambers, the rat was removed, and the trial was considered a failure. If the rat approached the correct bowl first (bowl containing the reward item) the trial was deemed successful. After the completion of a trial, the rat was removed from the testing apparatus and returned to the home cage. In between trials the apparatus was cleaned with 70% ethanol solution. Rats were eligible for promotion to phase D after they had successfully completed 5 consecutive days with 3 out of 4 trials achieving success per day. Rats took an average of 21 (±5.2) days to reach criterion for phase C.

The protocol for phase D was identical to phase C, with the exception that the reward items were buried in the sand. For each extraction of the reward, the depth at which the reward was buried increased, until it was buried halfway into the sand. Different burial depths were utilised as to further encourage the rats to continue exhibiting foraging behaviours in order to obtain the reward. A trial was regarded as successful when the rats had foraged in the reward-containing bowl first. A "forage" was characterised as being a continuous and deliberate displacement of sand in order to obtain the reward (Fig. 4). The conditions for failure and promotion to phase E remained consistent from phase C. Rats took an average of 13 (±3.1) days to reach criterion for phase D.

Phase E followed the same procedures as phase D, however the reward items were always buried halfway into the sand, and one of the four trials per day (chosen at random) went unrewarded. Unrewarded trials were implemented to make the rats more resistant to the extinction effect, the phenomena of the rats learning the outcomes of the protocol and as such not performing the required

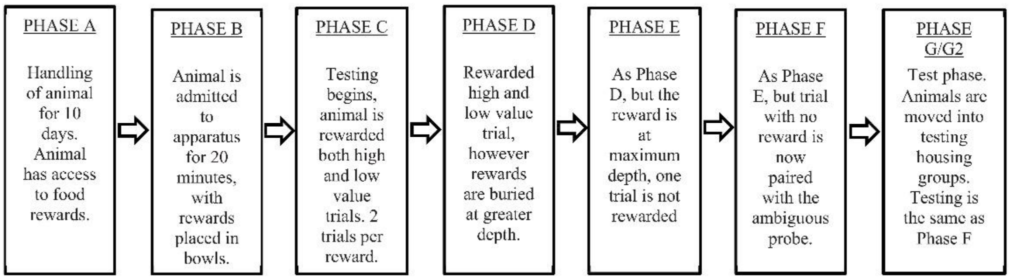

Fig. 2. Flow chart of experimental procedure with simplified experimental steps.

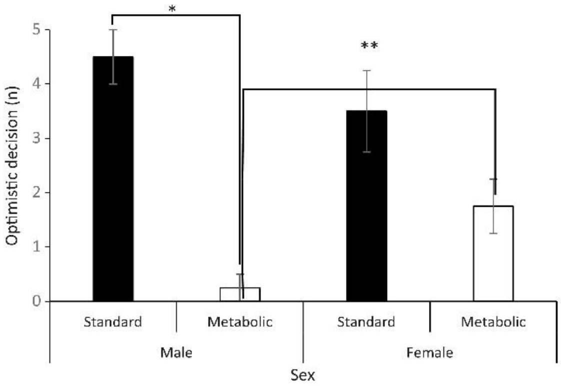

Fig. 3. Effects of sex and treatment on optimistic decisions.
The mean number of optimistic decisions to the ambiguous probe made over 5 days. Male*standard ($n = 6$), male*metabolic ($n = 6$), female*standard ($n = 6$), female*metabolic ($n = 6$). Error bars represent 1 SE. *denotes significance $p = < 0.001$, **denotes significance $p = 0.0079$.

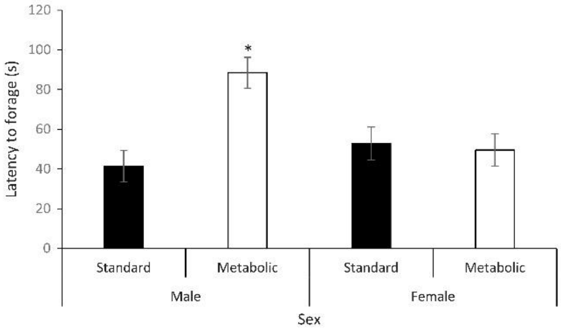

Fig. 4. Effects of the interaction between sex and housing.
Effects of the interaction between sex and housing treatment on the latency to initiate foraging behaviour for the unrewarded trial in phase G2. Male*standard ($n = 6$), male*metabolic ($n = 6$), female*standard ($n = 6$), female*metabolic ($n = 6$). Error bars represent 1 SE. *denotes significance $p = 0.001$.

actions to reach promotion criterion. For the unrewarded trials, success was determined when the rat foraged in the bowl that would usually correspond with a reward first. Animals were removed immediately after eliciting their initial foraging behaviour. Animals that had rewarded trials were still removed after consuming the reward item. The criteria for determination of success, failure and promotion were identical to phase D. Phase E took an average of 10 days (±2.6) for the rats to reach criterion.

Phase F was identical to phase E, however during the unrewarded trials, the sandpaper was removed and replaced with sandpaper of intermediate grade (P180). These intermediate, unrewarded trials continued for 5 days.

2.6. *Testing phase (phase G and G2)*

Phase G began immediately after the completion of phase F. Rats in this phase were sorted into their respective treatment groups; half of which were housed in standard, open-top group housing ($n = 12$) (control), with the remainder being housed in metabolic cages ($n = 12$). During this phase, rats continued to be trained according to the protocol in phase F. This continued for 3 days. This 3-day period was employed to habituate the rats to the metabolic cage conditions. After the 3-day habituation period, rats entered phase G2 and testing began, this phase followed the same protocol as for phase G. However, in addition to the standard recordings still being made, the experimenter also recorded the time for the rat to forage in any bowl during the unrewarded trial, and which bowl the rat approached, and foraged in first, this phase continued for 5 days. An optimistic decision was regarded as being so when the rat displayed foraging behaviours in the bowl that would normally correspond with a chocolate reward, whilst a pessimistic behaviour was identified when the rat displayed foraging behaviour in the bowl that would normally correspond with a cheerio reward.

2.7. *Data analysis*

Data were analysed using the IBM SPSS Statistics 22 (IBM, NY, USA) software package. In all cases normality was tested with a Shapiro-Wilk test. A mixed model was used with sex, sandpaper and bowl colour fitted as fixed factors, to analyse the number of days it took for the rats to complete phase C. Significance was not detected between any of these factors or their interactions and as such, the data has been omitted. A repeated measures 2-way Analysis of Variance (ANOVA) was used fitting sex and treatment to analyse the number of days featuring an optimistic decision. A repeated measures 3-way ANOVA fitting sex, treatment and reward, was used to analyse the time taken to elicit foraging behaviour for the 5 days in phase G2. Minimal models were produced by the removal of all non-significant factors and are presented within the study. Significance was determined at $p < 0.05$. Unless stated otherwise, results are presented as mean ± standard error of the mean.

3. Results

3.1. *Number of optimistic decisions over 5 days*

There was a significant effect of the number of days featuring an optimistic decision for the interaction between sex and housing treatment type $F(120) = 26.67$, $p = 0.018$. Males that remained in standard housing ($n = 6$) displayed greater numbers of optimistic responses than males moved to metabolic cage housing ($n = 6$) $F(110) = 45$, $p = <0.001$. Significance was also detected between males ($n = 6$) and females ($n = 6$) moved to metabolic cages F

(110) = 13.91, $p = 0.0079$. However, there were no significant differences observed between the females in standard ($n = 6$) versus those moved to metabolic cage housing ($n = 6$) F (110) = 3.478, $p = 0.0524$, and between males ($n = 6$) and females ($n = 6$) that remained in standard housing F(110) = 3.37, $p = 0.2375$ (Fig. 3).

3.2. Effects of housing type and sex on latency to initiate foraging behaviours

Moving male rats to metabolic cage housing ($n = 12$) resulted in a significantly greater time to initiate foraging behaviour for each probe trial during phase G2 testing, compared to female rats in metabolic cages ($n = 12$) F (1348) = 10.582, $p = 0.001$. Significance was not observed between male rats and female rats in standard housing (Fig. 4).

3.3. Effects of reward type on latency to initiate foraging behaviours

There was a significant effect of reward type, on the time to initiate foraging behaviour during phase G2 F(1348) = 36.41, $p = <0.001$. However, there were no significant effects observed on the time to initiate foraging behaviour for sex F(1348) = 3.166, $p = 0.076$, or the interaction between sex and reward type F(1348) = 0.016, $p = 0.984$ on time to initiate foraging behaviours in phase G2 (Fig. 5).

4. Discussion

It was hypothesised that rats moved into metabolic cages from standard cages would experience a negative affective state, and would respond with a decrease in the number of optimistic responses to the ambiguous probe. This hypothesis is supported by the data from the male rats. Males moved to the metabolic cages responded with fewer optimistic judgement biases to the ambiguous stimulus than males that remained in standard housing, as evidenced by their more frequent foraging in the location that had been previously associated with the low-positive reward. Therefore, they can be said to have exhibited a pessimistic judgement bias (response). Housing in metabolic cages has been demonstrated to cause multiple negative physiological responses in rodents, including weight loss, (Eriksson et al., 2004) and up to ten-fold increases in corticosterone concentrations (Kalliokoski et al., 2013) and other non-homeostatic secretions of the hypothalamic-pituitary-adrenal (HPA) axis (Gil et al., 1999; Eriksson et al., 2004). Furthermore, sociability as an evoked behaviour has also been shown to be decreased, indicating an anxiogenic effect of the cages (Whittaker et al., 2015b). Whilst, these previous studies have demonstrated that rodents moved to metabolic cage housing experience significant physiological reactions, which are likely to be negatively valanced (Yeates and Main, 2008), our data establish that this is so from the animal's perspective. These findings provide new evidence to support the proposition that housing conditions which restrict behavioural and social freedoms can induce a pessimistic judgement bias in animals. Conversely, whilst this trend was observed in female rats moved to metabolic cages compared to standard housing (Fig. 3), the data was not significant, which suggests that metabolic cages do not exert the same affective response in female rats.

The choice of the positive versus low-positive reward paradigm was made under the assumption that the chocolate would be more highly valued than the cheerios. Some evidence has been provided for this claim from the study by Brydges et al. (2011), where it was demonstrated that there was a significantly greater latency to initiate consumption of the cheerio reward than the chocolate reward. This is consistent with the results from the current study, since latency to initiate foraging behaviour was significantly less for the chocolate reward compared to the cheerio reward during phase G2 (Fig. 5). This finding validates our assumption. Previous work has also demonstrated that shorter latencies to initiate foraging are associated with preference (Bateson and Kacelnik, 1995).

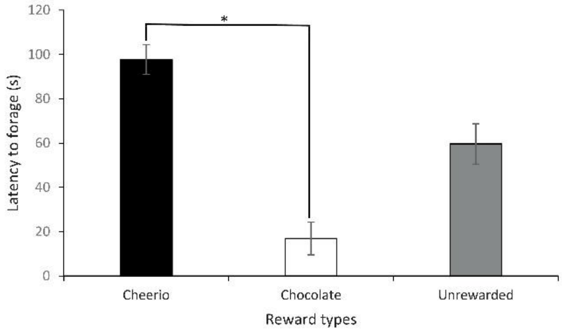

Fig. 5. Effects of reward type on the latency to initiate foraging.
Effects of reward type on the latency to initiate foraging behaviour for each rat and every trial in phase G2 ($n = 24$). Significance was detected between all three reward types ($p = < 0.001$). Error bars represent 1 SE. *denotes significance $p = < 0.001$.

It could be argued that over-exposure to the non-reinforced ambiguous grade of sandpaper results in the rats learning that the ambiguous probe was always paired with no reward. The consequence being that the animals displayed an increased latency to approach the reward bowls, which could be misconstrued as taking a pessimistic action. This potential confounding factor has been discussed previously in cognitive bias experiments involving sheep, (Doyle et al., 2010a,b) and starlings (Brilot et al., 2010). In both of these studies, the animals demonstrated an extinction effect, namely that animals learnt that the ambiguous locations were unrewarded and responded with a decreased response to these probes. However in refutation of the possibility of extinction having occurred in our study the following are asserted: firstly, unlike the work of Doyle et al. (2010a,b) and Brilot et al. (2010), the animals were only subjected to the absolute minimum number of ambiguous probes, as they did not act as their own controls, secondly, as Fig. 4 illustrates there was a difference in the latency to initiate foraging behaviour for the ambiguous probe trial between males housed in metabolic versus standard caging implying that extinction did not occur. However, significance was not observed in the female sample and extinction could be argued to have occurred. This is unlikely however as the female data was insignificant, as to be discussed.

This experiment represents one of the first to specifically demonstrate that males and females do not react in the same way to an imposed stressor, during judgement bias experimentation. This result has seldom been reported within the literature, indicating that previous studies did not find a significant sex effect or did not analyse the interaction between sex and treatment in a subject cohort that included both males and females (Matheson et al., 2008; Mendl et al., 2010; Scollo et al., 2014).

This interaction between sex and an imposed stressor has perhaps been overlooked in the multiple studies in which only a single sex sample was used (Harding et al., 2004; Burman et al., 2008, 2009, 2011; Doyle et al., 2010a,b, 2011; Brydges et al., 2011; Salmeto et al., 2011; Bethell et al., 2012; Douglas et al., 2012; Wichman et al., 2012; Destrez et al., 2013; Dupjan et al., 2013; Neave et al., 2013; Papciak et al., 2013; Destrez et al., 2014; Rygula et al., 2014). However, Burman et al., 2008 did find that male rats housed in conditions known to cause depressive-like symptoms exhibited an increased latency to respond to tasks (Burman et al., 2008). Further, Briefer and Briefer and McElligott (2013), reported an effect of sex in a study of female goats. They determined that female goats previously exposed to conditions of poor welfare responded with more optimistic judgement biases compared to male goats. The authors postulated that this finding may have been subject to the individual temperament of the goats. However, given the small sample sizes ($n = 4$), the observed difference in optimism between sexes may have arisen as a result of a relatively low study power.

Despite this lack of previous characterisation of sex effect, the data from the female rats in the current study are consistent with previous cognitive bias work using a female-only sample. Doyle et al. (2010a,b) conducted a study using restrained ewes with the hypothesis that the animals would be in a negative affective state, as identified by a greater latency to approach the ambiguous probe. The hypothesis was refuted as the animals displayed a decreased latency to approach the ambiguous probe. The study's authors proposed that female animals had a greater capacity to feel optimistic after previously experiencing, or being released from, conditions of poor welfare (Doyle et al., 2010a,b). It was also suggested that females were capable of responding to neutral conditions with greater displays of optimism compared to their male counterparts once the imposed negative condition had been removed.

It could also be argued that the change in housing and social structure that the current study employed activated a different stress response in the females compared to the male rats. Studies have concluded that social isolation in juvenile female rats leads to a decreased latency to immobility in the forced swim test, and a decreased preference for sucrose in the sucrose preference test, both of which are indicators of a stress response in rodents (Hong et al., 2012). In contrast to this, male rats in the same study exhibited no significant change, indicating that social isolation as a stressor results in significantly greater changes in female rats compared to male rats. This is contrary to the findings in the current study, in that the female animals demonstrated more resistance to the stress created by the metabolic cage. However, this phenomenon is also discussed by Hurst et al. (1998), who identified that singly-housed female rats displayed significantly greater escape tendencies than singly-housed males. Compared to singly-housed male rats, females responded with increased bar-chewing and investigation of the top and sides of the cages; behaviours that are considered escape-oriented. It could be argued that when the females in the current study were taken from the metabolic cage in order to be tested in the judgement-bias paradigm they experienced a shift in affective state, resulting in their cognitive biases being recorded as optimistic. Therefore, removal of female rats from the metabolic cages for testing may be a confounding factor in the current study. This however remains a potential experimental design problem for all cognitive bias studies where a specialised test set-up outside of the home environment is required.

There is another possible confounding influence in the current study. The decrease in the number of optimistic responses made by the rats to the probe may not have been due to the metabolic cage, but due to the act of moving the animal from an established housing environment to a foreign environment; namely the effect of novelty. This is a valid concern, yet the protocols as followed during the study remain consistent with laboratory use of metabolic cages, in that moving animals between housing systems in this manner is an established protocol. Therefore, these results whilst not being indicative entirely of the stress responses induced by metabolic cages per se, demonstrate how the act of moving a rat from standard housing to metabolic cage housing can lead to development of a negative emotional state in male rats.

A further limitation of the current study is that no correlation was made between the observed cognitive biases expressed by the rats and physiological measures of animal welfare, typically measured through secretions of the HPA axis. The main reason for this was that the gap in the literature regarding welfare consequences of metabolic cage housing was a lack of behavioural study. However, in addition, invasive procedures to acquire corticosterone data had previously been argued to cause continued detriment to an animal's welfare (Brydges et al., 2011). This could have influenced the animals' responses to the judgement bias paradigm and as such, were actively avoided. Faecal corticosterone measurement as performed by Kalliokoski et al., 2013, could have been utilised in the current study but would have been difficult to interpret in the group housing set-up utilised. However, in future studies we will consider use of this physiological measure since it is minimally invasive and hence unlikely to confound cognitive-bias data interpretation.

5. Conclusion

The current study demonstrated that male rats, when tested in a novel judgement bias paradigm, responded to an ambiguous stimulus with a significantly greater number of pessimistic responses when housed in metabolic cages as compared to group housing in standard open-top cages. Female rats however, showed no significant differences between treatment groups. Females responded with significantly greater numbers of optimistic decisions than the males when housed in metabolic cages. These findings provide further evidence that females and males do not respond in similar fashions in regards to response to an imposed stressor and support the use of both sexes in future cognitive bias studies. Confirmation of this result should be performed by increasing the study sample size.

These findings are the first to demonstrate that the valence of the emotional response to housing in metabolic cage is negative in male rats, thus potentially compromising their welfare and the metabolic data collected from these animals. The data could be adversely affected by the negative affective state of the animal, and therefore may not be representative of the treatment prescribed to that animal, but of the sub-optimal welfare state. These findings have profound implications on the continued use of metabolic cages in small-animal research.

Conflicts of interest

Authors declare that there are no conflicts of interest.

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CHAPTER 3.

Female rats display fewer optimistic responses in a judgment bias test in the absence of a physiological stress response

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Overall percentage (%)	80		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

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

iv. the candidate's stated contribution to the publication is accurate (as detailed above);

v. permission is granted for the candidate in include the publication in the thesis; and

vi. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	**Larisa Bobrovskaya**		
Contribution to the Paper	Performed analysis of tyrosine-hyrodoxylase. Provided guidance on content and structure of manuscript. Editing of manuscript.		
Signature		Date	27/03/2018

Name of Co-Author	**Gordon Stanley Howarth**
Contribution to the Paper	Principal supervisor. Provided guidance on content and structure of manuscript. Editing of manuscript.

Signature		Date	27/03/2018

Name of Co-Author	**Alexandra Louise Whittaker**
Contribution to the Paper	Principal supervisor. Assisted with experimental design and procedures. Provided guidance on content and structure of manuscript. Editing of manuscript.

Signature		Date	27/03/2018

3.2. Statement of Context

As identified in Chapter 2, female rats housed in metabolic cages responded with significantly more optimistic responses to the ambiguous probe then expected, and when compared to males. After performing a retrospective power calculation, the conclusion was reached that whilst statistical significance was achieved with the male cohort of animals (n=12), more female animals were required due to greater variability in the female response. It was therefore imperative, before investigating the nature of this female variability, to refine the methods of work described in Chapter 2 with a greater sample size of female animals.

In addition to increased animal numbers (n=30), Chapter 3 details an experimental methodology that included use of physiological correlates of a stress response. Faecal corticosterone and changes in adrenal tyrosine hydroxylase were measured to establish if a stress-response was physiologically experienced by animals. The animals were also subjected to the sucrose preference test to identify the presence of anhedonia.

As with findings of Chapter 2, females and males again failed to respond similarly, with females responding with significantly fewer optimistic biases than males when in control housing. However, in this instance female animals responded with significantly fewer optimistic biases in metabolic cages compared to control housing. In addition, a physiological stress response failed to be observed using the methods employed. This data demonstrated, for the first time, that metabolic cages encourage negative affect in both male and female rats. This highlighted variability in the female response and served as the basis for the exploratory studies presented in Chapters 4 and 6.

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Female rats display fewer optimistic responses in a judgment bias test in the absence of a physiological stress response

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HIGHLIGHTS

- Metabolic cages induce pessimistic biases in housed rats.
- Significant differences observed between male and female cognitive expression.
- No correlation observed between cognitive biases and physiological controls.

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ABSTRACT

Metabolic cages are a type of housing used in biomedical research. Metabolic cage housing has been demonstrated to elicit behavioural and physiological changes in rodents housed within them. The nature of this effect has been characterized as anxiogenic. However, few studies have evaluated positive affect in response to metabolic cage housing and the interaction between this, sex and traditional physiological measures of stress. Cognitive biasing, as measured through a judgment bias paradigm has proven a reliable measure of animal affective state, particularly through its ability to measure positive affect. The current study investigated differences in cognitive biasing between male and female rats when transferred from open-top, grouped housing to a metabolic cage. Rats (*Rattus norvegicus*) ($n = 60$) were trained in a judgment bias paradigm previously validated for use in the rat model. Upon exposure to an intermediate, ambiguous probe rats responded with either an optimistic or pessimistic decision. The animals were also subjected to the sucrose preference test to identify the presence of anhedonia. Faecal corticosterone and changes in adrenal tyrosine hydroxylase were also measured to establish whether a stress-like state was experienced. There was a significant interaction between sex and metabolic cage housing on the number of optimistic decisions made $F(1, 56) = 7.461$, $p = 0.008$. Female rats that remained in control housing responded with a reduced number of days featuring an optimistic decision compared to males in control housing ($p = 0.036$). However, both males and females responded with significantly fewer optimistic decisions in the metabolic cage compared to control ($p < 0.001$). There was a significant negative correlation between treatment and sucrose consumption ($r_{pb} = -0.654$, $n = 195$, $p < 0.001$). There was also a significant sex effect for faecal corticosterone concentrations $F(1, 30) = 6.305$, $p = 0.018$) with female rats (4.050 ± 1.285), displaying greater corticosterone concentrations than males (2.291 ± 0.495). No differences between treatment were observed for either corticosterone or tyrosine hydroxylase levels. This data demonstrates that movement into a metabolic cage resulted in rats displaying significantly greater pessimistic cognitive biases as determined through the judgment bias test. Interestingly, male rats that remained in control housing demonstrated cognitive biases that were not equivalent to female rats. Furthermore, despite a behavioural change being evident, a physiological change in corticosterone or tyrosine hydroxylase levels was not observed.

1. Introduction

The classification of affective state in animals is an attempt to quantify the subjective feelings, emotions and experiences that individual animals engage when performing reward-motivated behaviours [1]. The detection of a positive affective state has been

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most commonly achieved with cognitive bias tests, such as the judgment bias paradigm. This testing paradigm was first proposed by Harding et al. [2] and involved training animals to respond with two unique behaviours in response to two unique stimuli. These behaviours could be objectively classified as positive (rewarding or pleasurable) or negative (aversive or punishing). After the animal had learned these associations it was tested through introduction of a novel stimulus. If the animal responded to this ambiguity with the positive behaviour, the implication was that the animal behaved optimistically and vice versa. Optimism and optimistic-tendencies have been associated with animals being in a positive affective state, and the occurrence of optimistic judgments to an ambiguous probe identifies animals as such.

Negative affective states have been associated with the sensation of pain [3], restraint [4,5], threat [6], depression and anxiety [7–9]. Meanwhile environmental enrichment [10–13] and removal from a stressor [5,14] have been associated with the prevalence of positive affective states. The accurate assessment of these affective states has become increasingly important in animal welfare assessment [15].

Metabolic cages are commonly used in biomedical research [16]. Their utility in allowing the separation and collection of faeces and urine as well the controlled provision of food and water have made the cages popular choice for gastrointestinal and nutritional research in small rodents [17–20]. The employment of these cages however, has been associated with changes in rodent physiology, including increased corticosterone concentrations [21,22], reduced immunoglobulin A secretion [23] and increases in both catecholamine levels and monoamine-oxidase activity [24]. In addition, metabolic caged animals have responded with altered behavioural expression; when tested using a social interaction test, caged animals responded with a decreased movement time, fewer incidents of manipulating enrichment and rearing behaviours and were accompanied with a corresponding shift toward inactivity [16]. The use of these cages has therefore been strictly regulated by governing bodies through legislation [25]. Despite this legislative intervention, there has been little research into the effects of metabolic cage housing on animal affective state.

There has been one previous attempt to assess the impact of metabolic cage housing on positive affective state in rats [7]. This study indicated that female and male cognitive expression may not have been equivalent to one another, a finding scarcely reported within cognitive bias literature [14]. However, in the Barker et al. [7] study, no other correlative measures were used to assess if metabolic cage housing per se induced a stress response. This could suggest that the pessimistic biases expressed by the male metabolically housed animals may have been attributed to an external confounding factor. We aimed to determine if there was any difference in cognitive bias expression between male and female rats exposed to an identical stressor. We also wanted to compare the judgments made to an ambiguous probe with other, well-established controls indicative of a stress response. These included the concentrations of faecal corticosterone [22], adrenal tyrosine hydroxylase [26,27], and the sucrose preference test as an indicator of anhedonia [28]. Utilising a previously-validated judgment bias paradigm [12] it was hypothesised that animals moved to a metabolic cage from control housing would respond with a significant decrease in the number of optimistic biases expressed. It was also hypothesised that there would be no significant differences in the cognitive bias expression of the male rats compared to the females. We further hypothesised that animals moved to the metabolic cages would experience a significant increase in concentrations of faecal corticosterone and levels of tyrosine hydroxylase in the adrenal gland, and a significant decrease in the percentage of sucrose solution consumed in a sucrose preference test.

2. Methods

2.1. Ethics statement

All animal housing and experimental protocols were approved by the Animal Ethics Committee of the University of Adelaide and conducted in accordance with the provisions of the Australian Code for the Care and Use of Animals for Scientific Purposes [29].

2.2. Experimental design and apparatus

Treatment groups were randomly allocated; rats either remained in open-top group housing as controls ($n = 15$) or were separated and moved to singly-housed metabolic cages ($n = 15$). These allocations were counter-balanced between sexes. Rats were moved to the metabolic cages after demonstrating that they successfully completed the training phase (see below), and could be reliably tested in the judgment bias test. This training period took approximately 148 days, at which point the rats were approximately 21–23 weeks old. Whilst training in the judgment bias test, all animals were housed in open-top cages with two same-sex conspecifics.

The testing chamber and apparatus were identical to those utilised in the Barker et al. [7] study, adapted from the design of the original study by Brydges et al. [12]. They comprised a start box connected via a pipe to a goal box constructed from perspex. The pipe was lined with one of two-grades of sandpaper to act as the coarse (P80) and fine (P1200) training cues. The goal box contained a brown bowl in the right-hand corner filled with coriander scented sand (1% by weight of spice to sifted sand) and a blue bowl was placed in the left-hand corner filled with cinnamon scented sand (Fig. 1). Milk chocolate baking chips (Cadbury, London, England) and Cheerios (UncleToby's, Victoria, Australia) were used for the high-positive and low-positive reward items respectively.

Animals were randomly assigned an association to the reward items and rewarded locations as described in Barker et al. [7]. These associations were counter-balanced between sex and treatment and are summarised in Table 1.

2.3. Animals and housing

Male ($n = 30$) and female ($n = 30$) Hsd: Sprague Dawley rats were sourced at 3-weeks of age from a barrier-maintained Specific-Pathogen-Free facility (University of Adelaide, Laboratory Animal Services, Adelaide, Australia). This sample size was calculated to have a power of 80% based on the data obtained from the Barker et al. [7] study. All animals were housed in same sex groups of three, per their future testing parameters (e.g. metabolic cage or control) in standard polycarbonate open-top rat cages (415 mm × 260 mm × 145 mm, Tecniplast, NSW, Australia). Cages were lined with paper-based bedding (Animal Bedding, Fibercycle Pty Ltd., Qld, Australia) and furnished with cardboard boxes and PVC tubes as enrichment. All enrichment items were standardized between cages. Standard rat chow (Rat and Mouse Cubes, Specialty Feeds, WA, Australia) and water purified by reverse osmosis were provided ad libitum. All rats were identified by marking the base of the tail with a unique identifying number with a non-toxic marker pen. During the testing phases, 30 rats (15 male, 15 female) were moved into metabolic cages (220 mm in diameter × 120 mm tall, Tecniplast, NSW, Australia), with a metal grid floor and no shelter. Room temperature remained between 21 and 23 °C. The photoperiod was set on a reversed 12 h light/dark cycle.

2.4. Judgment bias test

Definitions of important procedures and milestones have been included in Table 2. Prior to training in the judgment bias test, rats were handled by the researchers for 5 days to habituate them to human

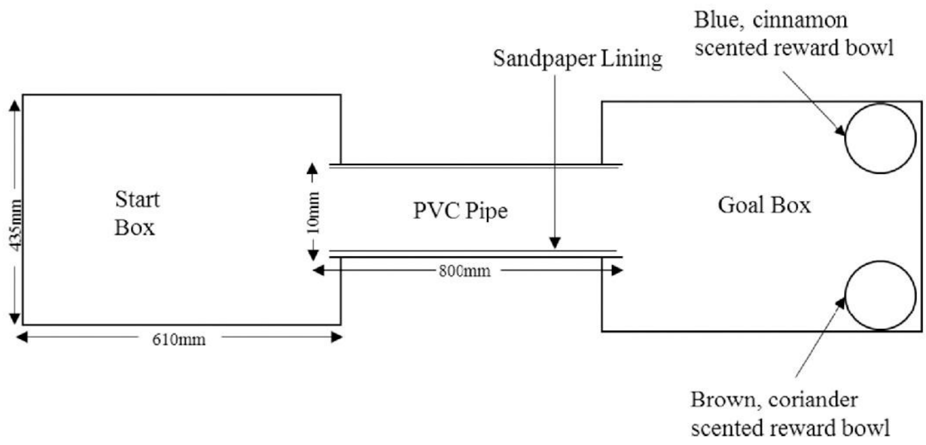


Fig. 1. Diagram of testing apparatus utilised.

contact (phase A). Rats were handled for 10 min from 0900 and 1200 h, and a further 10 min between 1400 and 1700 h.

Following phase A, rats were placed into the testing apparatus, four times a day for 5 min intervals (phase B). The food bowls contained the reward items appropriate to the individual rat, these rewards were placed on the surface of the sand in the reward bowls.

In phase C, testing with the sandpaper stimuli began. Animals had two training trials between 0900 and 1200 h and two from 1400 to 1700 h, one chocolate trial and one cheerio trial were completed per period in a random order. For each trial, a single chocolate or cheerio reward was placed on the surface of the appropriate reward bowl, to the appropriate sandpaper that was present in the apparatus. Rats were placed in the start box, and a timing commenced. Latency for the rat to leave the start box, enter the goal box, approach any reward bowl, approach the correct reward bowl and start to consume the reward was recorded. Approach and consumption behaviours have been defined in Table 2, as has the criteria to determine a failure or successful trial. The rat was immediately removed from the apparatus following consumption of the reward or after classification of the trial as a failure and the apparatus was cleaned with 70% ethanol solution. Rats were promoted to phase D after having completed 5 consecutive days with 3 out of 4 trials being deemed successful.

Phase D was identical to phase C, except that the reward items were buried in the sand. The rats were required to forage for the reward item in the sand and extract the reward (Table 2). Following extraction of the reward from the reward bowl, the depth at which the reward was buried for the next trial increased. Depth of burial increased with each successive trial until the reward was completely buried in the sand. The conditions to determine a successful or failing trial were consistent from phase C, as were the promotion conditions to permit the rat entering phase E.

Phase E was identical to phase D, but the reward items were always completely buried in the reward bowls, and one of the four trials per day was randomly selected to go unrewarded. A successful, unrewarded trial was defined when the rat foraged in the bowl that would usually correspond with a reward first, following the first elicitation of foraging behaviours the rats were immediately removed. The conditions to determine a successful or failing trial were consistent from phase D as were the promotion conditions to permit the rat entering phase F.

The protocol for phase F was identical to phase E, except that for each unrewarded trial the sandpaper was replaced with the intermediate sandpaper (P180). Phase F continued for 5 days.

During phase G1, half of the rats were moved into metabolic cages ($n = 30$) while the remainder stayed in the control open-top group housing ($n = 30$). For three days, the rats were habituated to the metabolic cage conditions and continued to be trained as per the protocol in phase F. Following the three-day habituation, rats entered phase G2 and testing began. The animals received one test per day, as per phase G1, a test comprised of the sandpaper in the pipe being the intermediate, ambiguous grade (P180) and no reward being present in the food bowl. During these unrewarded testing trials, the experimenter recorded the time for the rat to forage in any bowl, and which bowl the rat approached, and foraged in first, this phase continued for five days. An optimistic decision was defined when the rat displayed foraging behaviours for these intermediate ambiguous trials, in the bowl that would normally contain the chocolate reward. A pessimistic behaviour was defined when the rat displayed foraging behaviour in the bowl that would normally contain the cheerio reward. A flowchart of the judgment bias test has been included as Fig. 2.

2.5. Sucrose preference test

During the five-day testing period, the animals had access to two water bottles. Of these two bottles, the first contained standard reverse osmosis water, and the second contained a 2% sucrose solution. These bottles were presented to the animals at 0900 on the final day of phase G1 and were present for all 5 days of phase G2 testing. At 0900 each day of phase G2 testing the volume remaining in each bottle for every cage was recorded, and the bottles were topped up with an appropriate solution back to the original volume. The location of the bottles

Table 1
The associations to reward items and rewarded locations. Associations were randomly assigned and were counter-balance between sex and treatment.

	Chocolate stimulus	Chocolate location	Cheerio stimulus	Cheerio location
Association 1 $n = 15$	Coarse sandpaper	Brown bowl right side	Fine sandpaper	Blue bowl left side
Association 2 $n = 15$	Coarse sandpaper	Blue bowl left side	Fine sandpaper	Brown bowl right side
Association 3 $n = 15$	Fine sandpaper	Brown bowl right side	Coarse sandpaper	Blue bowl left side
Association 4 $n = 15$	Fine sandpaper	Blue bowl left side	Coarse sandpaper	Brown bowl right side

Table 2
The definitions of common methods and milestones used in the Judgment Bias Paradigm [7].

Term	Definition
Approach	When the rat actively and intentionally placed its forelimbs and face into a reward bowl in an attempt to extract the reward.
Forage	When the rat continuously and deliberately displaced the sand in the food bowl in order to obtain the reward.
Consumption	When the rat actively and intentionally interacted with the food by bringing it to its mouth.
Success	A successful trial was determined to be so after the animal had approached and foraged in the correct (reward containing) food bowl before approaching or foraging in the incorrect food bowl.
Promotion	Animals were promoted to the succeeding trial (where appropriate) after achieving ¾ successful trails per day, for 5 consecutive days.
Failure	If the rat failed to consume the reward within 10 min of being placed into the testing chamber.

within the cage was randomised every day to prevent the onset of location-bias forming. The animals had ad lib access to both bottles, and the percentage of sucrose consumed by each cage over a 24-hour period was calculated using total sucrose solution ingested / (total water ingested + total sucrose solution ingested).

2.6. Faecal corticosterone

During days 1, 3 and 5 of phase G2 testing, faeces were collected from individual animals for analysis of corticosterone. Faeces stored at −80 °C after being snap-frozen in liquid nitrogen. Prior to analysis, the faeces were dried using a conventional drying oven for 24 h at 50 °C. Faecal concentration of corticosterone was measured by commercial competitive ELISA, supplemented with standard corticosterone prepared in ethanol solution. Testing was done at a commercial testing laboratory (University of Western Australia, Perth). The sensitivity of the assay was 5 ng/ml.

2.7. Tyrosine-hydroxylase

Following testing on day 5 of phase G2 animals were euthanased via CO_2 asphyxiation and cervical dislocation. The adrenals were removed and snap frozen in liquid nitrogen before being stored at −80 °C. The adrenal glands were processed as previously described [30] and changes in tyrosine hydroxylase protein levels between experimental groups were measured using western blotting technique.

2.8. Statistical analysis

All data were analysed using the IBM SPSS Statistics 22 (IBM, NY, USA) software package. The Shapiro-Wilk test was used in all cases to test for normality of the data set, all data was found to be parametric and therefore parametric tests were used. A mixed model fitting sex, sandpaper and reward association was performed to analyse the number of days it took for the rats to learn the judgment bias paradigm (defined as days to complete Phase C). Significance was not detected and the data has been omitted. A repeated measures two-way ANOVA examined the effects of sex and housing on the number of days that an optimistic interpretation to the ambiguous cue was recorded and was performed fitting sex and treatment on the corticosterone output for each day. This test was also performed to examine the effects of sex and treatment on the percentage of sucrose consumed over time. A two-way ANOVA was performed fitting sex and treatment on the adrenal TH expression of the rats. A point-biserial correlation was also run on this data to determine the strength of the relationship between treatment and percentage of sucrose consumed. Significance was determined at $p < 0.05$. Post hoc tests were performed on the data where appropriate using the Tukey correction Unless stated otherwise, results are presented as mean ± standard error of the mean.

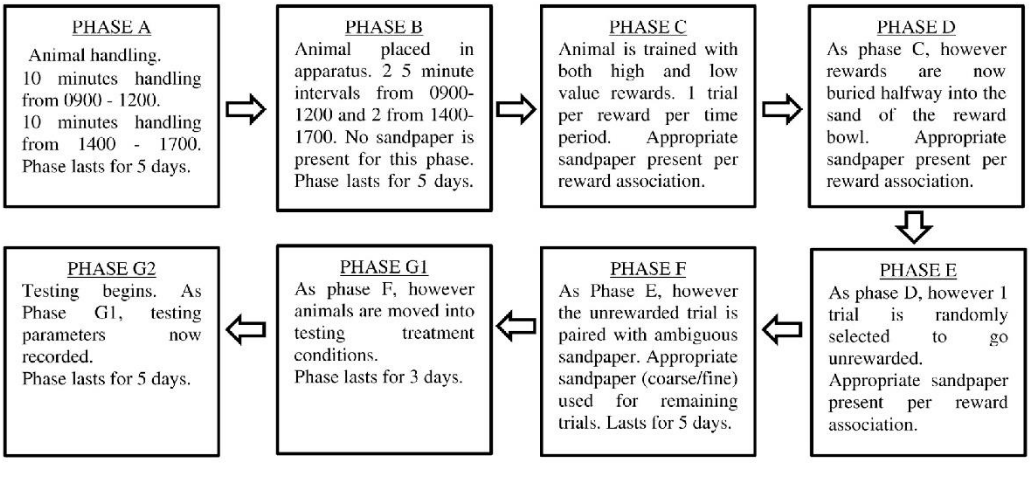
3. Results

3.1. Effects of metabolic cage housing on cognitive expression

There was a statistically significant interaction between sex and housing on the number of days featuring an optimistic decision, $F(1, 56) = 7.461$, $p = 0.008$. Simple main effects analysis showed that female rats in the control housing responded with a significantly reduced number of days featuring an optimistic decision compared to males in control housing ($p = 0.036$). No differences between sex were observed when housed in metabolic cages ($p = 0.092$). Significance was also detected between housing types. Both males and females responded with a significantly greater number of days featuring an optimistic decision in control housing ($p < 0.001$) Fig. 3.

3.2. Corticosterone analysis

Female rats displayed increased corticosterone production corticosterone (4.050 ± 1.285) compared to male rats (2.291 ± 0.495), $F(1, 30) = 6.305$, $p = 0.018$). No significance was observed for either treatment on corticosterone output, $F(1, 30) = 0.389$, $p = 0.538$, or day $F(1, 30) = 0.390$, $p = 0.537$, and hence data has been combined in Fig. 4.



PHASE A
Animal handling.
10 minutes handling from 0900 - 1200.
10 minutes handling from 1400 - 1700.
Phase lasts for 5 days.

PHASE B
Animal placed in apparatus. 2 5 minute intervals from 0900-1200 and 2 from 1400-1700. No sandpaper is present for this phase. Phase lasts for 5 days.

PHASE C
Animal is trained with both high and low value rewards. 1 trial per reward per time period. Appropriate sandpaper present per reward association.

PHASE D
As phase C, however rewards are now buried halfway into the sand of the reward bowl. Appropriate sandpaper present per reward association.

PHASE E
As phase D, however 1 trial is randomly selected to go unrewarded. Appropriate sandpaper present per reward association.

PHASE F
As Phase E, however the unrewarded trial is paired with ambiguous sandpaper. Appropriate sandpaper (coarse/fine) used for remaining trials. Lasts for 5 days.

PHASE G1
As phase F, however animals are moved into testing treatment conditions.
Phase lasts for 3 days.

PHASE G2
Testing begins. As Phase G1, testing parameters now recorded.
Phase lasts for 5 days.

Fig. 2. Flowchart of judgment bias paradigm.

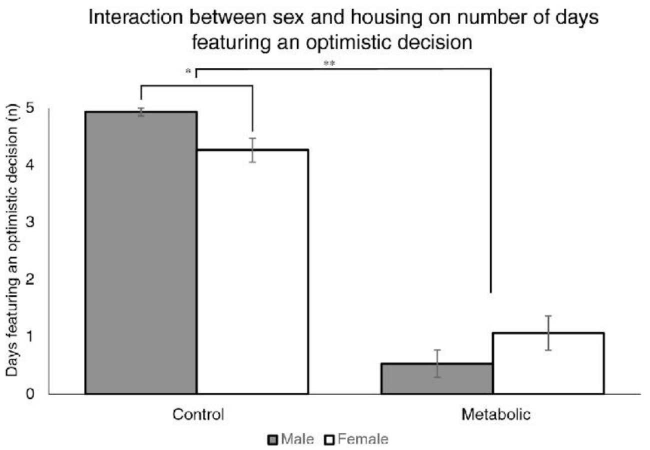


Fig. 3. The interaction between sex and housing on number of days featuring an optimistic decision. There was a significant interaction between sex and housing on number of days featuring an optimistic decision. Females in control housing responded with a significantly reduced number of days with an optimistic decision compared to males in control housing. All values depict mean ± SEM. * denotes significance at $p < 0.05$. ** denotes significance at $p < 0.001$.

3.3. Tyrosine-hydroxylase analysis

A two-way ANOVA was conducted that examined the effects of sex and treatment on the adrenal TH expression. No significance was detected for sex $F(1, 30) = 0.034$, $p = 0.855$, or treatment, $F(1, 30) = 2.733$, $p = 0.109$.

3.4. Sucrose preference test analysis

There was a statistically significant interaction between day and treatment on percentage of sucrose consumed, $F(1, 35) = 12.241$, $p < 0.001$). Post hoc tests using the Tukey correction identified that metabolic cage housing resulted in a significant reduction in the percentage of sucrose consumed for each day, compared to control housing ($p = 0.001$). Significance was also detected between days for only metabolic cage housing, each subsequent day of metabolic cage housing resulted in a statistically significant decrease in the percentage of sucrose consumed, Fig. 5. This trend was not significant for rats in control housing. A point-biserial correlation was run to determine the strength of the relationship between treatment and percentage of sucrose consumed. There was a negative correlation between treatment and sucrose consumption, that was statistically significant ($r_{pb} = -0.654$, $n = 195$, $p < 0.001$). No significance between sex was observed, $F(1, 35) = 0.047$, $p = 0.829$).

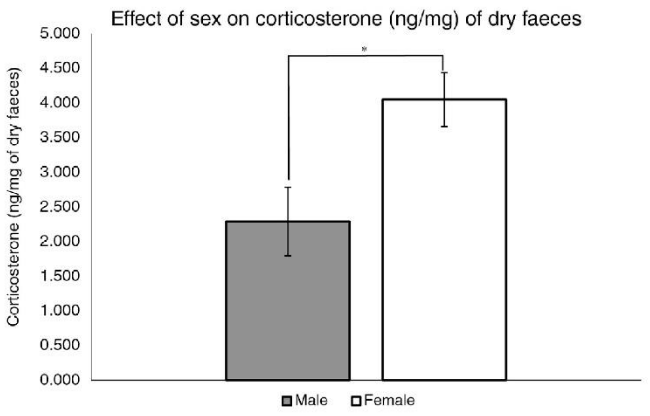


Fig. 4. The effect of sex on corticosterone (ng/mg) of dry faeces. There was a significant effect of sex on the corticosterone output, measured as ng/mg of dry faeces. Females responded with significantly greater corticosterone output then males. All values depict mean ± SEM. * denotes significance at $p < 0.05$.

4. Discussion

The widespread use of metabolic cages has prompted the need to better understand the ethical and scientific impact these housing modes have on laboratory animals. It was hypothesised that when rats were moved to a metabolic cage they would exhibit significant behavioural and physiological adaptions in contrast to conspecifics in open-top caged housing. Metabolic cage housing resulted in rats responding with fewer optimistic judgment biases and significantly reduced sucrose preference ratios. However, no significance was detected between housing and the physiological measures of corticosterone or tyrosine hydroxylase levels.

4.1. Effects of metabolic cage housing on cognitive bias expression

The data presented in this study confirms the hypothesis that both male and female rats, when moved to a metabolic cage responded with significantly fewer optimistic biases than those that remained in control housing, Fig. 3. This finding contradicts the reports made in the Barker et al. [7] and Briefer and McElligott [14] studies. In these studies, it was reported that female animals, having experienced stressful conditions responded with significantly greater optimistic decisions than males. The results from the current study are the first to demonstrate that metabolic cage housing does induce negative affective states in both male and female rats as demonstrated through their responses to the judgment bias test. However, sex does still have an influence on cognitive bias expression, as shown by the significant interaction between sex and treatment in Fig. 3. Female rats in control housing responded with significantly fewer optimistic biases than males in control housing. This interaction supports the growing body of evidence to suggest that female and male cognitive bias expression is not equivalent [7,14,31]. Previous results have identified that female animals seemingly respond with greater optimistic responses than male counter-parts after experiencing a stressful condition [7,14]. However, both studies report an under-sized power and these results could be attributed to variability in the female sample. A more recent study identified that female animals are more likely to make continuous pessimistic decisions following a failure to be rewarded when making an optimistic decision [31]. Where the current study differs, is that a significant difference was not observed between the metabolic caged males and females, but those in control. We hypothesise that the females that remained in the control group responded with significantly fewer optimistic responses due the modulating effect of the oestrous cycle phases. However, the lack of difference in valence of bias responses between males and females in metabolic cages appears to contradict this hypothesis. Considering this result, we propose that the negative environment of the metabolic cages resulted in a significant pessimistic shift in cognitive biasing, that was severe enough to mask any subtle fluctuation in response caused by the physiological changes of oestrous. Thus, the male and female behavioural response to metabolic cages was equivalent.

Changes in the rat hypothalamic-pituitary axis (HPA) have already been associated with the different oestrous phases [32–34], with some authors suggesting that the oestrous phase needs to be taken into consideration when designing specific experiments using a female cohort [32]. There have also been documented cases of behavioural adaptations to oestrous phase. Female rats in the late dioestrous phase were reported to experience a rapid onset of hyperalgesia when compared to male rats or females in different oestrous phases [35]. Furthermore, females in the dioestrous phase responded with significantly longer latencies to re-enter the 'inner-zone' of the Open Field Test, when

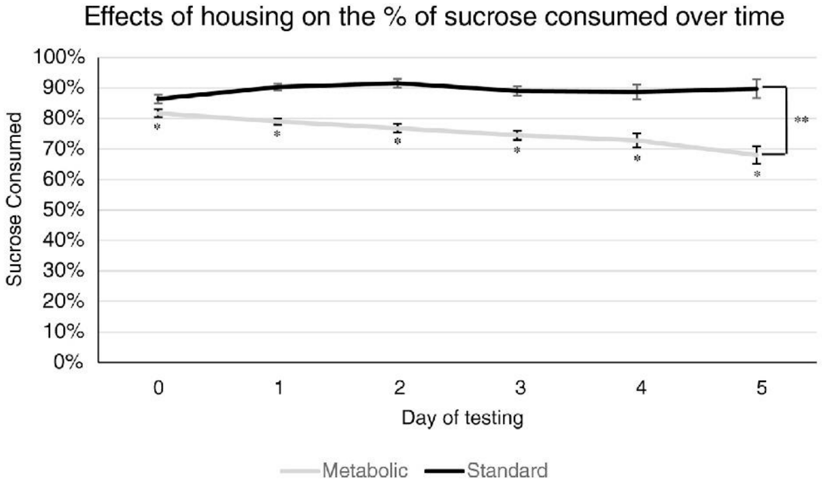


Fig. 5. The effects of housing on the percentage of sucrose consumed over time. There was a significant interaction between treatment and day on the percentage of sucrose consumed. Metabolic cage housing resulted in significantly reduced sucrose consumption for each day compared to control housing. Rats in metabolic cages responded with a significant reduction in sucrose consumption for each subsequent day of testing. This significance was not observed in the control animals. All values depict mean ± SEM. * denotes significance at $p < 0.05$. ** denotes significance at $p < 0.001$.

compared to male rats and females in other oestrous phases [35]. Similar findings have also been reported in rats undertaking the forced-swim test, females in the proestrous phase responded with significantly reduced rates of immobility (i.e. displayed fewer incidences of depression-like behaviour) compared to females in the dioestrous phase [36]. As described by Devall et al. [35], the phase of oestrous, rather than individual trait differences appears to be the important determining factor of the response of female rats to stress. Therefore, the apparent significant difference we observed between male and female animals in the control housing may be due to oestrous-phase predisposing females to respond pessimistically when the expected outcome would be optimism. Effects of oestrous phase on cognitive biases is an area for future research.

4.2. The disconnect between physiology and behaviour

Rats housed in metabolic cages did not exhibit any significant changes in either faecal corticosterone or adrenal tyrosine hydroxylase levels, rejecting one of the hypotheses of the study. These findings are unexpected, as previous literature has demonstrated an increase in faecal corticosterone concentration in metabolic-caged housed animals [22]. Similarly, many stressors have been identified to induce tyrosine hydroxylase expression in the adrenal gland, including immobilization [37], isolation [38] and foot-shock [39]. In addition, increased tyrosine-hydroxylase activity in the brain has been associated with mice considered to have increased levels of 'high-trait' anxiety compared to rats with 'low-trait' anxiety [26]. 'High-trait' anxiety mice that experienced a chronic stress (exposure to rat) demonstrated significant increases in mRNA expression of corticotrophin-releasing hormone (CRH) [26]. CRH has been shown to be upregulated by increased concentrations of circulating corticosterone [40].

It has been established that housing in a metabolic cage is a severe stressor for laboratory animals [16,22,23], and the lack of change in the physiological parameters observed is in stark contrast to previous studies. Our data, contrary to expectation, showed that metabolic cages induced a cognitive/behavioural change in the housed animals, but not a physiological change. In contrast, multiple studies have demonstrated a rodent physiological response to metabolic-cage housing. As discussed previously, increased corticosterone concentrations [21, 22], reduced immunoglobulin A secretion [23] and increases in both catecholamine levels and monoamine-oxidase activity [24], have all been associated with housing rodents in metabolic cages. It would be dishonest to state that metabolic cage housing does not induce physiological change in rats as observed. The lack of physiological response may have arisen due to differences in the period of metabolic-cage housing between the studies. Previous studies housed animals in metabolic cages for 3 [23], 7 [24], 20 [21] and 21 [22] consecutive days. In comparison, animals in this study were only housed for 7 days. Importantly, as we utilised a housing period for an equal or greater period of time than some of the aforementioned studies, the animals in the current study were removed from the cages at least 4 times a day for a maximum of up to 40 min per day to undergo cognitive bias testing.

It has been discussed previously that cognitive bias testing through judgment bias paradigms may be a positive event for the animal. Keen et al. [41] observed that the familiar testing area, coupled with the interaction of food rewards and novel stimuli contributed to judgment bias testing being considered a positive event. This would suggest that this judgment bias paradigm has little utility in testing animals outside of a stressful environment and there is a need to devise a test that can work in a home cage. However, as significant differences in cognitive biases were observed during this study this claim is disputed. It is postulated that in the current study, the repeated removal of the animals from the metabolic cage into a positively-considered environment may have negated any changes in physiological condition that were expected to occur. This indicates that changes in cognitive/behavioural measures of a stress response may be more sensitive than changes in physiological indicators. Future research is required to validate such a claim.

4.3. Effects of metabolic cage housing on sucrose preference

Fig. 5 reports a significant negative correlation between treatment and sucrose preference (as a percentage) over time. This demonstrates that both male and female rats, when housed in metabolic cages responded with a significant decrease in percentage of sucrose consumed for each subsequent day of metabolic cage housing. Sucrose preference testing has been established to be a reliable indicator of anhedonia in animals. Anhedonia is defined as the physical inability for that animal to experience pleasure [28], for example, stressed animals consistently consume decreased levels of sucrose compared to non-stressed animals [28]. As a significant correlation was not observed in the control animals, it suggests that metabolic cage housing causes a significant onset of anhedonia in the rats. This finding would also suggest that conditions known to encourage pessimistic biases in animals also result in those animals responding with increased incidences of anhedonia as measured through a sucrose-preference test. This association has been reported previously, with anhedonia occurring faster and lasting longer in rats that displayed more pessimistic biases to an active-choice judgment bias paradigm [42]. However, we have difficulties in encouraging this line of association between cognitive biases and anhedonia as there seems to be a previously unidentified confounder, being sweetness, within the nature of the judgment bias paradigm utilised. In both the current and Rygula et al. [42] study, the positively rewarded item was a sweetened food-stuff (chocolate and 20% sucrose solution). Therefore, it could be argued that the pessimistic biases observed were attributed to the animals no longer being physically capable of being 'positively rewarded' as the inherent value of the positive reward would be diminished. Therefore, pessimistic responses may be encouraged through the onset of anhedonia through altering of the reward value. This suggests that pessimistic responses may not predict stress-induced anhedoia, but instead may be a symptom of stress-induced anhedonia. To explore this issue further, a judgment bias paradigm must be utilised that does not use reward items based on food, but on another characteristic, that will not be influenced by anhedonia.

5. Conclusion

This study is the first to demonstrate that metabolic cage housing induces a negative affective state in both male and female rats as observed through judgments made to an ambiguous probe. This finding adds to the growing literature on the negative effects that metabolic cage housing has on welfare.

This study failed to provide evidence that pessimistic biases were correlated with either significant increases in the concentration of faecal corticosterone or adrenal tyrosine hydroxylase. However, this was likely attributed to the constant removal of the animal from the metabolic cage into a 'positive' location to undergo cognitive bias testing. This demonstrated that cognitive/behavioural indicators of a stress response may be more sensitive than physiological indicators.

Although correlation was found between conditions to encourage pessimistic biases and sucrose preference, we are hesitant to suggest that pessimistic biases can predict stress-induced anhedonia. Instead we suggest that pessimistic biases to a judgment bias paradigm utilising sweetened reward items may be a symptom of stress-induced anhedonia. Future research efforts focused on studying anhedonia using a judgment bias paradigm without food rewards would assist in identifying this concern. There is also justification for future studies to be focused on the effects that oestrous phase has on cognitive output in female animals.

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CHAPTER 4.

Assessment of housing density, space allocation and social hierarchy of laboratory rats on behavioural measures of welfare

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Contribution to the Paper	Experimental design and procedure. Statistical analysis. Wrote manuscript. Acted as corresponding author.		
Overall percentage (%)	80		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

vii. the candidate's stated contribution to the publication is accurate (as detailed above);

viii. permission is granted for the candidate in include the publication in the thesis; and

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4.2. Statement of Context

A major discussion point of Chapter 3 was that social hierarchy and the hierarchal position of a rat may influence affective state. Subordination in rats has been previously associated with significant physiological (Blanchard et al., 1993; Lucas et al., 2004; Tamashiro et al., 2005) and behavioural (Inagaki et al., 2005; Davis et al., 2009) adaptations consistent with chronic stress. In addition, rodents with low hierarchal rank have been evidenced to try and gain a greater rank by challenging other lowly ranked animals (Van Loo et al., 2001). These findings suggest that subordinate rats experience greater social stressors than their dominant cage-mates, with female rats being more sensitive to these stressors than males (Hurst et al., 1996; Hurst et al., 1999).

With this understanding, the study presented in Chapter 4 was established to identify if social hierarchy could be a significant modifier of judgement bias expression in male rats. As discussed in section 1.4, males were utilised in this study as, until the effects of oestrous were understood in females, a male cohort was the most sensible option.

Prior to commencement of this study it became clear that social hierarchy and associated stressors are closely linked to two other factors, being housing density (animals per cage) and space allocation (floor area allowance for any given animal). Therefore, the study was expanded to investigate the effects that these factors had on judgement bias expression in male rats. These judgement biases were compared with other behavioural measures of welfare, being the open-field, social-interaction and novel-object recognition tests to validate that a significant stress response was being experienced.

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RESEARCH ARTICLE

Assessment of housing density, space allocation and social hierarchy of laboratory rats on behavioural measures of welfare

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Abstract

Minimum space allowances for laboratory rats are legislated based on weight and stocking rates, with the understanding that increased housing density encourages crowding stress. However, there is little evidence for these recommendations, especially when considering positive welfare outcomes. This study consisted of two experiments which investigated the effects of housing density (rats per cage), space allocation (surface area per rat) and social rank (dominance hierarchy) on the ability to perform simple behavioural tests. Male Sprague Dawley (SD) rats (n = 64) were allocated to either high-density (n = 8) or low-density (n = 8) cages. The second experiment investigated the effects of surface area. SD rats (n = 40) were housed in dyads in either the large (n = 10) or small (n = 10) cage. In both experiments, animals were tested on a judgment bias paradigm, with their responses to an ambiguous stimulus being ascribed as optimistic or pessimistic. Animals were also tested on open-field, novel-object recognition and social-interaction tests. Recordings were taken from 1700-2100h daily for rat observation and social rank establishment. Dominant animals responded with significantly more optimistic decisions compared to subordinates for both the housing density ($p<0.001$) and space allocation ($p = 0.0015$) experiment. Dominant animals responded with increased social affiliative behaviours in the social-interaction test, and spent more time in the centre of the open-field test for both experiments. No significance was detected between housing density or space allocation treatments. These findings suggest that social rank is a significantly greater modifier of affective state than either housing density or space allocation. This finding has not yet been reported and suggests that future drafts of housing guidelines should consider animal social status in addition to floor space requirements.

Introduction

International standards for the care and housing of Lab Animals provide relatively uniform guidelines regarding stocking rates and surface area allowance afforded to all rodents used for scientific purposes. The *Guide for the Care and Use of Lab Animals* [1] (United States) and the

EC Directive 2010/63/EU [2] (Europe) are among a few of the regulatory bodies that provide guidelines for rodent housing. These guidelines usually state specific measurements regarding the weight of the animal and the floor area allocated per animal to reduce the effects of crowding-related stressors. However, it has been recently noted that these guidelines rarely cite scientific literature to support these space requirements [3]. It has also been noted in the *Resolution on Accommodation and Care of Lab Animals* [4] that evidence-based data is lacking on this specific subject. While the scientific community have identified this knowledge gap, recent literature has focussed primarily on mice [5]. Few publications have identified the need to establish the effects of housing density and space allocation in rats (*Rattus norvegicus).*

Investigations of space use by rats have been historically well-researched, however the primary focus was conducted in wild rats [6, 7]. Cage floor area and space allocation provide logistical limitations to the types of research activities that can occur [5] which perhaps prompted early research methods to study the effects that crowding can elicit [8]. It was first noted by Calhoun [8] that when a population of laboratory rats was allowed to increase in a confined space, abnormal behavioural patterns began to occur. It was argued that these behaviours could lead to the extinction of the entire caged population. This crowding effect has led to the prominence of guidelines and legislative documentation that encourage strict space allowances for each caged animal. However, this may not be an accurate portrayal of the multitude of factors that interact. Housing density is defined hereafter as the number of animals that occupy the same caged floor area while space allocation is defined as the surface area allocated to each animal within a shared cage. Housing density and space allocation are two separate, yet closely linked factors that interact to produce 'crowding'. Crowding is the operational word used by these regulatory bodies that defines the motivational state that occurs when spatial and social factors interact [9]. Many studies that have previously discussed the effects of housing density or space allocation on rodent behaviour have been confounded by their inability to successfully separate these two factors [10, 11]. Studies in which cage size is kept constant and animals are added or subtracted are not designed to investigate either housing density or space allocation, instead they report the effects of crowding.

We sought to assess the effects of both housing density and space allocation on the performance of rats in an array of simple behavioural tests. We also aimed to determine if the social class (as determined through a dominance hierarchy) of the animals would interact with these factors, as this interaction had been significantly underreported in the literature, despite the knowledge of social composition contributing to crowding stress [9]. The behavioural tests utilised included the open-field test (OFT), the novel-object recognition test (NORT), the social-interaction test (SIT) and cognitive bias detection through a judgment bias paradigm (JBP). These tests were chosen due to their repeatability and their reliability at providing evidence of anxiety-like behaviours. The JBP has the added advantage of being able to identify positive welfare outcomes.

Materials and methods

Animals and housing

This study was separated into two distinct experiments. The first studied the effects of housing density and used 64 male Hsd: Sprague Dawley (SD) rats. The second study studied the effects of space allocation and used 40 male SD rats. All animals were sourced from a barrier-maintained, specific pathogen free production facility (University of Adelaide, Laboratory Animal Services, Adelaide, Australia). Upon arrival at the testing facility, animals were housed in their treatment groups (discussed later) in commercially available cages (Tecniplast, Exton, PA, USA). Cage design and specifications are discussed below. Each cage was provided with a paper-based bedding substrate (Animal Bedding, Fibrecycle Pty Ltd, Yatala, Queensland, Australia). Standard rat chow (Rat and Mouse Cubes, Speciality Feeds, Western Australia, Australia) and reverse-osmosis water were provided *ad libitum*. Room temperature was maintained between 21˚C and 23˚C and a reversed 12-hour light/dark cycle (lights on at 1800h, off at 0600h) was used. The animals were acclimatized to the facility environment for 5 days before behavioural training on the judgment bias paradigm commenced. All animal use and housing protocols were approved by the Animal Ethics Committee of the University of Adelaide and conducted in accordance with the provisions of the *Australian Code for the Care and Use of Animals for Scientific Purposes* [12].

Housing density experiment

Upon arrival at the testing facility, rats (n = 64) were housed in groups of either 6 (high-density), or 2 (low-density) rats per cage. The high-density cages (n = 8, total of 48 rats) were the Eurostandard type IV (Tecniplast, Exton, PA, USA) with dimensions of 59.8cm by 38cm with 26cm of vertical space at the lowest point (Fig 1A). These cages had an area of 2,280cm^2 and were appropriate to house 6 rats of approximately 450 grams, per the guidelines as prescribed in the eighth edition of the *Guide for the Care and Use of Lab Animals* [1]. Low-density cages (n = 8, total of 16 rats) were the Eurostandard type IIL (Tecniplast, Exton, PA, USA) with dimensions of 36.5cm by 20.7cm with 22cm of vertical space at the lowest point (Fig 1B). These cages had an area of 755.55cm^2 and were appropriate to house 2 rats up to 450 grams.

Space allocation experiment

The second experiment used 40 male SD rats and occurred immediately following the completion of all behavioural testing of the housing density experiment. Animals were randomly housed in either the large cage (Eurostandard type IV) or the small cage (Eurostandard type IIL) with one other conspecific and allowed to acclimatize to these conditions for 3 weeks. Animals housed in the large cages were the large surface area treatment group (n = 10, total of 20 rats) and those housed in the small cages were the small surface area treatment group (n = 10, total of 20 rats).

Cognitive bias test

Cognitive bias detection has been used as an indicator of animal affective state (emotional state) [13]. These biases were measured using a judgment bias paradigm (JBP), that was based on an earlier JBP design [14]. Commonly used terminology of the JBP has been defined in

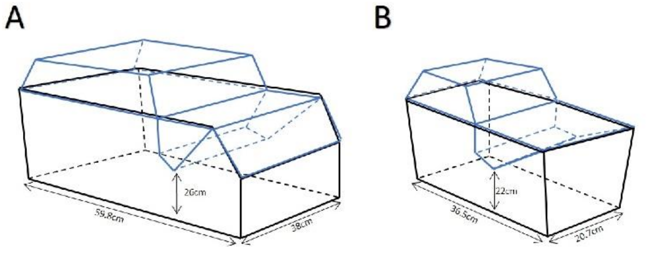

Fig 1. Diagram of cage sizes used. A) The Eurostandard type IV cage drawn to approximate 1:10 scale. This cage is appropriate to house up to 6 rats of approximately 450 grams. B) The Eurostandard type IIL cage drawn to approximate1:10 scale. This cage is appropriate to house up to 2 rats of approximately 450 grams. The black lines represent the cage bottom while the blue lines represent the cage lid.

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Table 1. This JBP makes use of a single testing/training apparatus that comprises two Perspex boxes (610mm x 435mm x 500mm) connected via a PVC pipe with a 100mm diameter. During certain training phases, the pipe was lined with one of two-grades of sandpaper, one being coarse (P80) the other being a fine sandpaper (P1200). Inside one box (henceforth referred as the 'goal box') were two reward bowls positioned in the two far corners. These reward bowls were filled with either a coriander or cinnamon scented sand (1% by weight of spice to sifted sand). The coriander scented reward bowl remained in the right-hand corner for each trial, while the cinnamon scented reward bowl remained in the left-hand corner (Fig 2). Milk chocolate baking chips (Cadbury, London, England) were used as the high-positive reward items whilst Cheerios (Uncle Toby's, Victoria, Australia) were considered a low-positive reward items. Every animal was randomly assigned a sandpaper association to the reward items and rewarded location for both the housing density experiment (Table 2) and the space allocation experiment (Table 3). This paradigm has been divided into phases where different experimental outcomes are expected. A summary of these phases is included in Table 4. The animals would learn to associate the different type of sandpaper with the type of reward item and where that reward item was located. During the testing phase, the sandpaper in the PVC pipe was replaced with sandpaper of an intermediate grade (P180) and no reward items were present in the reward bowls. This sandpaper type behaved as the intermediate, ambiguous probe and the responses to this probe could be considered either optimistic or pessimistic. An optimistic decision was defined when the rat displayed foraging behaviours for these intermediate ambiguous trials in the bowl that would normally contain the chocolate reward. A pessimistic behaviour was defined when the rat displayed foraging behaviour in the bowl that would normally contain the cheerio reward [15]. Testing in the JBP occurred once a day, for 5 days (Table 4).

Open-field test

On day 2 of the five-day testing period, each animal was subjected to the open-field test (OFT) after recording a cognitive bias decision for that day. Testing was performed as described by the methods of Wallace [16] and utilised a square testing arena (100cm by 100cm by 100cm) made from black corflute in a homogenously illuminated arena (150 lux) away from where the animals were normally housed. Animals were placed individually into the centre of the arena upon which a video camera (Logitech HD Webcam C525, Lausanne, Switzerland) suspended

Table 1. Definitions of commonly used terminology for the judgment bias paradigm.

Term	Definition
Approach	When the rat actively and intentionally placed its forelimbs and face into a reward bowl to extract the reward.
Forage	When the rat continuously and deliberately displaced the sand in the food bowl to obtain the reward.
Consumption	When the rat actively and intentionally interacted with the food by bringing it to its mouth.
Success	Successful trial was determined after the animal had approached and foraged in the correct (reward containing) food bowl before approaching or foraging in the incorrect food bowl.
Promotion	Animals were promoted to the succeeding trial (where appropriate) after achieving successful trails per day, for 5 consecutive days.
Failure	If the rat failed to consume the reward within 10 minutes of being placed into the testing chamber.

First described by Barker et al. (15)

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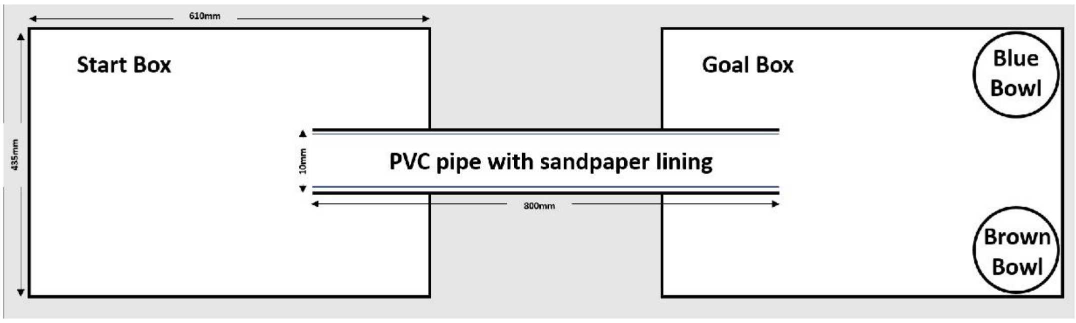

Fig 2. Diagram and size of judgment bias testing apparatus utilized. The judgment bias test was comprised of two Perspex boxes connected via a PVC pipe. Two reward bowls were placed in either corner of the goal box containing scented sand.

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over the centre of the arena began recording. The experimenter then immediately left the room and the animals stayed in the arena for 10 minutes. After 10 minutes the animal was removed from the arena and placed back into its home cage. The arena was then cleaned with a 70% ethanol solution. OFT video analysis was conducted manually utilising the CowLog open source software. Three zones were superimposed to the open field test video files (Fig 3) and the time spent in each zone was recorded, as was the number of transitions into each zone. Observers also recorded the time spent inactive during the test, time spent rearing, and number of defecation or urination incidents. A rat was considered to have entered a 'zone' when its centre of gravity had crossed into the new zone.

Novel-object recognition test

On the third day of testing, after recording a cognitive bias result, each animal was subjected to the novel-object recognition test (NORT). Testing was performed according to the methods presented by Bevins and Besheer [17], the arena utilised was a barren (no bedding), 'high-density' home cage, with accompanying wire-lid. Testing involved two behavioural phases, during phase 1 (Fig 4A) an individual animal was placed into this testing arena with two identical objects (stainless steel, water bottle tops) in either corner. The experimenter than left the room after starting the video recording from the suspended camera. After 10 minutes, the animal was removed from the arena and placed back into the home cage and the arena was cleaned with a 70% ethanol solution. After one hour, the animal was ready to be tested again in the

Table 2. Associations of reward items and locations for treatments of the housing density experiment.

	Cage Density	Chocolate Stimulus	Chocolate Location	Cheerio Stimulus	Cheerio Location
Association 1 (n = 12)	High	Coarse Sandpaper	Brown Bowl / Right	Fine Sandpaper	Blue Bowl / Left
Association 2 (n = 12)	High	Coarse Sandpaper	Blue Bowl / Left	Fine Sandpaper	Brown Bowl / Right
Association 3 (n = 12)	High	Fine Sandpaper	Brown Bowl / Right	Coarse Sandpaper	Blue Bowl / Left
Association 4 (n = 12)	High	Fine Sandpaper	Blue Bowl / Left	Coarse Sandpaper	Brown Bowl / Right
Association 5 (n = 4)	Low	Coarse Sandpaper	Brown Bowl / Right	Fine Sandpaper	Blue Bowl / Left
Association 6 (n = 4)	Low	Coarse Sandpaper	Blue Bowl / Left	Fine Sandpaper	Brown Bowl / Right
Association 7 (n = 4)	Low	Fine Sandpaper	Brown Bowl / Right	Coarse Sandpaper	Blue Bowl / Left
Association 8 (n = 4)	Low	Fine Sandpaper	Blue Bowl / Left	Coarse Sandpaper	Brown Bowl / Right

Each association was randomly assigned and counter-balanced between treatments.

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Table 3. Associations of reward items and locations for treatments of the space allocation experiment.

	Cage Size	Chocolate Stimulus	Chocolate Location	Cheerio Stimulus	Cheerio Location
Association 1 (n = 5)	Large	Coarse Sandpaper	Brown Bowl / Right	Fine Sandpaper	Blue Bowl / Left
Association 2 (n = 5)	Large	Coarse Sandpaper	Blue Bowl / Left	Fine Sandpaper	Brown Bowl / Right
Association 3 (n = 5)	Large	Fine Sandpaper	Brown Bowl / Right	Coarse Sandpaper	Blue Bowl / Left
Association 4 (n = 5)	Large	Fine Sandpaper	Blue Bowl / Left	Coarse Sandpaper	Brown Bowl / Right
Association 5 (n = 5)	Small	Coarse Sandpaper	Brown Bowl / Right	Fine Sandpaper	Blue Bowl / Left
Association 6 (n = 5)	Small	Coarse Sandpaper	Blue Bowl / Left	Fine Sandpaper	Brown Bowl / Right
Association 7 (n = 5)	Small	Fine Sandpaper	Brown Bowl / Right	Coarse Sandpaper	Blue Bowl / Left
Association 8 (n = 5)	Small	Fine Sandpaper	Blue Bowl / Left	Coarse Sandpaper	Brown Bowl / Right

Each association was randomly assigned and counter-balanced between treatments.

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same arena, however during phase 2 (Fig 4B) one of the two familiar items from phase 1, was randomly replaced with a novel item (red, large die), of approximate equal mass to the familiar item. After the animal was placed into the testing arena for phase 2, the video recording was again started and the experimenter left the room for 5 minutes. After 5 minutes, the animal was placed back into the home cage and the arena was cleaned with a 70% ethanol solution. Video footage from phase 2 was used to analyse the NORT data. Using the CowLog open source software, the observer documented the time each rat spent interacting with both the familiar and the novel object, as well as the number of interactions that occurred for each object. Other measures taken include the time the animal spent exploring the confines of the arena, time spent rearing and inactive, and the number of defecation and urination incidents.

Social-interaction test

On day 4 of the five-day testing period, each animal was subjected to the social-interaction test (SIT) after they had recorded a cognitive bias decision for that day. Testing was slightly modified from the methods of Sams-Dodd [18], and was performed in a circular arena with a diameter of 100cm made from hessian supported by flexible plastic sheeting. The arena was in a homogenously illuminated (150 lux) area in a different room to where the animals were normally housed. Test animals was marked with a nontoxic black marker immediately prior to being placed in the arena. The test animal was placed simultaneously into the arena with an unfamiliar SD rat (non-cage mate) that was not used as part of this study. This animal will henceforth be referred as the unfamiliar rat. Both test and unfamiliar rats were placed approximately 40cm apart in the arena. The video camera suspended over the arena began recording and the experimenter then left the room. Testing lasted for 10 minutes after which time the experimenter re-entered the room and returned each animal to its appropriate home cage before cleaning the arena with a 70% ethanol solution. Video analysis was performed manually with the CowLog open source software, animal behaviour was scored using a continuous sampling method. Behavioural expression was categorised using the ethogram as prescribed in Sams-Dodd [18] (Table 5). The time spent exhibiting each behaviour was totalled and a time-budget was generated. Each behavioural expression is presented as a percentage of the total time exhibited from the time-budget.

Social classification

Classification of the rats into their social classes was achieved through observing video footage recorded with CCTV cameras (OzSpy, Brisbane, Australia). Rats were recorded from 1500-

Table 4. Descriptions and promotion criteria of each training and testing phase involved in the JBP.

Phase	Description	Promotion Condition
A	Rats handled for two 10-minute periods. The first period between 0900 and 1200 hours, the second period between 1400 and 1700 hours.	Phase lasted for 5 days.
B	Rats placed into the testing apparatus, four times a day for 5-minute intervals. The food bowls contained the reward items appropriate to the individual rat, these rewards were placed on the surface of the sand in the reward bowls. No sandpaper was present within the PCV pipe.	Phase lasted for 5 days.
C	The testing apparatus now contained the appropriate sandpaper stimuli. Animals had two training trials between 0900-1200h and two between 1400–1700 hours. Each period had one chocolate trial and one cheerio trial that occurred in a random order. For each trial, a single reward item was placed on the surface of the appropriate reward bowl, to the appropriate sandpaper that was present in the apparatus. Rats were placed in the start box, upon which a timer was started. Latency for the rat to leave the start box, enter the goal box, approach any reward bowl, approach the correct reward bowl and start to consume the reward was recorded. The rat was immediately removed from the apparatus once it had consumed the reward or if it failed the test. The whole apparatus was then cleaned with 70% ethanol solution.	Promotion to phase D was achieved after the animals achieved success on 3 of their 4 daily trials for once a day, for 5 days in a row.
D	Identical to phase C, however during phase D the reward items were buried in the sand of the reward bowls. Each rat was required to forage for the reward item and extract it from the sand. Following the successful extraction of the reward, the depth at which the reward was buried for the next trial increased. Burial depth continued to increase with each successive trial until the reward was always completely buried in the sand.	Promotion to phase E was identical to the promotion conditions of phase C
E	Identical to phase D, but the reward items were always completely buried in the sand and one randomly selected trial per day contained no reward item. A successful, unrewarded trial was defined when the first bowl that the rat foraged in would normally contain a reward item.	Promotion to phase E was identical to the promotion conditions of phase C
F	Identical to phase E, except the unrewarded trial was now paired with a sandpaper of intermediate grade (P180).	Phase lasted for 3 days
G	Testing Phase. The rats received one test per day that involved the intermediate sandpaper (P180) being present in the pipe and no reward being present in the food bowl. During testing, the time was recorded for the rat to forage in any bowl, and the bowl the rat approached, and foraged in first was documented.	Phase lasted for 5 days

Adapted from Brydges *et al*. (14)

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2300h each day over the five day testing period. Each animal was observed for 10 minutes with the start time of each sample being randomly selected between 1500-1750h. Behaviours of each

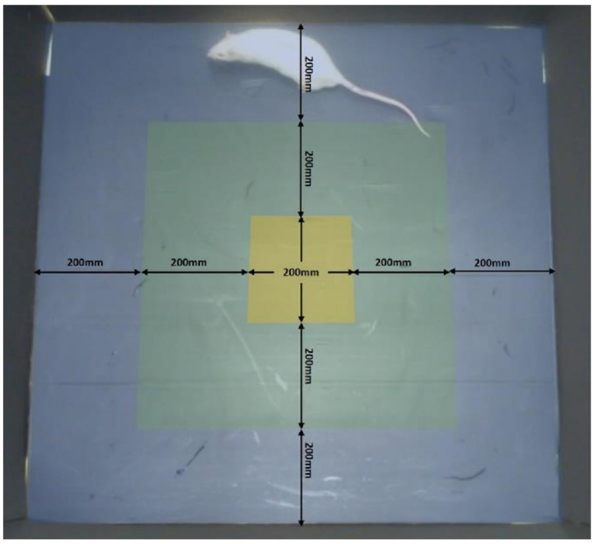

Fig 3. Diagram of the open-field test. Image of the video recording of the open-field test detailing the location and size of the zones used. Blue: Peripheral Zone, Green: Inner Zone, Yellow: Centre Zone. Arrows have been superimposed to indicate size.

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animal were scored per the ethogram as originally devised by Hurst et al. (1996) (Table 6.). Interactions between each individual with respect to each of its caged conspecifics were examined to determine the dominance relationship. Total numbers of aggressive encounters initiated were summed and compared against the number of aggressive encounters received, to assign the animal an agonistic score. As per the experimental design of Hurst et al. [19], dominance within a dyad was assigned if the animal initiated greater numbers of aggressive encounters than it received over each of the five 10-minute videos. Social class of rats for the housing density experiment is included in Table 7, and social class of rats for the space allocation experiment is included in Table 8.

Statistical analysis

All data were analysed using the IBM SPSS Statistics 22 (IBM, NY, USA) software package. Levene's test was used in all cases to test for normality of the data set, all data were found to be

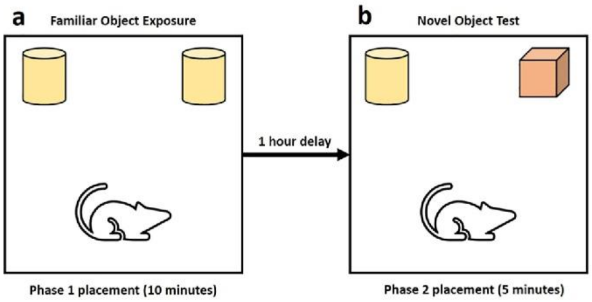

Fig 4. Novel object recognition test design. (A) Phase 1: Familiar object exposure. (B) Phase 2, Novel-object recognition test. From the methods of Bevins and Besheer [17]

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Table 5. Ethogram used for behavioural analysis during the social interaction test.

Behaviour	Description
Exploration	Movement in the arena, sniffing at floor, walls and inspecting arena.
Rearing	Raised on the hind legs, sniffing into the air.
Investigation	Sniffs at and investigates the unfamiliar rat.
Follow	Follows the unfamiliar rat.
Grooming	Cleaning the fur and/or scratching.
Inactive	No discernible action.
Stationary Stereotyped Behaviour	Stationary and performs circular head movements and/or head weaving.
Lateral Threat	Body is arched in a sideward posture towards the unfamiliar rat.
Upright	Standing on its hind legs and is facing the unfamiliar rat.
Stand Over	Standing on top of the unfamiliar rat.
Lie Under	Lying on its back beneath the unfamiliar rat.
Clinch	Active fighting with the unfamiliar rat.
Pursue	Runs after the unfamiliar rat during "Clinch"
Escape	Runs away from the unfamiliar rat during "Clinch"

Adapted from Sams-Dodd (18)

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parametric unless otherwise stated. Numerical data have been presented as mean ± standard error of the mean. Differences between means were considered significant when p was less than 0.05. All normally distributed data was analysed using a two-way multivariate analysis of variance (MANOVA) test, fitting housing density and social class on the test variable. Where the data were not normally distributed, they were analysed using the Kruskal-Wallis H test and/or the Mann-Whitney U test. Further details of statistical analysis are found in the results section.

A complete flowchart of the experimental procedure is illustrated in Fig 5.

Table 6. Ethogram used for behavioural analysis during social classification.

Behavioural category	Behavioural elements of the viewed rat
Sleeping	Lying or sitting unalert, eyes closed
Feeding/drinking	Eating food or faeces; drinking
Non-intake maintenance	Grooming; yawning; stretching; sneezing; urinating; defecating
Exploration	Sniffing air, floor, wall, water bottle, faeces, urine or bedding
Stationary	Alert (eyes open) but no directed attention while lying, sitting or leaning
Movement	Alert but no directed attention while walking, stretching, climbing or running
Other non-social behaviour	Chewing bedding; digging/scrabbling; jumping
Aggressive action	Bite; chase; aggressive over (pinning rat on its back); aggressive groom; aggressive sideways; upright; mounting; pull tail, pursuit of fleeing rat
Defensive action	Defensive over (on back, being pinned), defensive sideways, flight (with and without pursuit)
Social investigation	Sniffing nose, mouth, head, shoulders, back, flank, anogenital area, belly, tail
Other social behaviour	Attend; allogroom

Originally designed by Hurst *et al.* (19) This ethogram was used to assess social hierarchy within cages and to assign dominance to animals within dyads.

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Table 7. Social classes of caged rats for the housing density experiment.

Social Class	Definition	No.
(D) Dominant	Dominant over all cage mates–dominant in every dyad	n = 16
(DS) Dominant subdominant	Mostly dominant–dominant in most dyads but not all	n = 14
(SS) Subordinate subdominant	Mostly subordinate–subordinate in most dyads but not all	n = 22
(S) Subordinate	Subordinate to all cage mates–subordinate in every dyad	n = 12

Definitions of each class and criteria for assignment into a social class for the housing density experiment. As designed by Hurst *et al.*(19).

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Results

Effects of housing density and social class on the cognitive bias test

The Shapiro-Wilk test determined that the data were not normally distributed. A Kruskal-Wallis H test showed that there was no significant effect or interaction involving housing density on the number of days featuring an optimistic decision $\chi2(3) = 0.082$, $p = 0.521$. However, there was a statistically significant difference observed in social class on number of optimistic decisions made, $\chi^2(3) = 26.95$, $p<0.001$. The Mann-Whitney U test was used to investigate the nature of this effect. Dominant (D) animals responded with significantly greater number of optimistic decisions (4.94 ± 0.25) compared to both Subordinate Subdominant (SS) animals (3.68 ± 0.22; $p<0.001$) and Subordinate (S) animals (2.77 ± 0.28; $p<0.001$). Likewise, Dominant Subdominant (DS) animals (4.5 ± 0.28) responded with significantly greater numbers of optimistic decisions compared to the SS (3.68 ± 0.22; $p = 0.044$) and S animals (2.77 ± 0.28; $p = 0.002$) (Fig 6A).

Effects of space allocation and social class on the cognitive bias test

The Shapiro-Wilk test determined that these data were not normally distributed. A Kruskal-Wallis H test showed that there was no significant effect or interaction between cage size on the number of days featuring an optimistic decision $\chi^2(1) = 0.044$, $p = 0.725$. However, there was a statistically significant difference observed between social class on number of optimistic decisions made, $\chi^2(1) = 5.865$, $p = 0.015$. D animals responded with significantly greater number of optimistic decisions (453 ± 0.23) compared to S animals (3.63 ± 0.28; $p = 0.015$). (Fig 6B).

Effects of housing density and social class on the social-interaction test

A two-way MANOVA was performed fitting housing density and social class against the percentage of time spent exhibiting each of the scored behaviours. There was a statistically significant interaction between housing density and social class on the percentage of time the animal spent 'investigating' the unfamiliar, F (1, 57) = 6.878, $p = 0.011$. Simple main effects analysis

Table 8. Social classes of caged rats for the space allocation experiment.

Social Class	Definition	No.
(D) Dominant	Dominant over conspecific	n = 20
(S) Subordinate	Subordinate to conspecific	n = 20

Definitions of each class and criteria for assignment into a social class for the space allocation experiment. As designed by Hurst *et al.*(19).

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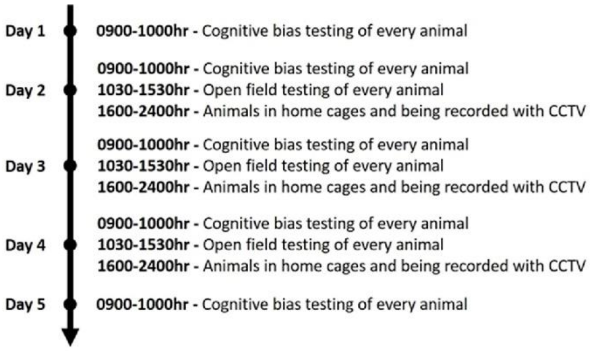

Fig 5. Flowchart of the experimental procedure and the days and times tests are performed. This flowchart details the steps used for both the housing density and the space allocation experiment. Despite the protocol remaining the same, it is important to note that these did not occur at the same time. The space allocation experiment occurred after the animals in the housing density experiment finished testing.

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showed that S rats housed in the high-density cages responded with a significantly reduced percentage of time investigating the unfamiliar rat (7.22% ± 1.91) compared to the S rats housed in the low-density cages (15.92% ± 1.85; p = 0.004) (Fig 7).

There was also a significant effect of social class on the percentage of time spent exploring the confines of the social-interaction test F (3,57) = 5.370, p = 0.003. D animals (49.98% ± 1.62) spent a significantly reduced percentage of time exploring the apparatus of the test when compared with both SS animals (56.81% ± 1.78; p = 0.004) and S animals (57.6% ± 1.44; p = 0.002) (Fig 8A). There was no significance detected for housing density on percentage time exploring, F (1,57) = 0.001, p = 0.992.

Finally, significance was also detected between social class and the percentage of time spent following the unfamiliar rat F (3,57) = 3.684, p = 0.017. D animals (3.27% ± 0.42) spent a significantly greater percentage of time following the unfamiliar rat then both SS animals (1.96% ± 0.43; p = 0.03) and S animals (1.37% ± 0.35; p = 0.002) (Fig 8B). There was no significance detected for housing density on percentage time following the unfamiliar rat, F (1,57) = 0.108, p = 0.744.

Effects of space allocation and social class on the social-interaction test

A two-way MANOVA was performed fitting space allocation and social class against the percentage of time spent exhibiting each of the scored behaviours. There were no statistically significant interactions between space allocation and social class on the percentage of time spent exhibiting any scored behaviour. However, there was a significant effect of social class on the

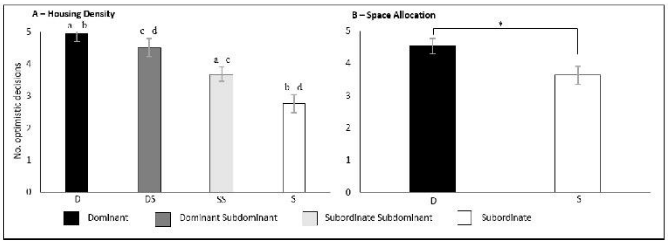

Fig 6. The effects of social class on the number of optimistic responses made in the JBP. A) For the housing density experiment. B) For the space allocation experiment. Significance is denoted at p< 0.05.

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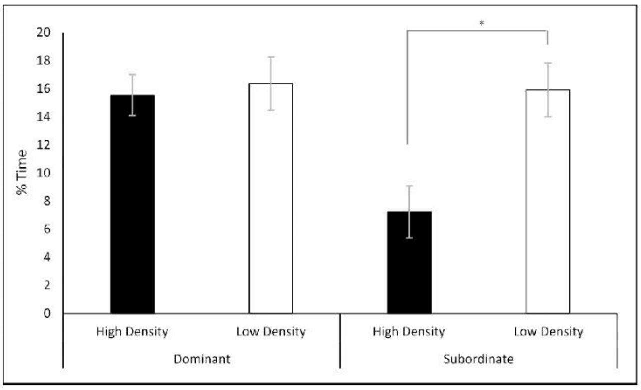

Fig 7. Interaction between density and class on the percentage time spent investigating the unfamiliar rat. Significance is denoted at p< 0.05.

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percentage of time a rat spent investigating the unfamiliar rat. Dominant animals (12.64% ± 1.03) spent significantly greater percentages of time investigating the unfamiliar animals than the subordinate animals (9.79% ± 0.93) F (1, 22) = 4.301, p = 0.049 (Fig 9). No significance was detected between space allocation on the percentage of time investigating F (1, 22) = 0.572, p = 0.891.

There was also a significant effect of space allocation on the percentage of time spent following the unfamiliar rat. Animals in the small cages (5.98% ± 1.45) spent a significantly greater percentage of time following the unfamiliar rat than compared to animals in the large cages (0.77% ± 1.23) F (1, 22) = 4.863, p = 0.038 (Fig 10). No significance was detected between social class on the percentage of time investigating F (1, 22) = 0.321, p = 0.613.

Effects of housing density and social class on the novel-object recognition test

A two-way MANOVA was performed fitting housing density and social class against the percentage of time spent interacting with the familiar and the novel objects as well as the time spent exploring the cage parameters. There was a statistically significant interaction between housing density and social class on the percentage of time the animal spent interacting with the novel object F (1, 57) = 4.798, p = 0.033. Simple main effects analysis identified that S animals in the high-density cages responded with a significantly reduced percentage of time (4.673% ± 3.26) interacting with the novel object compared to S animals in the low-density cages (17.9% ± 3.36) (p = 0.012) (Fig 11).

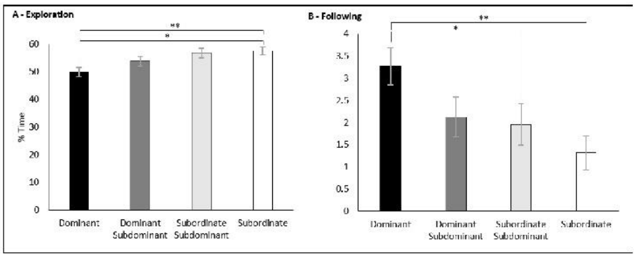

Fig 8. Effects of social class on the percentage of time spent exploring in the SIT. A) For the housing density experiment. B) For the space allocation experiment. Significance is denoted at p<0.05.

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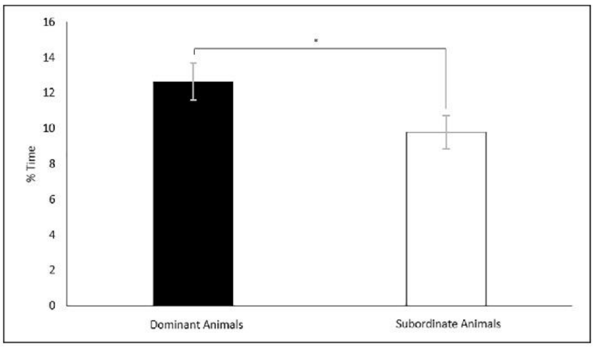

Fig 9. Effects of social class on the percentage of time spent exploring in the SIT. Results of the space allocation experiment. Significance is denoted at $p < 0.05$.

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Effects of space allocation and social class on the novel-object recognition test

A two-way MANOVA was performed fitting space allocation and social class against the percentage of time spent interacting with the familiar and the novel objects as well as the time spent exploring the cage parameters. There was no statistically significant interaction or effect between these parameters on the test variables and therefore the data has been omitted.

Effects of housing density and social class on the open-field test

A two-way MANOVA was performed fitting housing density and social class against the percentage of time the animal spent in each of the open field testing 'zones' as well as the percentage of time spent defecating/urinating, rearing and being inactive. There was a significant interaction between housing density and social class on the percentage of time the animal spent in the peripheral zone of the OFT F (1,57) = 10.396, p = 0.002. Simple main effects analysis showed that S rats (81.99% ± 3.81) in the high-density cages responded with a significantly increased percentage of time in the peripheral zone compared to D rats (62.08% ± 3.01; p< 0.001), DS rats (64.1% ± 2.33; p< 0.001) and SS rats (69.43 ± 1.97; p = 0.003) also housed in the high-density cages. Likewise, S animals in the high-density cages also responded with increased percentage of time compared to S rats (65.82% ± 3.92; p = 0.009) housed in the low-density cages. SS rats of the high-density cages also responded with a significantly increased

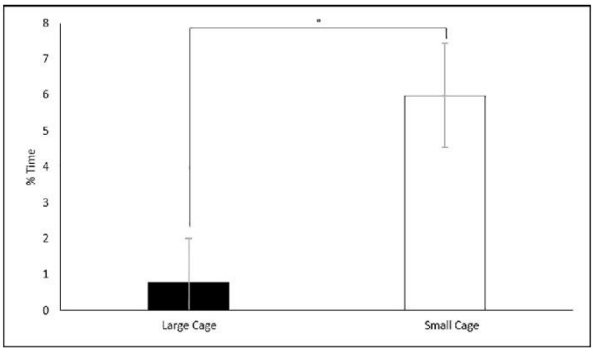

Fig 10. Effects of space allocation on the percentage of time following the unfamiliar in the SIT. Significance is denoted at $p<0.05$.

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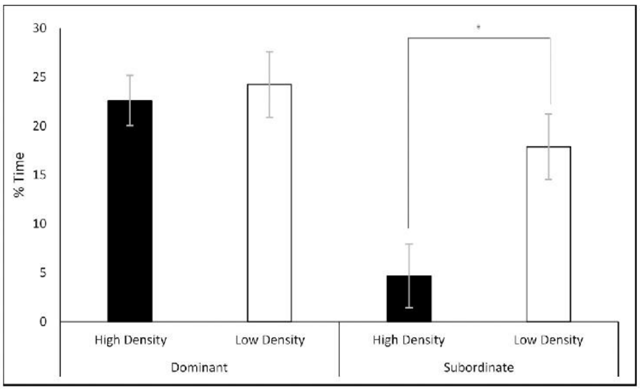

Fig 11. Interaction between housing density and class on percentage of time interacting with the novel object. Significance is denoted at $p<0.05$.

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percentage of time in the peripheral zone compared to the D rats (p = 0.034) in the same cages. (Fig 12A). No significance was detected between social class for the low-density cages.

There was also a significant interaction between social class and housing density on the percentage of time the animals spent in the centre zone of the OFT, F (1,57) = 5.123, p = 0.027. S rats in the high-density cages (2.09% ± 1.6) responded with statistically significant decreases in the percentage of time spent in the centre compared to D rats (10.18% ± 1.27; $p<0.001$) and DS rats (7.34% ± 0.98; p = 0.005) of the high-density cages. Similarly, SS rats in the high-density cages (4.88% ± 0.83) also responded with a significantly reduced percentage of time in the centre zone compared to both D rats ($p<0.001$) and DS rats (p = 0.042) also in the high-density cages. Once again, there was no significance detected between social classes for the rats in the low-density cages (Fig 12B).

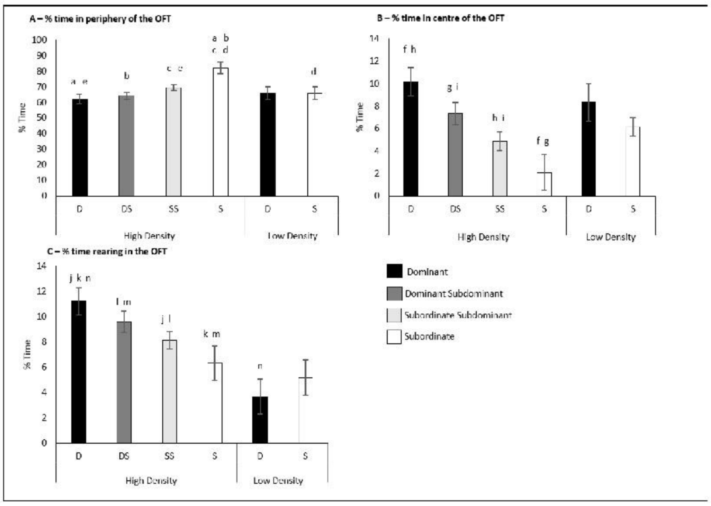

Fig 12. Results of the open field test for the housing density experiment. A) The interaction of housing density and social class on the percentage of time spent in the peripheral zone of the OFT. B) The interaction of housing density and social class on the percentage of time spent in the centre zone of the OFT. C) The interaction of housing density and social class on the percentage of time spent rearing in the OFT. Significance is denoted at $p<0.05$.

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The final significant interaction between social class and housing density was between the percentage of time the animals spent rearing in the OFT, F (1,57) = 8.478, p = 0.005. S rats in the high-density cages (6.292% ± 1.35) once again responded with statistically significant decreases in the percentage of time spent rearing in the OFT compared to both the D rats (11.19% ± 1.07) ($p<0.001$) and the DS rats (9.57% ± 0.83) ($p<0.001$) also housed in high-density cages. SS rats in the high-density cages also responded with decreased percentage of time rearing compared to both D rats ($p<0.001$) and DS rat (p = 0.042). Finally, D rats housed in the low-density cages (3.66% ± 1.39) responded with a statistically significant decrease in the percentage of time rearing compared to D rats in the high-density cages ($p<0.001$). (Fig 12C).

Effects of space allocation and social class on the open-field test

A two-way MANOVA was performed fitting space allocation and social class against the percentage of time the animal spent in each of the open field testing 'zones' as well as the percentage of time spent defecating/urinating, rearing and being inactive. The data for the percentage of time spent defecating/urinating was found to be non-parametric. Consequently, a Kruskal-Wallis H test was performed which revealed no significant effects or interactions between space allocation $\chi^2(1) = 0.042$, p = 0.838, and social class $\chi^2(1) = 3.101$, p = 0.078, on the percentage of time spent defecating/urinating in the open field test, and therefore this data has been omitted.

There was a significant interaction as observed from the two-way MANOVA of space allocation and social class on the percentage of time the rats spent in the peripheral zone, F (1,33) = 7.725, p = 0.009. Simple main effects analysis was employed to investigate this interaction. D rats in the large cages (56.74% ± 4.03) responded with a significantly reduced percentage of time in the peripheral zone compared to the S rats in the large cages (75.93% ± 4.03) ($p<0.001$) (Fig 13A).

There was another significant interaction between space allocation and social class on the percentage of time the rats spent in the inner zone, F (1,33) = 9.410, p = 0.004. Simple main effects analysis identified that S rats in the large cages (10.74% ± 2.42) responded with a significantly reduced percentage of time in the inner zone compared to D rats in the large cages (20.53% ± 2.41) (p = 0.001). S rats in the large cages also responded with a significantly reduced percentage compared to S rats in the small cages (22.53% ± 2.61) (p = 0.009) (Fig 13B).

The final significant interaction between space allocation and social class was on the percentage of time the animal spent in the centre zone, F (1,33) = 8.907, p = 0.005. Simple main effects analysis was used to investigate this interaction and showed that the S rats in the large cages (2.73% ± 1.5) responded with a significantly reduced percentage of time in the centre zone compared to the D rats in the large cages (11.36% ± 1.5) ($p<0.001$) (Fig 13C).

Discussion

The study aimed to investigate the effects of housing density and space allocation on the behavioural performance of rats. No significant effects of housing density, and only one significant effect of space allocation was observed. There were several interactions between these factors and social status. Animal performance in the open-field [20], novel-object recognition [21] and social-interaction tests [18] are all highly repeated methods to observe anxiety-like behaviours in the rat. As hypothesised, subordinate animals regularly responded in these behavioural tests with significantly higher numbers of behaviours considered anxiety-like. Subordinate animals responded with significantly fewer optimistic decisions compared to their more highly ranked conspecifics. These significant effects and interactions indicated that cage size and

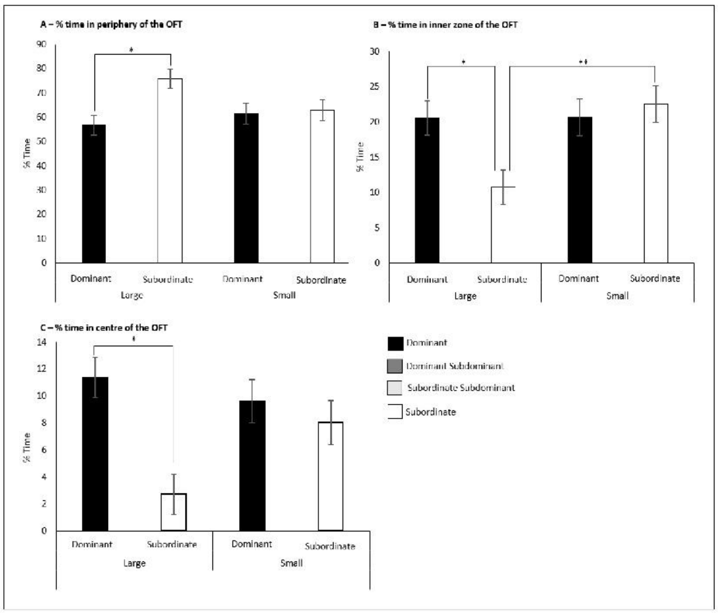

Fig 13. Results of the open field test for the space allocation experiment. A) The interaction of space allocation and social class on the percentage of time spent in the peripheral zone of the OFT. B) The interaction of space allocation and social class on the percentage of time spent in the inner zone of the OFT. C) The interaction of space allocation and social class on the percentage of time spent in the centre zone of the OFT. Significance is denoted at $p < 0.05$.

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social structure could potentially play a significantly greater role in the minimisation of social stressors than previously identified.

Subordinate status is a source of anxiety in group-housed male rats

For both the housing density and space allocation experiments subordinate and subordinate-subdominant animals routinely responded with greater numbers of behaviours associated with an anxiogenic state. A reduced expression of optimistic cognitive biases (Fig 6A and 6B) has been associated with anxiety-like states in multiple animal species [13]. Subordinate animals demonstrated a decrease in the percentage of time spent following the unfamiliar rat in the SIT (Fig 8B) in both experiments, and a decreased percentage of time investigating the unfamiliar rat (Fig 9). A decrease in social investigatory behaviours has been reviewed and is associated with conditions known to cause anxiogenic responses [22]. Finally, a decrease in the amount of time an animal spent in the centre zones of the OFT (Fig 12B and 12C) was associated with anxiety and/or depression like conditions [20].

Subordination has been correlated with significant physiological changes, including decreases in bodyweight [23], reductions in dopamine activity of the brain [24], and teste weight, and decreased plasma testosterone and corticosterone levels [25]; changes consistent with chronic stress responses. Behavioural adaptions have also been associated with social class, with dominant animals spending significantly more time in the open arms of an elevated plus maze compared to subordinates [26]. Subordinate rats housed with a dominant conspecific also responded with an increase in the number of ultrasonic vocalisations (response to aversive stimuli) being emitted and displayed greater freezing responses than dominant conspecifics [27].

The results of the current study suggest that social status of rats can be a reliable cause of anxiety. Whilst there were several interactions of either housing density or space allocation with social status, there was no significant effect of housing density alone on any of the observed parameters and only one significant effect of space allocation (Fig 10). These interactions are discussed in more detail below. Investigations into housing density and space allocation on animal welfare are often confounded. The current study provides evidence that social status needs to be considered as an independent variable when studying the effects of either housing density, space allocation or the effects of crowding.

Subordinate stress varies with housing density

The results suggested that the social stressors associated with subordination were not as severe in the low-density cages compared to the high-density cages. Subordinate animals of the high-density cages responded with a significantly decreased percentage of time investigating the unfamiliar rat in the SIT (Fig 7) and a decreased percentage of time interacting with the novel-object in the NORT (Fig 11). Reduced novel-object recognition is a sign of cognitive impairment in animals suffering from chronic stress [21]. In addition, subordinate animals of the high-density cages responded with significantly greater anxiety-like responses to the OFT (Fig 12A–12C) Previous study findings conducted in mice [28–30] and rats [10] support this observation in which aggression was intensified by increasing group size. In the current study, subordinate animals in the high-density cages experienced greater numbers of aggressive acts initiated upon them compared to the subordinates of the low-density cages. However, these discussed studies did not separate space allocation (surface area per animal) and housing density (animals per cage) this is discussed in greater detail later.

Studies that successfully decoupled space allocation from housing density have reported that aggressive encounters increased in larger groups of caged mice [31]. Another study reported that the number of aggressive encounters between the dominant and subordinate of highly dense cages (8 animals per cage) were significantly greater than animals housed in low density cages (3 animals per cage) [32]. A larger population size encourages the dominant animal to display greater levels of aggressive behaviour to sustain its dominant status. Meanwhile, subordinate animals of the high-density cages showed increased aggressive behaviours to possibly earn a higher social status within the hierarchy [32]. As summarised by Poole and Morgan [29] the greater the population size per cage, the more unstable the hierarchy, increasing the likelihood that dominance status would change between individuals.

Most previous studies have investigated mice, and given inherent species differences, it could be argued that these studies have limited relevance to the current study. However, many common behavioural tests of anxiety [33], learned helplessness [34] and general cognitive ability [35] report mouse and rat behaviour as being ‘equivalent’. This suggests that comparisons between the two species are valid when using tests to identify an anxiety or depressive like state. Therefore, while we cannot state that the subordinate stress experienced by the rats in the current study was equivalent to mice, or that the stress was caused in the same mechanistic manner, we can confidently state that the behavioural tests we employed to detect subordinate stress were appropriate.

The current study has shown that larger group sizes of rats lead to increases in the number of anxiety-like behaviours expressed by subordinate rats, and an overall increase in social stressors. This finding has been reported in mice, but has yet to be reported in rats. This encourages future research to focus on understanding the mechanisms underlying subordination stress in rats. Housing recommendations for lab animals are currently based on weight, with few guidelines based on experimental observations of appropriate group size. The findings of the current study should therefore be considered in future guidelines and legislative drafting.

Subordinate stress varies with space allocation

As illustrated in Figs 6B and 13A–13C, subordinate animals in the large cages responded with significantly more anxiety-like behaviours compared to subordinate animals in the small cages. Furthermore, there were no significant differences observed at all, between dominant and subordinate animals in the small cages compared to those in the large cages. This suggested that subordinate stress was amplified with a larger area which the subordinate shared with the dominant. Guidelines and legislation tend to promote larger cages on the belief that an increased space allocation reduces crowding stress [3]. However, as discussed previously, this may stem from a failing of much peer-reviewed literature to successfully separate housing density and space allocation.

When provided with different cage sizes, rats would preferentially choose the larger cage (1620cm^2 of usable floor space) that housed four other rats over a small cage (540cm^2 of useable floor space), despite the larger cage providing less physical space for the rat to occupy than the small cage [36]. This suggested that rats preferentially chose conditions with greater crowding stressors than lone housing with a greater surface area allowance. Monogamous breeding pairs of Dahl salt-sensitive rats housed in small cages (922.6cm^2 of useable floor space) showed no significant differences in breeding parameters compared to similar pairs housed in larger cages (1355cm^2 of useable floor space) [37]. The 3rd edition of the *Guide for the Care and Use of Agricultural Animals in Research and Testing* [38] states that reproductive parameters are an important indicator of animal welfare, suggesting that the use of smaller rat cages for breeding purposes is acceptable, despite it being considered 'over-crowded'.

Crowding is often confounded with housing density, as an increase in housing density invariably leads to a crowding effect. Density has been defined as the number of animals occupying the same floor area. Crowding is defined as the motivational state that occurs when spatial and social factors interact, which influences behaviour to mitigate the effects of the restricted space [9]. The current study demonstrated that the larger the space allocation per animal, the greater the effect of the stressors associated with subordination. Van Loo, Mol [32] found both dominant and subordinate male mice housed in a small cage responded with fewer acts of aggression compared to those in larger cages. These authors concluded that a small cage was associated with dominant animals having a smaller defendable territory that reduced the number of aggressive acts needed to maintain control over this territory [32]. Therefore, it was hypothesised for the current study that the subordinate rats in the larger cages were subjected to more acts of aggression from their dominant cage-mates compared to the subordinate rats of the small cages.

This discussion highlights the need to consider the effects of crowding stress versus subordination stress, and future research should identify if the stressors from crowding produce more anxiety-like responses than subordination stress. Whilst increasing the surface area per animal will reduce the stressors associated with crowding, it may in turn increase the stressors associated with subordination.

Social class and the effects of motivation in the judgment bias paradigm

Subordinate-subdominant and subordinate rats responded with fewer optimistic decisions then both the dominant and dominant-subdominant rats, when exposed to the JBP for both the housing density (Fig 6A) and space allocation experiments (Fig 6B). Likewise, these rats responded with increased time exploring the confines of the SIT (Fig 8A) and spent less time following the unfamiliar rat (Fig 8B) during the housing density experiment, signs previously associated with compromised sociability [39]. This suggested that an individual animal having a lower social rank not only encouraged pessimistic cognitive biases, but also encouraged social dissonance. A similar finding of sociability on cognitive bias expression reported that bottlenose dolphins that displayed more social affiliative behaviours (synchronous swimming) responded with greater numbers of optimistic decisions to a JBP [40]. This may provide an explanation as to why subordinate animals, which are the subjects of more acts of aggression and therefore fewer social affiliative behaviours, responded with significantly fewer optimistic decisions. We hypothesised that dominant animals experience a more harmonious social standing and therefore experience fewer anxiety-like effects associated with group housing. As discussed previously, large group sizes encourage animals of lower social standing to challenge other low ranked animals in order to gain a higher social status [32]. This finding is important as it suggests that animal status in a social hierarchy is an important covariate that needs to be taken into consideration when assessing animals on their cognitive bias expression using a JBP.

Dominance has also been correlated with an increased motivation for food reward, when housed in the visible burrow system (VBS), a model in which unfamiliar rats form dominance hierarchies. Dominant rats have been reported to respond to a palatable food reward with an increase in operant responses [26]. Therefore, it could be argued that a dominant social status in rats can significantly augment their ability to respond to palatable food rewards. Rats experiencing chronic social-stress have also shown reduced motivation-related behaviours. Using an indirect marker of dopamine activity (dopamine transporter binding density), non-responsive subordinate rats displayed long-lasting (3-week) changes in their mesolimbic dopaminergic system after experiencing a chronic-social stressor (VBS housing) [24]. This is significant when discussing the JBP as used in the current study, which relied on the motivating factor of food that encouraged the expression of a cognitive bias. Dominant animals have an innate increased motivation to attain a food reward, whilst subordinates experience physiological changes that decrease their ability to be 'rewarded'. This implies that animals experiencing chronic-social stressors are less-motivated to perform reward-motivated behaviours, as they no longer receive "pleasure" by doing so.

If dominance status and subordination stress significantly alter behaviour [24] and food-motivation [26], then a food rewarded JBP to assess affective state of group-housed rats was a significant limitation of the study. Food-motivation has been established as a confounding variable when discussing animal behaviour in general [41, 42]. The possibility that coupling of food-motivation and social status acts as a confounding factor in the JBP is a novel theory. This suggestion renders many studies involving group-housed animals and a food-rewarded JBP confounded unless social status is included as a covariate in design. Logically, future work should then avoid a JBP that relies on animal motivation to food. This may prove difficult as the majority of previous JBP designs have utilised the presence or absence of food as the motivating factor [43]. Few JBP studies have reported success using location or social based rewards as the motivator to express a cognitive bias [44]. These tests may prove superior and should receive attention in future research.

Conclusion

This study used an array of behavioural tests to explore the effects of housing density and space allocation on common laboratory rats. We were unable to confirm the hypothesis that increased housing density or a decreased space allocation would result in increased numbers of anxiety-like behaviours. However, we confirmed the hypothesis that subordinate rats would respond with greater anxiety-like behavioural traits compared to dominant rats. Furthermore, it was concluded that subordination stress in rats could be exacerbated by housing a greater number of rats in the same cage and by providing a greater surface area per animal. These findings are novel, being the first to successfully dissociate the commonly confused factors of space allocation and housing density in rats. Future work should include treatment groups of variable densities than those utilised in the current study, and a greater range of differently sized, commercially available cages.

Furthermore, the continued use of a reward-based JBP to assess the affective state in at least a group-housed rat model is discouraged. The combined factors of motivation and status within the social hierarchy can significantly augment behavioural expression of the rat. Future studies using a JBP in group-housed animal models should consider controlling for the social status of the animals.

This study has challenged the notion that rats have a greater standard of welfare when housed in larger cages, with more surface area per animal, a common presumption of rodent housing guidelines. An increased surface area does lead to a decrease in the negative effects associated with crowding. However, increasing the surface area also encourages the prevalence of anxiety-like behaviours associated with subordination. Therefore, simply increasing the surface area per rat may not lead to increased animal wellbeing. Furthermore, even when rats are housed with an approximately equivalent floor area per animal, those housed with more conspecifics experience greater levels of social stressors than those housed with a single cage-mate. Therefore, the data encourages the drafting of guidelines and legislative documents that do not simply increase the surface area of cages in which animals can be legally housed. Consideration of other factors such as cage complexity, housing density and social status will provide a higher standard of welfare for caged laboratory rats.

Supporting information

S1 File. Raw behavioural data used in the analysis. Data is presented as one excel spreadsheet. data has been separated into sheets by experiment (housing density or space allocation) and by behavioural test.
(XLSX)

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CHAPTER 5.

Imposed subordination in female rats impedes learning as determined by the judgment bias test.

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5.1 Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	**Timothy Hugh Barker**		
Contribution to the Paper	Experimental design and procedure. Statistical analysis. Wrote manuscript. Acted as corresponding author.		
Overall percentage (%)	80		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

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

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	**Gordon Stanley Howarth**		
Contribution to the Paper	Principal supervisor Provided guidance on content and structure of manuscript Editing of manuscript		
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Name of Co-Author	**Alexandra Louise Whittaker**		
Contribution to the Paper	Principal supervisor. Assisted with experimental design and procedures. Provided guidance on content and structure of manuscript. Editing of manuscript.		
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5.2. Statement of Context

The study presented in Chapter 5 is a short communication resubmitted for publication following revision, that details work conducted in tandem with the published study presented in Chapter 6, using the same cohort of female animals.

As social status was identified as a significant modifier of affective state in male rats, this factor was studied using a female cohort (presented in Chapter 6). However, whilst animals were being trained on the JBT, as per methods detailed in section 1.3, it was considered that social stressors associated with subordination may also negatively impact on learning rates of the animals. Therefore, this study was designed to assess how hierarchal rank and stressors associated with having low hierarchal rank affect animal learning on the JBT. This study was the first applied study conducted during candidature to specifically identify a common hindrance of judgement bias testing, being extended and often impractical training times.

5.3. Abstract

Legislative direction has encouraged the standard laboratory practice of group-housing rats used for scientific purposes. It has been demonstrated that this type of housing causes subordinate animals to be exposed to chronic psychosocial stressors through imposed subordination, with resultant induction of anxiety-like behaviours. Despite previous studies documenting the negative effects of stress on learning, there has been relatively little attention given to the effects of imposed subordination on animal learning. The aim of this study therefore, was to assess the effects of social stress through imposed subordination on rat learning aptitude. Twenty, female, Sprague-Dawley rats were exposed to three training trials of a commonly employed judgment bias test. The results showed that dominant animals took significantly fewer days (42.50 ± 5.15) to learn the training criteria than their subordinate-subdominant (68.60 ± 4.61) ($p = 0.003$) and subordinate cage-mates (64.60 ± 4.61) ($p = 0.015$). This implied that subordination, as imposed by standard group-housing could impede the ability of subordinate animals to learn. In conclusion, at least in group-housed female rats, researchers should modify experimental design to account for social status when learning parameters are a critical study outcome.

Keywords

Judgment Bias Test, Cognitive Bias, Psychosocial Stress, Animal Learning, Imposed Subordination

5.4. Introduction

Detection of cognitive biases has become a commonly employed measure of rodent affective state. As primarily observed using a judgment bias paradigm, the judgment of rodents to ambiguity can be objectively categorised as either optimistic or pessimistic, with optimism and optimistic tendencies associated with positive affect (Mendl et al., 2009). However, a major limiting factor for the practical use of common judgment bias tests are the significant training times required (Brydges and Hall, 2017). In addition, for a judgment bias test to avoid common confounding factors such as motivation or response latency, an active choice design is preferable (Bethell, 2015; Roelofs et al., 2016; Barker et al., 2018). However, active choice judgment bias paradigms have inherently longer training times than go/no-go paradigms (Bateson and Matheson, 2007; Brilot et al., 2010)

Psychosocial stressors associated with subordination can also significantly impact on the behavioural response to a judgment bias paradigm (Papciak et al., 2013; Barker et al., 2017b). Subordination in mice has been linked with an impairment in general cognitive ability, including the ability to learn (Colas-Zelin et al., 2012). Subordinate mice also displayed impaired spatial learning on a T-maze (Fitchett et al., 2005). The effects of stress on learning are extremely varied and often contested in the scientific community, with stress being reported as both facilitative and aversive to learning (Joëls et al., 2006). Studies investigating the effects of psychosocial stress on learning ability in rodents have yielded variable results (Colas-Zelin et al., 2012). This variation could be due to the differences between imposed and innate subordination, and how these subordination archetypes impact on the different forms of learning and memory employed by the rodent. Furthermore, to date, only a single study has investigated the specific effects of imposed subordination (Colas-Zelin et al., 2012); imposed subordination being defined as the psychosocial stress experienced by a subordinate animal caused by being continuously housed with the same dominant cage-mates. Many studies have imposed psychosocial stress in rats through constantly changing cage-mates (Touyarot et al., 2004; Alzoubi et al., 2009) or social defeat through learned helplessness in the resident-intruder paradigm (Buwalda et al., 2005). However, imposed subordination presents a scenario that is likely to occur in all group-housed rats used for scientific purposes and therefore is argued to be a much more applicable and relevant stressor to study. In addition, of the aforementioned studies, no female animals were investigated. Therefore, the effects of social-stress on learning aptitude in a female, group-housed cohort is critically under-studied. The current study therefore aimed to identify the effects of imposed subordination on ability to learn a commonly employed judgment bias testing protocol in group-housed, female rats. This experiment was also the first to utilise a frequently employed training protocol for use as a learning metric; the judgment bias test. It was hypothesised that rats identified to be subordinate would take a significantly greater number of days to complete the training protocol compared to their dominant cage-mates.

5.5. Materials and Methods

5.5.1. Ethics Statement

Animal use, housing protocols and experimental design were approved by the Animal Ethics Committee of the University of Adelaide and conducted in accordance with the provisions of the *Australian Code for the Care and Use of Animals for Scientific Purposes.*

5.5.2. Animals and Housing

Twenty female, hsd: Sprague Dawley rats were used in this study. Animals were sourced from a barrier-maintained, specific pathogen free production facility (The University of Adelaide, Laboratory Animal Services, Adelaide, Australia) at 3 weeks of age. These animals were utilised for an additional study performed by this lab. As such, only the training data of the animals has been presented. Upon arrival in the facility, animals were housed in groups of four, in the commercial Eurostandard type IV cage (Techiplast, Exton, PA, USA). Each cage was provided with a paper-based bedding substrate (Animal Bedding, Fibrecycle Pty Ltd, Yatala, Queensland, Australia) and shredded paper material for nesting. Enrichment was provided in the form of PVC pipes loose on the floor of the cage, and affixed to the cage roof and walls. Chewing objects were also available (Nylabone Products, NJ, USA). Rat chow (Rat and Mouse Cubes, Speciality Feeds, Western Australia, Australia) and water (reverse-osmosis purified) were freely available in each cage. The facility maintained an internal temperature of 21-23°C and lighting was on a reversed 12-hour light/dark cycle (on at 1800, off at 0600). All training procedures took place during the dark photoperiod under red-light.

5.5.3. Judgment Bias Training

The training apparatus used was identical to that designed by Brydges et al. (2011) and comprised two Perspex boxes connected via an 80cm PVC pipe. The training methods employed were identical to those presented in Barker et al. (2017b). In summation, the ‘goal’ box contained a brown bowl filled with coriander scented sand (1% spice to weight of sand) in the far-right hand corner, and a blue bowl filled with cinnamon scented sand in the left-hand corner. The pipe connecting the two boxes was lined with either a coarse (P80) or fine (P1200) sandpaper, which acted as the training cues. Milk chocolate baking chips (Cadbury, London, England) and Cheerios (UncleToby’s, Victoria, Australia) were utilised as the positive and less-positive reward items, respectively. Animals were randomly assigned an association, such that sandpaper type was paired with reward type (chocolate or Cheerio) and reward location (blue or brown bowl). These associations were counter-balanced. Each animal was trained on the judgment bias paradigm as described in the methods of Barker et al. (2016), however only three training phases were utilised. Every animal experienced four training trials per day; two with the positive reward and corresponding sandpaper, and two with the less-positive reward and corresponding sandpaper.

5.5.3.1 Phase 1

In phase 1, a single reward item was placed on the surface of the sand in the appropriately corresponding bowl. The sandpaper present in the pipe was appropriate according to the association of the tested rat. The rat was placed into the start box and a timer started. Every rat was given five-minutes to find and consume the reward item. If the animal did not consume the reward item, the trial was considered a failure. The animal was then removed from the apparatus which was subsequently cleaned with a 70% ethanol solution. If the animal approached the correct (reward containing) bowl first, then that trial was considered a success. The animal eventually learnt that the sandpaper present in the pipe indicated the reward present, and thus the reward-containing bowl was approached first. If a rat was successful in three of its four daily trials, that day was considered successful. Every animal was required to complete five successful days of training before it could be promoted to phase 2.

5.5.3.2. Phase 2

Phase 2 was similar to phase 1, however the reward was buried into the sand of the appropriate bowl For description and burial depth guide see Barker et al. (2017a). To be promoted to phase 3, rats were required to both approach and forage in the correct bowl first, within the five-minute allotment. All other promotion criteria remained identical to phase 1.

5.5.3.3. Phase 3

Phase 3 was similar to phase 2, however one trial chosen randomly went unrewarded. This trial was included to observe the effects, if any, that an absence of reward item had on the ability of the rat to perform the test. Completion of phase 3 was recorded when a rat had achieved five successful days in a row.

5.5.4. Social Status Identification

Identification of the social hierarchy of the rats was performed as per the methods of (Barker et al., 2017b). CCTV cameras (OzSpy, Brisbane, Australia) were set-up to record the home cage behaviour of each rat. Recordings were taken from 1400-1800 hours for a 5-day period during the final phase 3 training. Of every recording made, each animal was observed over a 10-minute period, on each of the 5-day viewing periods. The start time of the 10-minute viewing window was randomly selected between 1400-1750 hours. Behaviours were recorded using a continuous sampling method using the ethogram and methods as described by Hurst et al. (1996). Social classification was then determined as described in the methods of Barker et al. (2017b). Due to this classification technique, not every social class was represented in each cage. This is expanded on in the discussion.

5.5.5. Statistical Analysis

All statistical analyses were conducted in the IBM SPSS Statistics 22 (IBM, NY, USA) software package. Levene’s test was used to test for normality of the data set. Significance was assumed when $p < 0.05$. All data were found to be normally distributed ($p < 0.05$ at each level) and reported henceforth as mean (number of days to learn) ± standard error of the mean. Since data were parametric, they were analysed using a two-way ANOVA fitting the phase of training and the social status of the animals on the mean number of days (d) taken to reach promotion criteria.

5.6. Results

Levene's test indicated that variances for every training phases were equal, Phase 1 (F = 0.46, $p = 0.71$); Phase 2 (F = 0.30, $p = 0.83$); Phase 3 (F = 1.34, $p = 0.29$). Analysis of variance showed significant main effects for both phase of training, F (2,48) = 12.052, $p < 0.001$, and social status F (3,48) = 5.33, $p = 0.003$. There was no significant interaction between training phase and social status F (6,48) = 0.38, $p = 0.89$. This data has therefore been presented separately. Post-hoc tests using the Bonferroni adjustment revealed that the mean number of days required to complete phase 1 of training were significantly greater (24.91 ± 1.58d) then the days taken to complete both phases 2 (19.2 ± 1.50d, $p = 0.012$) and 3 (15.60 ± 1.05d, $p < 0.001$). No significance was detected between the number of days taken to complete training for phases 2 and 3, $p = 0.39$. (Figure 1).

Further post-hoc analysis using the Bonferroni adjustment identified that dominant animals (n=4) took a significantly reduced mean number of days to complete all training phases (42.50 ± 5.15d) compared to both subordinate subdominant animals (n=5) (68.60 ± 4.61d) ($p = 0.003$) and subordinate animals (n=5) (64.60 ± 4.61d) ($p = 0.015$). No significance was detected between dominant subdominant animals (n=6) (59.67 ± 4.21d) and any other social status (dominant, $p = 0.78$; subordinate subdominant, $p = 0.95$; subordinate, $p = 1.0$). No significance was detected between subordinate subdominant animals and subordinate animals, $p = 1.0$. (Figure 2).

5.7. Discussion

A stressful stimulus evokes a physiological response in the rat. This response allows rats to adapt accordingly to the new stimulus resulting in the adoption of different cognitive strategies (Starcke and Brand, 2012). Decision making (Starcke and Brand, 2012), and learning (Joëls et al.,

2006) are two such cognitive factors that have been identified to significantly change under the effects of stressful stimuli. These cognitive functions are both critical in the training paradigm of the judgment bias test. As hypothesised, subordinate, and subdominant subordinate rats both took significantly longer to learn the training paradigm than their dominant cage-mates (figure 2).

Rats identified to be subordinate using this behavioural ethogram have previously shown significant anxiety-like behaviours in the open-field, social-interaction, novel-object recognition and judgment bias tests (Barker et al., 2017). These behavioural tests are all highly repeated methods used to assess a stress response in rats (Walsh and Cummins, 1976; Sams-Dodd, 1995; Antunes and Biala, 2012; Barker et al. 2016). Therefore, we hypothesise that the subordinate rats in the current study experienced a stress response due to this subordination. This subordination stress is suggested to have negatively impacted on the ability of these rats to learn the judgment bias paradigm. In the only known previous study of imposed subordination in rats, subordinate males responded with impaired learning, as evidenced by increased step-down latencies in the passive avoidance task (Colas-Zelin et al., 2012), and impairment in the animal’s general cognitive ability. Subordinate male rats have also responded with increased displays of neophobia in the novel-object recognition test, a common test to identify cognitive deficits (Barker et al., 2017b). These findings highlight that psychosocial stress as derived from subordination causes significant impairment to the ability of rats to learn simple spatial tasks. A possible mechanism for this impairment could be due to corticosterone release as a result of the imposed subordination. Male Long-Evans rats treated subcutaneously with corticosterone to produce a concentration sufficient to mimic the conditions of mild stress, demonstrated impaired learning in a Morris water maze (Bodnoff et al., 1995). Similarly, when tested on the rat Iowa Gambling Task (Rivalan et al., 2011) rats responded with impaired decision making after experiencing a corticosterone injection directly into the infralimbic cortex (Koot et al., 2014). The current study did not include a measure of stress response such as corticosterone. Whilst this is a limitation, invasive collections of plasma corticosterone were deliberately withheld. Non-invasive collection methods, such a faeces examination (Barker et al. 2017a) were also unavailable since interpretation is challenging in the group-housed scenario critical to the study design. It could be theorised that dominant animals were in fact more stressed than their subordinate cage-mates; with this stress facilitating learning on the judgment bias paradigm. Whilst this is a possibility, previous investigations that studied dyadic interactions between rats to identify social hierarchy, do not suggest that dominant animals experience a greater psychosocial stress than subordinates (Popova and Naumenko, 1972; Militzer and Reinhard, 1982; Hurst et al., 1996). In order to fully characterise the true association of social status with stress, further study utilising a range of behavioural and physiological measures of the 'stress response' would need to be performed. Previous studies conducted in rats have identified similar findings to the current study. Psychosocial stress as imposed through the daily changing of cage-mates, led to impaired learning in a spatial water-maze test in males (Touyarot et al., 2004; Alzoubi et al., 2009). Whilst these studies did not test imposed subordination as in the present study, the mechanism of effect is argued to be caused in similar ways. The disruption of an established social hierarchy results in increased aggressive behaviours to re-establish a new hierarchy (Burman et al., 2008). However, even in stable hierarchies, low-ranked animals challenge other lowly-ranked cage-mates in order to gain a higher social standing (Van Loo et al., 2001). This also explains why not every social class was represented equally in each cage. Therefore, whilst these caged hierarchies remain 'stable', the subordinate animals of these cages are still exposed to increased aggressive acts. The psychosocial stress associated with the daily changing of cage-mates could be argued to result from the increased number of aggressive behaviours exhibited to re-establish the dominance hierarchy. Subordinate animals in these environments are therefore the subjects of increased aggressive acts, thus causing a psychosocial stress similar to imposed subordination as described in the current study.

The current study is the first to identify the effects of imposed subordination on the ability of rats to learn a simple, commonly employed training paradigm. Imposed subordination is a significant issue since it is commonplace to house rats for scientific purposes in groups, in accordance with legislative direction (*The Guide for the Care and Use of Lab Animals*, 2011). However, these housing conditions, whilst still superior over single housing (Barker et al. 2017b), do create significant psychosocial stress to subordinates housed with dominant cage mates. The findings of the current study therefore encourage future studies to account for this social rank in the analysis of data particularly when conducting research related to cognitive impairment or learning. The results also indicate that subordination stress may play a role in extending the training times associated with judgment bias testing. One potential solution to overcome this issue could be the use of other affective state detection methods that are not burdened with training times such as attention biases (Bethell et al., 2012; Brilot and Bateson, 2012; Lee et al., 2016), or by using a judgment bias paradigm with fewer spatial components (Rygula et al., 2015).

Conflict of interest statement

The authors declare that no conflicts of interest exist. This research was funded by the University of Adelaide.

Authorship statement

The idea for the paper was conceived by Timothy Hugh Barker, Gordon Stanley Howarth and Alexandra Louise Whittaker

The experiments were designed by Timothy Hugh Barker and Alexandra Louise Whittaker

The experiments were performed by Timothy Hugh Barker

The data was analysed by Timothy Hugh Barker

The paper was written by Timothy Hugh Barker, Gordon Stanley Howarth and Alexandra Louise Whittaker.

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HAPTER 6.

Oestrous phase cyclicity influences judgment biasing in rats

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Co-Author Contributions

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

iv. the candidate's stated contribution to the publication is accurate (as detailed above);

v. permission is granted for the candidate in include the publication in the thesis; and

vi. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	**Karen Lee Kind**		
Contribution to the Paper	Provided guidance on content and structure of manuscript Assisted with oestrous cycle detection		
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Name of Co-Author	**Peta Danielle Groves**		
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Name of Co-Author	**Gordon Stanley Howarth**		
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Contribution to the Paper	Principal supervisor. Assisted with experimental design and procedures. Provided guidance on content and structure of manuscript. Editing of manuscript.		
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6.2. Statement of Context

The exploratory study described in Chapter 3 discussed that oestrous phase cyclicity and associated changes in hormonal concentrations could be a significant determining factor of judgement biases in the female rat cohort. In addition, the study presented in Chapter 4 identified that having low social hierarchal rank is a significant modifier of judgement biases in male rats. These factors, or an interaction between social status and oestrous phase, could provide explanation as to why the female response was significantly more variable than the response of males.

Chapter 6 presents a study designed to identify the effects that oestrous phase and social status may have on judgement bias expression of female rats. An interaction between oestrous phase and social stress had been previously identified in female mice (Palanza et al., 2001), suggesting that oestrous phase may modulate social stress. This presented the opportunity to identify if this relationship existed and if such a relationship would encourage presentation of specific judgements in the judgement bias test.

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Oestrous phase cyclicity influences judgment biasing in rats

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Anxiety

ABSTRACT

The identification of cognitive bias has become an important measure of animal welfare. Negative cognitive biases develop from a tendency for animals to process novel information pessimistically. Judgment-bias testing is the commonplace methodology to detect cognitive biases. However, concerns with these methods have been frequently-reported; one of which being the discrepancy between male and female cognitive expression. The current study assessed the factors of social status and oestrus, to investigate whether oestrous cycle rotation, or subordination stress encouraged an increase in pessimistic responses. Female Sprague-Dawley rats (n = 24) were trained on an active-choice judgment bias paradigm. Responses to the ambiguous probe were recorded as optimistic or pessimistic. Oestrous phase was determined by assessing vaginal cytology in stained vaginal cell smears. Rats in the dioestrous phase and those rats considered to be subordinate demonstrated an increased percentage of pessimistic responses. However, no interaction between these factors was observed. This suggests that oestrous cyclicity can influence the judgment biases of female animals; a previously unreported finding. On this basis, researchers should be encouraged to account for both oestrous phase cyclicity and social status as an additional fixed effect in study design.

1. Introduction

Evaluation of the affective experience has become a critical component in welfare assessment. Positive affective (emotional) states are now considered to be associated with improved animal welfare, whilst negative affect is often associated with poor welfare standards (Yeates and Main, 2008). Pre-clinical studies that use animal models of affective disorders have also been argued to greatly benefit from the increased understanding of positive affective states such as contentment, and the differences between negative affective states such as anxiety and depression (Panksepp, 2015). However, accurately determining animal affect relies on proxy measures of animal behaviour. The most common technique employed to observe changes in animal affect has been identification of a cognitive bias, in particular, judgment biases (Mendl et al., 2009; Bethell, 2015). Judgment bias testing has been developed based on insight into human psychology, where subjects in negative affective states (anxiety) would make pessimistic judgments more often than control (positive affect) subjects (Amir et al., 2005). This testing paradigm was first employed in an animal model by Harding et al. (2004) where it was demonstrated that animals suffering mild depression (negative affect) would respond with more pessimistic judgments to ambiguity than animals with positive affect. Since this foundational study, the judgment bias test has been employed in a wide-range of animal species, successfully identifying that pessimistic judgment biases are commonly associated with negative affect (Mendl et al., 2009; Bethell, 2015).

As discussed in Brown et al. (2016), much of the previous research on animal performance on a judgment bias test has focused on either a male or female subject. Few studies have controlled for both sexes in experimental design and statistical analysis (Briefer and McElligott, 2013; Asher et al., 2016; Barker et al., 2016; Brown et al., 2016; Carreras et al., 2016; Takeshita and Sato, 2016; Barker et al., 2017a; Roelofs et al., 2017). Whilst some studies have reported no difference between male and female cognitive expression (Asher et al., 2016; Carreras et al., 2016; Roelofs et al., 2017), discrepancies have been reported (Briefer and McElligott, 2013; Barker et al., 2016; Brown et al., 2016; Takeshita and Sato, 2016; Barker et al., 2017a). Of these studies in which a difference was reported, female animals responded with greater numbers of optimistic biases after experiencing a stressful treatment compared to males (Briefer and McElligott, 2013; Barker et al., 2016). However, a later study by Barker et al. (2017a), with increased statistical power, identified that females responded similarly to males after experiencing the stressful treatment, but responded with fewer optimistic biases in the control treatment (Barker et al., 2017a).

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Alternately, female Lister-hooded rats displayed decreased latencies to respond to ambiguous locations in a spatial judgment bias test compared to rewarded locations, whilst males recorded no difference, indicating optimism (Brown et al., 2016). Differences have also been observed in non-mammalian cognitive bias studies; compared to males, female Japanese pygmy squid (*Idiosepius paradoxus)* are more likely to make continuous pessimistic decisions following a failure to be rewarded after displaying behaviours that would normally go rewarded (Takeshita and Sato, 2016). Whilst scientific consensus as to the difference between male and female performance in judgment bias testing has yet to be reached, it is apparent that male and female performance is not always equivalent, with female performance being recorded as seemingly more variable, than that of males (Barker et al., 2017a). This presents practical issues for the continued use of the judgment bias technique as a tool for welfare assessment. As reviewed by Girbovan and Plamondon (2013), at least in rodents, there is a need to include sex as a predictor variable in the analysis of data collected from both males and females. An understanding of the factors contributing towards differences in male and female bias expression would improve utility of the judgment bias test.

One suggested reason for this observed sex difference in rat cognitive bias expression is oestrous cyclicity. The rat oestrous cycle lasts 4–6 days and is comprised of 4 unique phases (proestrus, oestrus, metoestrus and dioestrus), each of which can be categorised by the vaginal cell types present and the expression of different ovarian hormone concentrations (Goldman et al., 2007; Paccola et al., 2013; Levine, 2015). The influence of these ovarian hormones has been strongly correlated with variations in rodent behaviour (Kastenberger et al., 2012) with relevance observed in both female rats (Agrati et al., 2005; Devall et al., 2009) and mice (Plappert et al., 2005; Walf et al., 2008). Female rats in the dioestrous phase have responded with reduced exploratory behaviours, a common behavioural sign associated with anxiety, as observed through behavioural tests such as the elevated plus maze (Marcondes et al., 2001) and open-field test (Devall et al., 2009). Meanwhile females in the proestrous phase have responded with behaviours described as being anti-anxiety, social, exploratory and sexual (Frye et al., 2000) as indicated by their responses to the open-field and elevated plus maze (Frye et al., 2000), novel-object recognition (Walf et al., 2006) and forced swim tests (Walf et al., 2008). These behaviours promote greater sociability and exploration during the proestrous stage (Frye et al., 2000), and as females are approaching the sexually receptive stage of the cycle, are behaviours that are compatible with the need to facilitate successful mating. Variability in concentrations of the ovarian hormones progesterone and oestrogen, as well as elevated levels of these hormones at specific stages of the cycle, have been implicated in these behavioural changes (Toufexis et al., 2006). Evidence for hormonal mediation of anxiety behaviour was provided by Marcondes et al. (2001), who reported that female rats spent a reduced percentage of time in the open arms of the elevated plus maze during the dioestrous phase of the cycle, compared to rats in the proestrous phase. However, when rats in dioestrus were treated with oestradiol to produce oestradiol plasma concentrations similar to those reported in the proestrous phase, this difference in anxiety behaviour disappeared (Marcondes et al., 2001). This has led to some studies stating that the phase of the oestrous cycle, and associated hormonal changes, appears to be a more significant determining factor in responsiveness to stress compared to any individual trait differences (Devall et al., 2009), with the levels of oestradiol influencing stress responsiveness (Marcondes et al., 2001).

As identified in previous research conducted by this laboratory (Barker et al., 2017b) the social status of group-housed rats can be another significant external factor that impacts on the occurrence of certain biases being expressed. Subordinate rats are subjected to significantly increased aggressive behaviours compared to their dominant conspecifics (Van Loo et al., 2001). Subordinate animals therefore respond with reduced numbers of optimistic biases to a judgment bias paradigm (Barker et al., 2017b). An interaction between oestrous cyclicity and social stress has been previously identified in a rodent model (Palanza et al., 2001). CD-1 Swiss albino mice were individually housed, or housed in groups with 2 other same sex cage-mates. Mice that were individually housed showed fewer anxiety-like behaviours in a free-exploratory paradigm when in the proestrous phase, compared to those in both oestrus and dioestrus (Palanza et al., 2001). This suggests that this oestrous phase may modulate social stress. As such, subordinate females experiencing proestrus may respond with more optimistic decisions than subordinate females in dioestrus and oestrus.

Therefore, the current study was designed to investigate if the dioestrous phase would be associated with an increased percentage of pessimistic responses to an ambiguous probe. If present, this would be indicative of these animals being in a negative affective state. It was hypothesised that rats in the proestrous phase would respond with a significantly reduced percentage of pessimistic responses to the ambiguous probe (and therefore an increase in optimistic responses) which would indicate that these animals are in a comparatively more positive affective state. It was also hypothesised that dominant animals would respond with significantly fewer pessimistic biases than subordinate animals in dioestrus. If these differences were observed, it would highlight the need to include oestrous cycle phase and social status as predictor variables in study design when assessing cognitive biases in female rats.

2. Methods

2.1. Animals and housing

Twenty-four female Hsd: Sprague Dawley (SD) rats were sourced from a barrier-maintained, specific pathogen free production facility (University of Adelaide, Laboratory Animal Services, Adelaide, Australia). After arriving at the testing facility, the animals were randomly separated into groups of 4 using a random number generator. Each rat was identified by a marking made at the base of their tail with a non-toxic permanent marker. This marking was re-applied when necessary. Rats were housed in the Eurostandard type IV (Techniplast, Exton, PA, USA) commercially available cage. Every cage was furnished with paper-based bedding (Animal Bedding, Fibrecycle Pty Ltd, Yatala, Queensland, Australia), PVC pipes both loose and attached to the roof of the cage, chewing objects (Nylabone Products, NJ, USA) and shredded paper for foraging. Standard rat chow (Rat and Mouse Cubes, Speciality Feeds, Western Australia, Australia) and reverse-osmosis water were freely available in each cage. The animals were housed in a temperature maintained room (21 °C–23 °C) with a reversed 12-hour light/dark cycle (on at 1800, off at 0600). All procedures using the animals took place in the dark photoperiod under red-light conditions. Animals were acclimatised to the testing facility and their new housing arrangements over 14 days, after which, the rats began training on the judgment bias discrimination task. All animal use, housing protocols and methodology were approved by the Animal Ethics Committee of the University of Adelaide and conducted in accordance with the provisions of the *Australian Code for the Care and Use of Animals for Scientific Purposes.*

2.2. Judgment bias apparatus and associations

The test apparatus was originally designed by Brydges et al. (2011) and is presented as Fig. 1. The apparatus comprises two Perspex boxes connected via a PVC pipe. This pipe would be lined with either a coarse sandpaper (P80) or a fine sandpaper (P1200) dependant on the conditions of the test. One box, henceforth referred to as the 'goal' box, contained a brown bowl in the far-right hand corner filled with a coriander scented sand (1% spice to sifted sand) and blue bowl in the left-hand corner that contained cinnamon scented sand. Milk-chocolate baking chips (Cadbury, London, England) served as the high-positive

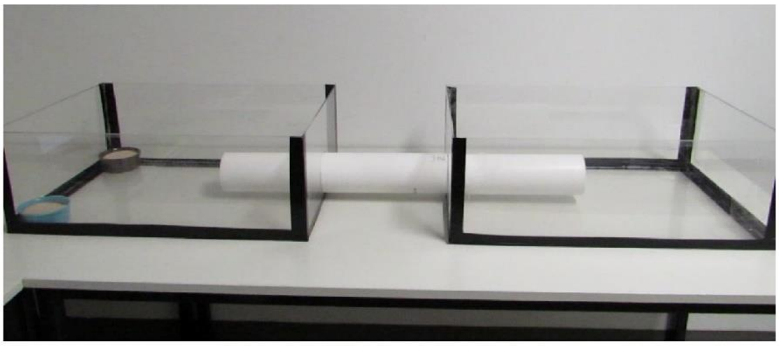

Fig. 1. Judgment Bias Testing apparatus utilised in the study.
Originally designed and developed by Brydges et al. (2011).
The start and goal box measured 610 mm × 435 mm × 250 m m. The PVC pipe was 800 m m long and had a diameter of 100 mm.

Table 1
Association of reward item to sandpaper stimulus and reward location. Each association was randomly assigned and counter balanced.

	Chocolate		Cheerio	
Association (n)	Sandpaper	Bowl/Location	Sandpaper	Bowl/Location
1 (n = 6)	Coarse	Brown Bowl/ Right	Fine	Blue Bowl/Left
2 (n = 6)	Coarse	Blue Bowl/Left	Fine	Brown Bowl/ Right
3 (n = 6)	Fine	Brown Bowl/ Right	Coarse	Blue Bowl/Left
4 (n = 6)	Fine	Blue Bowl/Left	Coarse	Brown Bowl/ Right

reward item, while Cheerios (UncleToby's, Victoria, Australia) were used as the low-positive reward items. These items were chosen as the high-positive and low-positive reward items respectively, as identified through preference testing as detailed in Brydges et al. (2011). Animals were randomly assigned an association between reward item, reward location and sandpaper that was counter-balanced between associations (Table 1).

2.3. Training on the judgment bias discrimination task

Animals received two training trials between 0700 and 1000 h and two between 1100 and 1400 h every day. For each trial a single reward item was present (chocolate or cheerio) in the appropriately corresponding reward bowl, with the correct sandpaper lining the PVC pipe. Rats were placed in the start box and allocated 5 min to proceed through the pipe to find and then consume the reward item. Animals were eligible for testing once they had approached the bowl contacting the hidden reward first, on at least 3 out of 4 daily trials for 5 days in a row. Once every rat in a cage had reached these criteria, the cage could then be tested. The full methodology for training on the judgment bias discrimination task has been included in the Supplementary Material and is based on the methods from Barker et al. (2017b).

2.4. Judgment bias testing

Testing occurred over 6 days, with each animal receiving a single test in the judgment bias apparatus per day. The time of judgment bias testing and assessment of oestrous phase remained consistent for each individual rat, to account for natural variation in the diurnal rhythm. The sandpaper in the PVC pipe was replaced with an ambiguous grade (P180) directly intermediate between the two learned stimuli used in the training phases and no reward item was present in the reward bowls. As the animals had not encountered this stimulus before, their responses to the probe could be interpreted as either optimistic or pessimistic. Optimistic decisions were defined when the rat foraged in the bowl that would normally contain the high-positive reward, while a pessimistic decision was defined when the rat foraged in the bowl that would normally contain the low-positive reward. Testing was performed by an observer blinded to the associations of the animals and the oestrous phases in which they would later be assigned. Immediately after being tested on the judgment bias paradigm, the animals were removed from the arena. During testing the animals were not exposed to the 'training' cues between ambiguous probe exposures as previously performed by this research group (Barker et al., 2016, 2017a; Barker et al., 2017b). Reinforcement of the training cues between ambiguous probe exposures, has been shown to reduce the effects of extinction from occurring in rats (Burman et al., 2009; Brydges and Hall, 2017). However, in defence of the omission of this reinforcement step from the methods utilised in the current study, recent findings (Barker et al., 2018) support up-to 6 days of consecutive ambiguous probe exposure without experiencing any effects of extinction. Moreover, as the animals continued to make the active response to the ambiguous probe, we can be confident that extinction of learning did not occur.

2.5. Oestrous cycle stage identification

Identification of oestrous stage was performed by a researcher blinded to the rats' performance in the judgment bias test. The vaginal smearing technique for oestrous identification was used; vaginal smearing is a routine husbandry technique used in animal facilities for time-mating of rodents (Cora et al., 2015). Animals were restrained and the sterile inoculation loop was washed with sterile saline and then gently introduced to the rat's vagina, rotated slightly, and withdrawn. Rats were then placed back into their home cage while a smear was made on a microscope slide using the inoculation loop. The smear was fixed using the commercially available staining procedure Quick Dip (Thermofisher Scientific Australia Pty Ltd, Riverstone, NSW, Australia) and allowed to dry before being observed to determine the cell type and morphology as an indicator of oestrous phase as described in the review by Cora et al. (2015).

Animals experienced at least one cognitive bias test per phase of the oestrous cycle. Six days of testing was utilised to account for any natural variation in the duration of the cycle where individual phases lasted for greater than 24 h. The judgment bias data analysed and presented were that of every animal for the days which coincided with their first display of each oestrous stage i.e. n = 24 for each oestrous stage, 4 tests per animal.

2.6. Social Status identification

Classification of the rats into their social classes was achieved

Table 2
Social classes of the caged rats. Definitions of each class and the criteria for class assignment. Every social class was represented in each cage of 4 grouped rats.

Social Class	Definition	No.
(D) Dominant	Dominant over all cage mates – dominant in every dyad	n = 6
(DS) Dominant subdominant	Mostly dominant – dominant in most dyads but not all	n = 6
(SS) Subordinate subdominant	Mostly subordinate – subordinate in most dyads but not all	n = 6
(S) Subordinate	Subordinate to all cage mates – subordinate in every dyad	n = 6

through observing video footage recorded with CCTV cameras (OzSpy, Brisbane, Australia). Recording took place from 1400 to 1800 hours each day, over the six-day testing period. Of these recordings, each animal was observed for 10 min each day, with the start time being randomly selected between 1400–1750 h. The behaviours of the animals were scored as per the ethogram described by Hurst et al. (1996) which has been included in the Supplementary material. The interaction between each individual with respect to each of its caged conspecifics were examined to determine the dominance relationship in each dyad (unique animal pairing). The total number of aggressive behaviours initiated were summed and compared against the number of aggressive encounters received. These were used to assign an animal an agonistic score for that unique dyad. Dominance in a dyad was assigned if the rat initiated more aggressive encounters than it received over every 10–minute recording of the six-day testing period. Table 2 details the social classes recorded and how these were identified, and the number of animals that fell into each class.

2.7. Statistical analysis

Data were analysed using the IBM SPSS Statistics 22 (IBM, NY, USA) software package. All data has been presented using the model estimates expressed as the percentage of pessimistic interpretations made to the probe ± the confidence interval. Data were analysed based on the reports of Gygax (2014), using a generalized linear mixed-effects model with interpretation to the probe as the response variable. The model was specified with a binary variable with a logit link function. Zero was used to identify an optimistic interpretation of the probe and 1 for a pessimistic interpretation. The fixed effects of Cycle and Social Hierarchy were included in the model with the interaction also included where $P < 0.05$. Animal ID and Cage were included as random terms to account for the repeated measures on each animal and the group housing of the rats.

3. Results

There was no significant interaction identified between the effects of Cycle and Social Hierarchy (F (15, 104) = 1.57, $p = 0.883$). The model was therefore run again excluding the interaction between Cycle and Social Hierarchy as a fixed effect, to identify the main effects of both Cycle and Social Hierarchy. There was a significant effect of both Social Hierarchy (F (3, 113) = 6.45, $p < 0.001$) (Fig. 2) and cycle (F (3, 113) = 3.77, $p = 0.013$) (Fig. 3) on the percentage of pessimistic interpretations to the ambiguous probe.

Pairwise contrasts were performed on the estimated marginal means using the least significant difference method in SPSS for both main effects of Social Hierarchy and Cycle. For Social Hierarchy, subordinate animals responded with an increased percentage of pessimistic interpretations to the probe compared to dominant animals ($p = 0.001$), dominant subdominant animals ($p = 0.001$) and subordinate subdominant animals ($p = 0.002$). For Cycle, animals in the dioestrous phase responded with an increased percentage of pessimistic interpretations to the probe compared to animals in proestrus ($p = 0.008$), oestrus ($p = 0.037$) and metoestrus ($p = 0.01$) (Fig. 3).

4. Discussion

This study investigated the differences in judgment bias expression between female rats in different phases of the oestrous cycle. As hypothesised, female rats in the dioestrous phase responded with a significantly increased percentage of pessimistic decisions compared to each other phase of the cycle. As previously observed and discussed in Barker et al. (2017b), subordinate rats of the current study also responded with significantly increased percentages of pessimistic decisions. There were no significant interactions between oestrous phase and social status.

Contrary to the hypothesis, however, proestrus was not associated with a significant decrease in the percentage of pessimistic decisions (and thus, an increase in the percentage of optimistic decisions) compared to the other phases. Application of a stressor such as metabolic cage housing (Barker et al., 2016) or subordination stress (Barker et al., 2017a) have routinely resulted in rats responding with pessimistic biases. However, only a handful of studies have applied seemingly pleasurable stimuli, such as environmental enrichment (Brydges et al., 2011) observing that this increased the optimistic responses of treated animals. Based on previous studies we would have expected rats in the proestrous phase to respond similarly to the Brydges et al. (2011) study, in which rats housed in enriching environments responded with fewer pessimistic biases. Given this data, it suggests that the judgement bias test employed is more sensitive to negative changes in cognitive state compared to positive; as expected in the proestrous phase (Frye et al., 2000). With the example of Brydges et al. (2011), the provision of enrichment could be argued to always encourage significantly more optimistic biases when compared against a treatment group that has no, or reduced, numbers of enrichment items (Matheson et al., 2008; Brydges et al., 2011; Douglas et al., 2012; Bethell and Koyama, 2015). These tests are therefore not a comparison of a positive treatment versus neutral control, but positive treatment versus negative treatment. Greater success has been achieved in discrimination tasks where rats have needed to respond with active behaviour to a clearly positive stimulus and a clearly negative stimulus (Anderson et al., 2012; Papciak et al., 2013; Rygula and Popik, 2016). In these studies, if the animal responds incorrectly they are actively punished (mild foot-shock). This encourages animal learning to display the correct behaviours to the correct stimulus (Rygula et al., 2013). Unlike the test employed in the current study, there is no discouragement for making an incorrect decision (approaching the unrewarded bowl first) whilst training. This may encourage healthy animals to behave optimistically upon exposure to the ambiguous stimulus as there is no 'punishment' for behaving as such. It is only when the rats experience a significant stressor that they begin to behave more pessimistically. Therefore, whilst previous behavioural tests have identified proestrus to be associated with other behaviours known to be indicative of positive affect (Frye et al., 2000; Walf et al., 2006, 2008), the judgment bias discrimination task employed lacks the practical sensitivity when attempting to identify a change in positive affect.

Cyclicity of the oestrous phase has been strongly correlated with an increase in anxiety-like behaviours expressed by female rats. Female rats in the dioestrous phase responded with a decreased latency to immobility in the forced swim test (Walf et al., 2008), spent less time in the open arms of the elevated plus maze (Marcondes et al., 2001; Brunton and Russell, 2010) and spent greater periods of time in the periphery and corners of the open-field test (Devall et al., 2009). These behavioural observations have all been associated with anxiety-like tendencies in animals. The results of the current study are supported by these previous studies in which animals in the dioestrous phase responded with fewer optimistic biases. Therefore, by logical progression, previously reported differences in male and female cognitive bias

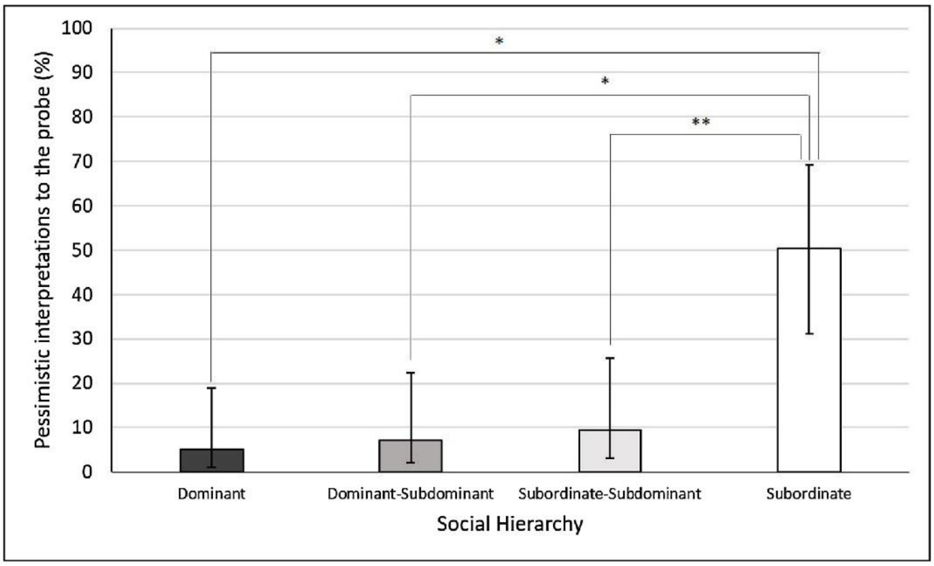

Fig. 2. Effect of social hierarchy on the percentage of pessimistic interpretations made to the ambiguous probe. All data has been presented using the model estimates ± the confidence intervals.
* indicates significance $p = 0.001$.
** indicates significance $p = 0.002$.

expression, in rats at least, could be attributed to the variability in responses that oestrous phase cyclicity encourages.

Oestrous cycle rotation has also been reported to influence animal cognition as evidenced through changes in the novel-object recognition test (Sutcliffe et al., 2007; Paris and Frye, 2008; Walf et al., 2008). Sutcliffe et al. (2007) identified that in a novel-object recognition test female hooded Lister rats responded with improved object recognition compared to males, whilst males responded with greater spatial recognition. It was noted that spatial memory of females can be influenced by the phase of the oestrous cycle, being weakest at both proestrus and dioestrus. Females in both these phases responded with no significant discrimination between the familiar object and the moved (novel) object in the novel-object recognition test, whilst significance was observed for rats in oestrus (Sutcliffe et al., 2007). The authors postulated that the elevated concentrations of progesterone which accompany the dioestrus and proestrus phases (compared to oestrus), have an inhibitory effect on location memory. However, contradictory results have been reported in earlier studies that identified a difference between male and female rats in their performance in a water maze test (Frye, 1995). Male rats performed this test significantly quicker than both dioestrous and oestrous females. However, these differences were greater when females were in oestrus compared to

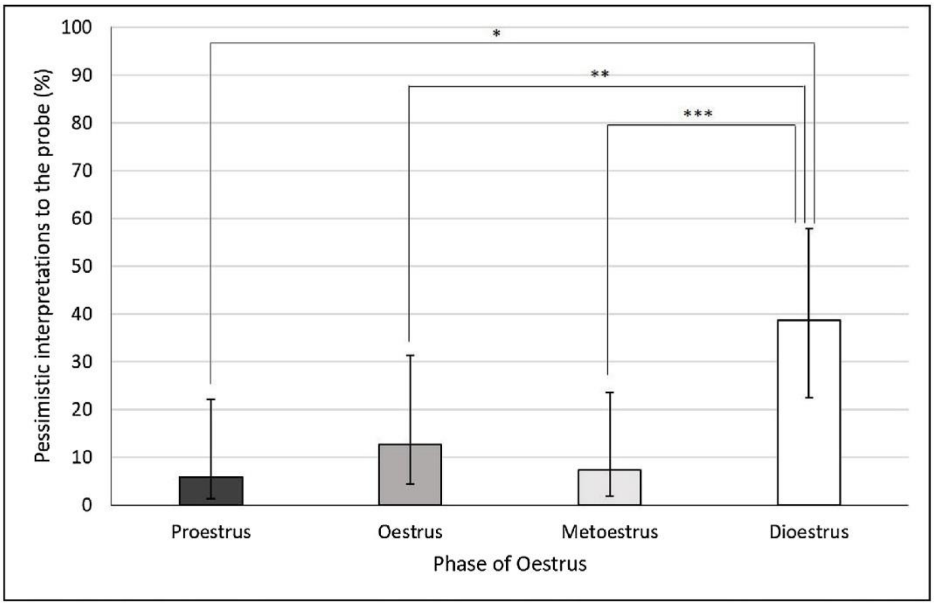

Fig. 3. Effect of phase of the oestrous cycle on the percentage of pessimistic interpretations made to the ambiguous probe. All data has been presented using the model estimates ± the confidence intervals.
* indicates significance $p = 0.008$.
** indicates significance $p = 0.037$.
*** indicates significance $p = 0.01$.

diestrus. Whilst Sutcliffe et al. (2007) speculate that the increased progesterone concentration, as observed in both proestrus and diestrus, may inhibit rat spatial memory, the findings of Frye (1995) suggest that oestrogen concentrations, which are decreased during dioestrus and oestrus compared to proestrus (Goldman et al., 2007; Levine, 2015) may also contribute to this effect. It is postulated that the decreased concentrations of oestrogen coupled with the fluctuating concentrations of progesterone that rats are subjected to during dioestrus negatively impacts their ability to recall the spatial components of the judgment bias test employed in our study. This raises a significant criticism of the methodology employed in the current study; with a compromised ability to recall the test, it could be argued that responses to the probe made in dioestrus are not true reflections of the affective state of the animal, but instead are an indication of the animal failing to remember the correct spatial locations of the reward, and thus responding in a predictable manner. However, our discrimination task does not only associate spatial location with the reward item and reward cue, but includes other associative factors being the colour of the bowl, and the scent of the sand. Oestrogen has been shown to have an inhibitory effect on spatial memory (Pompili et al., 2010). However, the same study also reported that oestrogen had no effect on reference memory and selectively improved working memory (Pompili et al., 2010). Therefore, even if animals in dioestrus did fail to recall the spatial location of the reward item, they are equipped with the other associative factors of scent and bowl colour, to locate and consume the reward item. This highlights the need for future studies to identify the effects of oestrous phase cyclicity using judgment bias discrimination tasks that do not have a sole spatial component.

Decreased oestrogen concentrations associated with the dioestrous phase could provide another potential reason as to why the percentage of pessimistic responses were significantly greater during this phase. Decreased oestrogen concentrations have been previously reported to be associated with the display of greater signs of anxiety or depressive-like behaviours in both humans and rodents (McCarthy et al., 1997; Dalla et al., 2004; Wharton et al., 2012). The hippocampus, an area of the brain important in both learning and memory (Jarrard, 1993), contains both receptors for oestrogen (ER), ERα and ERβ (Birzniece et al., 2006; Cahill, 2006; Wharton et al., 2012). When an agonist is bound to the ERα receptor, rats have responded with increased anxiolysis and anti-depressant like behaviours, as identified through the elevated plus maze and forced swim tests (Furuta et al., 2009), suggesting that increased concentrations of oestrogen produce fewer-anxiety like behaviours. This was pharmacologically tested when rats in the dioestrous phase were treated with oestradiol the predominant oestrogen produced by the ovary. These animals had plasma concentrations of oestradiol similar to females in the proestrous phase. These treated females responded with fewer anxiety-like behaviours compared to the dioestrous, untreated females (Marcondes et al., 2001).

Whilst the mechanistic basis for our study findings are yet to be elucidated, there were clear differences in response between dioestrous phase rats and those in other phases of the oestrous cycle. This is the first study to specifically identify a significant effect of oestrous phase cyclicity on cognitive bias expression. This finding supports monitoring of stage of the oestrous cycle in any future cognitive bias studies using female rats or inclusion of an alternative method of control of this factor. This observation may explain the variability in the female cognitive bias response previously reported in a number of studies. Future studies should determine mechanisms for this response, for example by including an ovariectomised control group, blocking the effects of oestrogen and progesterone, or treating the dioestrous group with oestradiol.

The authors declare they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.beproc.2018.03.020.

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CHAPTER 7.

Increased latencies to respond are not associated with pessimistic biases in rats

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

vii. the candidate's stated contribution to the publication is accurate (as detailed above);

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ix. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Principal supervisor Provided guidance on content and structure of manuscript Editing of manuscript		
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Name of Co-Author	**Alexandra Louise Whittaker**		
Contribution to the Paper	Principal supervisor. Assisted with experimental design and procedures. Provided guidance on content and structure of manuscript. Editing of manuscript.		
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7.2. Statement of Context

The final experiment conducted during candidature was designed to explore the effects that extinction of learning can have on the JBT employed. Extinction of learning is an extremely common limitation of JBT methodology. Extinction occurs when the animal subject learns that the ambiguous, intermediate stimulus is always unrewarded. This results in animals ceasing display of active behaviours.

The study presented in Chapter 7 was designed to assess if extinction of learning would occur in both male and female rats following consecutive exposures to the intermediate probe. Whilst extinction of learning was not observed, there was a significant effect on response latency for the interpretation made to the ambiguous probe. Whilst optimistic interpretations to the probe were associated with significantly decreased response latencies compared to pessimistic interpretations, these differences disappeared after seven days of consecutive probe exposures. This work is the first to identify that response latency is a weak measure of judgement bias testing, as increased latencies can falsely identify pessimism.

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Increased latencies to respond in a judgment bias test are not associated with pessimistic biases in rats

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ABSTRACT

Extinction of learning is a common, yet under-reported limitation of judgment bias testing methods Repeated exposure to the ambiguous probe of a judgment bias paradigm encourages the animal to cease display of the required behaviours. However, there remains a need to repeatedly test animals to achieve statistical power. A delicate balance therefore needs to be struck between over- and under-exposure of the animals to the test conditions. This study presents the data of rats, a common animal subject of judgment bias testing. Rats were exposed to the ambiguous probe of a common, active-choice judgment bias test for 11 consecutive days. There was a significant increase in the latency to respond to the ambiguous probe following day 8, with no significant increase experienced for either the positive or less-positive probes. Following day 8 there was a significant increase in both optimistic and pessimistic latencies in response to the ambiguous probe. Therefore, repeated exposure to the ambiguous probe caused an increased latency in response even though optimistic interpretations were recorded. This implies that the use of response latency alone as a measure in judgment bias testing can falsely identify pessimism. Researchers should modify experimental design to include both choice and latency measures.

1. Introduction

Detection of a cognitive bias through a judgment bias paradigm is an established measure of animal affect (Mendl et al., 2009). The cognitive bias technique allows a behavioural response to ambiguity to be objectively categorised as either optimistic or pessimistic, where pessimism and associated pessimistic tendencies have previously been associated with negative affect (Bethell, 2015). The use of judgment bias paradigms has found utility in animal welfare research; being repeatedly employed in rats (Harding et al., 2004), sheep (Doyle et al., 2010) and dogs (Mendl et al., 2010). However, as researchers' use of these methods increases, so does an awareness of the limitations and restrictions of these methods. A key limiting consideration when designing and implementing judgment bias testing in animals is whether, and when extinction of learning may occur.

Many judgment bias paradigms do not reward the animal for responding to the ambiguous stimulus (Roelofs et al., 2016). Through associative learning, the animals may then cease to exhibit the required behaviour as they no longer have the same motivation to perform the behaviours required (Jamieson et al., 2012). A delicate balancing act is required between testing the responses of animals to ambiguity an appropriate number of times without encouraging extinction. This balance being compounded by the need for repeat testing of animals to provide superior statistical power. The results herein present the data of rats, a frequent subject of the judgment bias test, that were exposed to the ambiguous cue of a commonly reported, active-choice, judgment bias paradigm repeatedly over 11 days. It was hypothesised that extinction of learning, categorised by the animals failing to make the active-choice on the ambiguous trial in the allotted 5 min would be present by at least day 11. It was further hypothesised that the latency to respond with this active behaviour to the ambiguous probe would significantly increase with each subsequent day.

2. Methods

2.1. Subjects and experimental design

All rats used in this study were sourced from a barrier-maintained, specific pathogen free production facility (The University of Adelaide, Laboratory Animal Services, Adelaide, Australia). Male (n = 21) and female (n = 21) hsd: Sprague-Dawley rats were used in this study after being trained on the judgment bias paradigm for other experiments

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performed in this laboratory. As such, animals were from different litters. Upon arrival at the testing facility, animals were housed in sex-separated groups of three in commerical cages (Tecniplast, Exton, PA, USA). Each cage was furnished with paper-based bedding substrate (Animal Bedding, Fibrecycle Pty Ltd, Yatala, Queensland, Australia), shredded paper and provided with a chewing toy (Nylabone Products, NJ, USA). Standard rat chow (Rat and Mouse Cubes, Specialty Feeds, WA, Australia) and reverse osmosis water were provided *ad libitum*. Animals were identified with a marking made at the base of the tail with a non-toxic marker and housed in a temperature controlled room (21–23 °C) on a reversed 12-h light/dark cycle. At time of testing, all animals were approximately 25 weeks old. Animals were trained on the judgment bias paradigm as per the methods of Barker et al. (2016). Coarse sandpaper (P80) and fine sandpaper (P1200) acted as the training cues, and chocolate and cheerio pieces were used as the positive and less-positive reward items, respectively. Each animal had a random association between reward item and training sandpaper, counter-balanced between sexes. The training paradigm consisted of animals learning to cross the sandpaper and to approach the reward containing bowl first. If the animal did not attempt to forage for the reward after 5 min, the animal was removed from the testing apparatus. After animals had completed these training phases (approximately 35 days of training per rat) they could begin testing. For full training and testing methodology see Barker et al. (2016).

During testing the ambiguous stimulus used was an intermediate grade of sandpaper (P180). Testing involved each animal being exposed to three trials per day that occurred randomly. Per day, rats underwent one unrewarded trial paired with the intermediate sandpaper, and one trial each for the positive and less-positive reward associations. Animal housing and experimental protocols were approved by the Animal Ethics Committee of the University of Adelaide and conducted in accordance with the provisions of the Australian Code for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2004).

2.2. Statistical analysis

A repeated measures two-way ANOVA was performed fitting sex and trial type as independent factors on the time it took the animals to initiate foraging behaviours. There was no significant effect of sex or interaction between sex and day on latency to display these behaviours. Therefore, the data for males and females have been combined in Fig. 1. A repeated measures ANOVA was also performed fitting decision and sex as fixed factors on the time it took the animals to display foraging behaviours during the ambiguous probe trial. Levene's test was used in all cases to test for normality of the data-set. All data were found to be normally distributed ($p < 0.05$ at each level) and reported henceforth as mean latency (seconds) to initiate foraging behaviours ± standard error of the mean. Mauchly's Test of Sphericity to test for the assumption of sphericity.

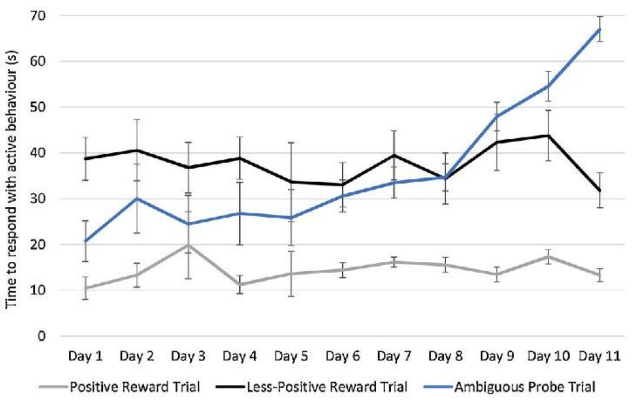

Fig. 1. The effects of day of testing on the latency to respond to the judgment bias test for the positively and negatively rewarded training trials, and the ambiguous probe test trials. No significant effect of day was observed for latency to exhibit behaviour for the positive reward trials or the less-positive reward trials ($P > 0.05$). There was a trend of overall increasing latency to exhibit behaviours for the ambiguous probe trials from day 8 onwards.

3. Results

Sphericity was not violated for the positive, $\chi^2(54) = 339.99$, $p = 0.289$; less-positive, $\chi^2(54) = 104.91$, $p = 0.607$; and ambiguous, $\chi^2(54) = 156.97$, $p = 0.510$ trials. The latency to forage between days were statistically different for only the ambiguous probe, F (5.098, 203.928) = 12.657, $p < 0.001$. There was no significance detected in latency to forage over days for the positive trials F (2.895, 115.792) = 1.050, $p = 0.372$, or for the less-positive trials F (6.074, 242.967) = 0.901, $p = 0.495$. Post-hoc tests using the Bonferroni adjustment revealed that there was no statistically significant difference between the average time to initiate foraging between any of the first 8 days. Latencies to forage for days 1 (20.74 ± 3.81s), 3 (24.42 ± 5.35s), 6 (30.55 ± 2.96s), 7 (33.51 ± 2.89s) and 8 (34.63 ± 2.47s) were all reduced compared to days 9 (47.88 ± 2.66s), 10 (54.54 ± 2.8s) and 11 (67.02 ± 2.32s) $p < 0.05$ for each comparison. Days 4 (26.75 ± 5.72s) and 5 (25 ± 5.18s) had reduced latencies to both days 10 and 11, $p < 0.05$ for both comparisons. Day 2 (29.96 ± 6.63s) was only reduced compared to day 11, $p = 0.001$(Fig. 1.)

There was a statistically significant interaction between the decision made and the day of testing on latency to perform the active response, F (10, 439) = 2.27, $p = 0.013$. Pairwise comparison using the Bonferroni correction identified that there was a significant difference in the latency to perform the active response for optimistic decisions compared to pessimistic decisions from days 2 to 6, $p < 0.05$ for each interaction. However, at day 1 and from days 7 to 11, no significance was detected ($p > 0.05$ for each comparison) (Fig. 2). Complete raw data is available with the supplementary material.

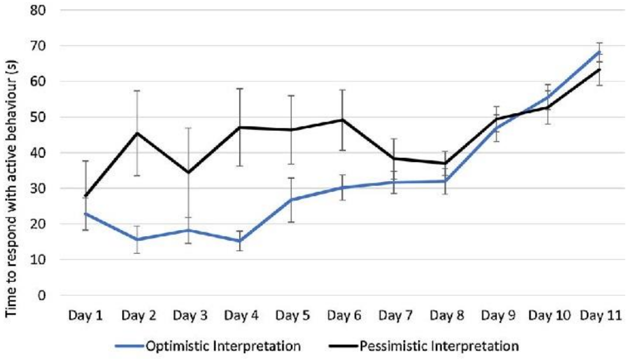

Fig. 2. The effects of day of testing and interpretation to the ambiguous probe on the latency to display the active behaviour. There was a significant difference in latency to perform the active response for optimistic decisions compared to pessimistic decisions to the ambiguous probe, from days 2 to 6 ($p < 0.05$). However, no significance was detected from days 7 onwards ($p > 0.05$).

4. Discussion

The effects of extinction have previously been discussed as being a significant limiting factor of the judgment bias paradigm (Doyle et al., 2010). Whilst some research has been conducted as to the effects of extinction in other animals (Bateson and Matheson, 2007; Matheson et al., 2008), few publications have made note of the effects in rats. In contradiction to one of our study hypothesis, extinction of learning was not observed. It was expected that by day 11 of consecutive exposures to the ambiguous probe, the animals would cease to display of the active-foraging behaviour. Instead, as hypothesised there was a significant increase in latency to perform the active behaviours during the ambiguous probe trial. No increase in latency was observed for the positive or less-positive rewarded trials (Fig. 1). There was also a significant interaction observed between day and decision the animals made to the ambiguous probe (Fig. 2). Latencies to display an optimistic response for days 2 through 6 were significantly reduced compared to the pessimistic responses to the probe. However, from days 7 to 11 no significance was observed. Despite this study failing to identify extinction, the data suggests that there is a need for animals to make an active response to judgment bias testing, as the use of latency alone can encourage the incorrect interpretation of results as pessimistic.

Increased latencies to respond have been previously correlated with pessimistic biases for both rats (Burman et al., 2008; Burman et al., 2009; Richter et al., 2012) and mice (Boleij et al., 2012; Kloke et al., 2014). However, as our data presents, the use of increased latency to detect pessimism is problematic only over time, and these studies did not employ repeated exposures to the ambiguous stimuli longer than 3 days. Whilst these studies employ sound experimental methodology, the lack of repeated testing can be argued to have produced low statistical power when compared to those that employed repeated testing over more days (Brydges et al., 2012; Chaby et al., 2013) or multiple expsoures to the ambiguous cure per session (Anderson et al., 2012). As observed in Fig. 1, it would be reasonable to assume that the increased response latency over the 11 days of testing implies that the animals were behaving more pessimistically over time. However, as identified in Fig. 2, even optimistic responses to the probe were associated with an increasing trend of response latency. Without the inclusion of the active response, this increase in latency would be statistically analysed as a pessimistic bias. The requirement for the animals to make the active-response removes this ambiguity and provides further support for the use of an active-choice judgment bias paradigm over go/no-go tests, particularly when the key measure of interest is latency.

There was also no significant difference in latency between optimistic responses and pessimistic responses to the ambiguous probe for day 1. This occurrence is hypothesised to have been a factor of the novelty of the first exposure to the new sandpaper stimulus, as significance was detected from day 2 onwards. Chaby et al. (2013), who utilised the same methods, noted that judgment biases were only detected after the initial ambiguous exposure. These authors further suggested that when analysing the data from judgment bias testing, the first exposure to the ambiguous stimulus should be analysed separately from each subsequent exposure, however this phenomenon warrants further study. This supports the argument for using an active choice paradigm since a difference between groups would not be observed on this first day of testing if latency was used as the primary measure.

Many judgment bias studies employed repeated testing on the ambiguous stimulus to achieve statistical power (Barker et al., 2017; Destrez et al., 2014). Our study demonstrated that extinction did not occur over 11 days of testing. It is hypothesised that if we continued to test the animals on this paradigm we would eventually observe total extinction of behaviour. However, the significant increases in animal latency to respond suggested that latency is a weak measure to identify animal cognitive bias. To counteract this concern, preventing animals from acting as their own controls has been utilised (Barker et al., 2016), and the introduction of training trials between ambiguous stimulus exposures has been suggested for use in sheep (Doyle et al., 2010) and has found success in rats (Burman et al., 2009; Brydges and Hall 2017). Combined with an active-choice paradigm, these suggestions remain the most pertinent option.

Conflicts of interest

The authors declare they have no conflicts of interest.

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CHAPTER 8.

General Discussion

8.1 Research Summary and Main Findings

This project began with the goal to identify the effects that metabolic cage housing have on the affective state of rats, as identified through rat response to a JBT. However, as deployment of the JBT continued multiple inconsistencies and confounding variables were encountered. Therefore, the majority of this thesis investigated common external and confounding factors that could significantly alter rat response to the JBT. Alterations in response could reduce accuracy of data collected from a JBT to assess an animal's welfare state. Findings presented in this thesis will improve the practicality and reliability of a JBT and the statistical legitimacy of data collected. The suggestions and provisions made throughout this body of work, when incorporated into test design, are argued to enhance the validity of the JBT as an accurate assessment technique of animal affect, and therefore animal welfare.

The initial studies in this research program were designed to determine how metabolic cage housing influences the judgement of male and female rats. Both male (Chapters 2 and 3) and female (Chapter 3) rats housed in metabolic cages responded with significantly fewer displays of optimistic judgements in response to the ambiguous stimulus of the JBT. These findings were the first in the literature to demonstrate metabolic cage housing to be a significant stressor capable of producing negative affect in rats. This is also consistent with the literature, in which it is identified that rodents in metabolic cages experience significant behavioural and physiological stress responses (Gomez-Sanchez and Gomez-Sanchez, 1991; Gil et al., 1999; Eriksson et al., 2004; Kalliokoski et al., 2013; Whittaker et al., 2016a). These findings are also in accordance with previous judgement bias studies that provide evidence that a lack of environmental enrichment produces pessimistic biases (as observed through decreased optimistic biases) in rats (Brydges et al., 2011).

Significance was observed between animals in metabolic cages and those in control caging (Chapter 3), however females in open-top caging responded with fewer optimistic biases than males in open-top caging. Of the JBT studies that utilised both sexes and controlled for this as a factor in analysis (Briefer and McElligott, 2013; Asher et al., 2016; Brown et al., 2016; Carreras et al., 2016; Takeshita and Sato, 2016), this dissimilarity had been identified only twice previously (at time of writing): in rats (Brown et al., 2016) and Japanese Pygmy Squid (Takeshita and Sato, 2016). Of these studies, females consistently responded with increased displays of pessimism compared to males. The significant lack of literature in this area prompted the need to evaluate why these differences between male and female judgement bias expression occurred. An improved understanding of the differences between male and female expression to the JBT is argued to improve the efficacy of the JBT for animal welfare assessment. The two factors identified and discussed to possibly contribute to this effect were social stress, as a result of social subordination, and oestrous cyclicity.

The studies presented in Chapters 2 and 3 involved moving animals from established, control open-top cage housing to the metabolic cage test housing. This is a contentious issue and can be argued to be a flaw in our experimental design. However, moving animals in such a way is common laboratory practice and is discussed in greater details in Chapters 2 and 3. It had been previously discussed that males are more resistant than females to changes in social structures (Hurst et al., 1998; Hong et al., 2012). It has also been discussed that rodents will consistently engage with one another to attempt to gain a higher social status (Van Loo et al., 2001). It could be argued that females, who are significantly more sensitive than males to changes in social hierarchy, are more likely to respond with more pessimistic biases in control housing compared with their male counterparts. This argument could perhaps provide some clarification as to why control female animals responded with significantly fewer optimistic biases than control males (Chapter 3). Whilst this assumption remains hypothetical, the study presented in Chapter 4 was designed to assess if social subordination is a significant modifier of animal affect. As discussed in sections 1.4 and 4.2, only males were utilised in this study as the effects of oestrous in a female cohort were yet to be elucidated.

Chapter 4 presented two experiments, both of which aimed to identify how social status and one of either housing density (number of rats per cage) or space allocation (floor area allowance per caged animal) would modify rat expression to the JBT. In addition to the JBT, a series of traditional behavioural tests were utilised, namely the open-field, social-interaction and novel-object recognitions tests. Animals considered to be subordinate in their cage responded with significantly reduced optimistic decisions compared to their dominant cage-mates. Subordinate animals also responded with significantly reduced percentages of time in the centre of open-field tests and displayed significantly fewer social-affiliative behaviours in the social-interaction test. No significance was observed for the main effects of social status on performance in the novel-object recognition test. Rodent performance in the open-field, social-interaction and novel-object recognition tests are all highly repeatable behavioural indicators of a stress response. Rats considered to be subordinate consistently responded to the tests with behaviours indicative of being in an anxiety-like state. Subordination, and the stressors associated with being a subordinate animal, have been previously correlated with significant physiological (Blanchard et al., 1993; Lucas et al., 2004; Tamashiro et al., 2005) and behavioural adaptations (Abel and Bilitzke, 1990; Inagaki et al., 2005; Davis et al., 2009) indicative of a stress response. The data presented in Chapter 4 are the first to specifically identify that social stressors associated with subordination are significant enough to encourage pessimistic biases in group housed male rats. It also provided evidence that this subordination stress can be exacerbated by increased housing density (more rats per cage) and increased space area allowance. The major findings of Chapter 4 provide evidence that future studies utilising a JBT in group-housed animals should consider social status as a fixed effect in statistical analysis of the JBT data.

With Chapter 4 providing evidence that social status can be a significant modifier of animal expression to the JBT, social status was considered as a fixed effect for analysis of data presented in Chapters 5 and 6. The studies presented in Chapters 5 and 6 were conducted in tandem with the main aim to investigate the effects of oestrous and oestrous cycle rotation on judgement bias expression. Chapter 5, presented as a short communication, details the effects that imposed subordination has on animal learning. This chapter was designed to investigate efficiency of the JBT training paradigm, as a major limitation of the continued and widespread use of the JBT are its considerably long training times (Brydges and Hall, 2017). At the time of writing, only one previous study had identified the effects of imposed subordination on animal learning. Imposed subordination being defined as the social stress experienced by a subordinate animal following continuous housing with a dominant cage-mate. As hypothesised, subordinate animals took a significantly increased number of days to learn the training requirements of the JBT compared to their dominant cage-mates. This finding suggests that the complex social-dynamics associated with group-housed animals can contribute to lengthy training times associated with use of a JBT. A potential solution to this issue is discussed in section 8.2.2.

Chapter 6 aimed to investigate how the specific effects of oestrous cycle rotation impact the judgement of female rats to the JBT. Social status was also considered as a factor for analysis, however, no interaction between oestrous phase and social status was observed. The oestrous cycle in rats is short, lasting only 4-6 days (Goldman et al., 2007). The JBT design utilised in this thesis involved testing animals once a day, over a 5-day period. This testing regime is a common theme of JBTs designed for use in rats (Brydges et al., 2011; Brydges et al., 2012; Chaby et al., 2013). It is therefore sensible to suggest that each day of testing can coincide with a new phase of oestrous. Chapter 6 details that rats in the dioestrous phase responded with a significantly increased percentage of pessimistic interpretations compared to each other phase (oestrus, metoestrus and proestrus). Each phase of the oestrous cycle can be categorised by the types and concentrations of ovarian hormones present (Paccola et al., 2013; Levine, 2015). These hormones have been associated with significant changes in behaviour for both mice and rats (Marcondes et al., 2001; Agrati et al., 2005; Plappert et al., 2005; Walf et al., 2008; Devall et al., 2009). Female rats in the dioestrous phase have previously responded with behaviours highly associated with anxiety and depression (Marcondes et al., 2001; Devall et al., 2009). This supports study findings of Chapter 6 that females in dioestrous are more inclined to respond pessimistically to a JBT. These findings are the first to identify a significant effect of oestrous phase cyclicity on judgement bias expression in rats. The effects of oestrous may provide explanation as to why male and female expression to the JBT is not equivalent. It is suggested that oestrous phase should be monitored or controlled for in future JBT studies that utilise a female rodent subject.

The final study, presented in Chapter 7, is another applied investigation to the mechanistic basis of the JBT methodology and was aimed to investigate the effects of learning extinction. Male rats were exposed to the ambiguous probe of the JBT for 11 consecutive days. It was hypothesised that the rats would eventually cease display of the active behaviour (digging in the sand) after they had learned that the ambiguous probe was never rewarded. However, extinction was not observed, every rat made the active response for each probe exposure, suggesting that extinction would not occur following 11 consecutive probe exposures. What was significant, however, was the latency of these active responses. Following day 8, there was a significant increase in time taken for rats to make the active response. This increase in latency was experienced for both an optimistic and pessimistic interpretation to the ambiguous probe. Despite this, response latency has been used in multiple JBT studies using both mice and rats, with pessimism commonly associated with increased response latency (Burman et al., 2008; Burman et al., 2009; Boleij et al., 2012; Richter et al., 2012; Kloke et al., 2014). The use of response latency alone can falsely identify a pessimistic bias.

8.2 Future Direction

8.2.1 Physiological Disconnect and Home Cage Testing.

As discussed in Chapter 3, judgement bias testing, in and of itself, could be a positive event for animals (Keen et al., 2014). The familiar testing arena utilised and propensity to being rewarded have previously been suggested to encourage optimistic responses to the ambiguous probe (Keen et al., 2014). Data presented in Chapter 3 disputes these assertions as pessimistic biases were observed. However, a correlation between pessimistic biases with a physiological indicator of stress (corticosterone or tyrosine-hydroxylase concentrations) failed to be observed. It was suggested that constantly moving animals from the stressful, metabolic cage housing into this assumed positive apparatus would encourage animals to be in a comparatively more positive affective state compared to remaining in metabolic cages indefinitely. Being housed continuously in metabolic cages would be typical of the routine use of these cages for research purposes. The constant removal of these animals can perhaps provide some explanation as to why no physiological correlates of stress were observed. This is despite multiple reports in the literature of a rodent physiological stress response being mounted to these cages (Gomez-Sanchez and Gomez-Sanchez, 1991; Gil et al., 1999; Eriksson et al., 2004). As pessimistic biases were still observed, we can also be certain that a behavioural indicator of stress was still being detected. The failure to correlate pessimistic biases with a physiological indicator of stress suggests that cognitive biases are perhaps more sensitive than physiological correlates of a stress response. However, without a JBT design that can be performed in the home cage and/or experimental unit this discussion remains hypothetical. Future JBT designs should be developed that do not require animals to be moved to a unique testing arena but can be performed in the natural home cage and/or environment of the animal.

8.2.2 Response Latency

In Chapter 7 it was demonstrated that response latency is a comparatively weak response variable for use in a JBT, as it can falsely identify pessimism. Response latency has been used in multiple JBT studies (Burman et al., 2008; Burman et al., 2009; Boleij et al., 2012; Richter et al., 2012; Kloke et al., 2014) to identify pessimistic bias. Data presented in Chapter 7 suggests that use of response latency as the only variable of interest in a JBT can be problematic over time. Without the active response component of the design utilised, it would be reasonable to assume that increased latency to respond (as seen from day 8 onwards) could be indicative of pessimism. However, as the optimistic decision was still made, it highlights the inaccuracy of response latency as the sole response variable in a JBT. This discussion further supports the argument for using an active choice JBT over a go/no-go design and encourages future JBT and cognitive bias studies in general to ensure that response latency is not the sole response variable of interest.

8.2.3 Applied Use and Attention Biases

Whilst use of a JBT to identify animal affect as a novel welfare assessment method has proven to be successful, the data presented in this thesis suggests the JBT has limitations as a practical indicator of animal welfare. The JBT is a reliable indicator of both positive and negative affect, however it is associated with extremely complex design, an involved training procedure and is labour and time intensive. The goal of this thesis was to improve the accuracy of JBT data by identifying and controlling for previously undiscussed external factors that could impact the judgement of rats. This goal was achieved and the JBT design used is still argued to have utility in a research and teaching scenario. The need to expedite JBT testing has been previously identified (Lee et al., 2016; Brydges and Hall, 2017), however, it is of the author’s opinion that the complex associative elements involved in animals learning a JBT make it an impractical candidate as an applied welfare assessment technique.

A new area of study in the cognitive bias literature involves identification of attention-biases. An attention bias can be demonstrated when an animal in a negative affective state directs more of their attention to a threatening stimulus compared to those animals experiencing positive affect (Lee et al., 2016). Whilst attentional biases have been well-associated with anxiety in humans (Mogg et al., 1995;

Bar-Haim et al., 2007), there have been limited validation studies in non-human animals. Attention biases have been identified in starlings (Brilot and Bateson, 2012), rhesus macaques (Bethell et al., 2012b), sheep (Lee et al., 2016) and cattle (Lee et al., 2017). Whilst these methods are still in their relative infancy, evidence suggests that attention bias testing may be a significantly more practical cognitive assessment technique with improved utility in applied settings. There is an immediate need to validate an attentional bias test in laboratory rodents.

8.3 Conclusion

The suggestions made throughout this thesis are argued to increase the quality of JBT experimental design and statistical accuracy of data collected from such a design. Factors such as social status have a requirement to be controlled for in future, as does the phase of oestrous cyclicity in a female cohort of animals. Whilst the JBT may have limited use as a welfare assessment technique in applied scenarios, it has been demonstrated to be an effective and repeatable measure of animal affect.

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APPENDIX 1.0.

A Judgement Bias Test to Assess Affective State and Potential Therapeutics in a Rat Model of Chemotherapy-Induced Mucositis

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

i. the candidate's stated contribution to the publication is accurate (as detailed above);

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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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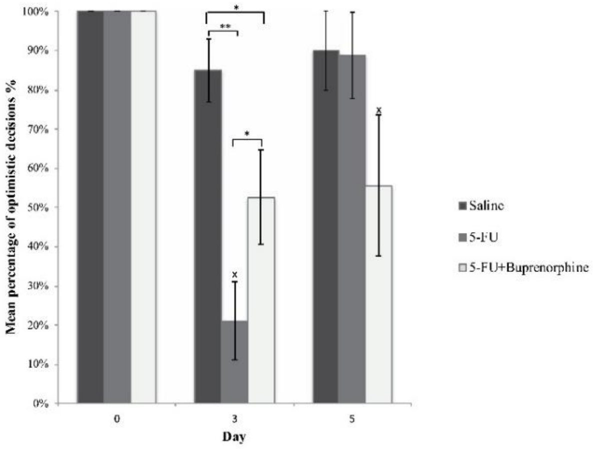

Figure 1. Mean percentage of optimistic decisions on days 0 (baseline prior injection, saline), day 3 (72 hours post injection) and day 5 (120 hours post injection) of rats injected with either saline, 5-FU, and 5-FU + buprenorphine treatments. Data expressed as mean ± estimated SEM. *Indicates significance $p < 0.05$, **indicates significance $p < 0.001$. X indicates significance $p < 0.05$ between time points within the same treatment group.

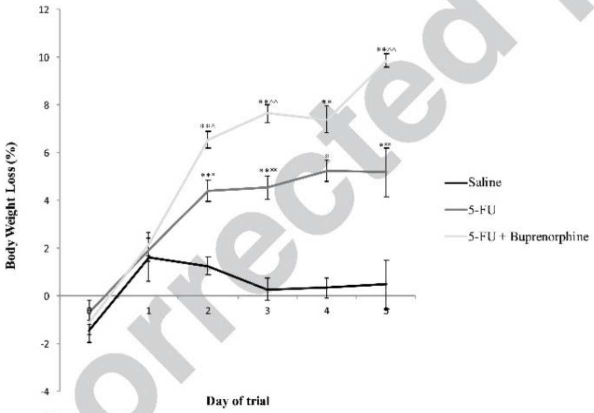

Figure 2. Effects of saline, 5-FU, and 5-FU + buprenorphine on body weight change in SD rats from days 0–5. Bodyweight change calculated from original weight recorded at day 0 prior to saline, 5-FU, buprenorphine injection. Data expressed as mean ± SEM. *$p < 0.05$, **$p < 0.001$ compared to saline, ^$p < 0.05$, ^^$p < 0.001$ compared to 5-FU, $^{\times}p < 0.05$, $^{\times\times}p < 0.001$ compared to 5-FU + buprenorphine.

improving patient quality of life and the impact on affective state[8,10]. Affective state being a subjective feeling state that encompasses different mood states, such as anxiety, depression, joy or happiness[11].

The majority of preclinical studies in mucositis have used rodents to investigate pathogenesis of the condition and effectiveness of novel therapeutics. These investigations typically assess pathological outcomes such as gut histological architecture or inflammatory response, yet fail to include a measure of affective state; the key therapeutic goal. Reliable assessment of affective state, integrated with investigation of therapeutic targets, is therefore required to improve transitional validity of these models in translating outcomes to human patients.

Since animals are incapable of verbally reporting their 'feelings', subjective experiences cannot be directly measured. Behavioural and physiological measures have traditionally been used to gauge an animal's affective state. Although these behavioural and physiological measures provide important information on the arousal of an emotion, they are simplistic and do not provide a complex interpretation of the positive and negative valence of an emotion[12–15]. One such method that has been derived from the human psychology field, and has been used as an indicator of affective state, is assessment of cognitive biases[16].

Cognitive bias testing is a novel approach to identify emotional states and establish an objective measure of cognitive performance in animals. Despite the promise of cognitive bias assessment methods as indicators of animal affective state these methods are yet to be employed in an animal disease model. It is pivotal for biomedical studies to find reliable assessment tools to evaluate various emotions experienced by animals to enhance

Day of trial	Saline	5-FU	5-FU + Buprenorphine
0	0	0	0
1	1.0	1.1	$1.5^{*,\wedge}$
2	1.1	1.5*	$2.7^{**,\wedge\wedge}$
3	1.1	1.5*	$3.0^{**,\wedge\wedge}$
4	1.2	1.6	$2.1^{**,\wedge\wedge}$
5	1.1	1.7*	$2.6^{**,\wedge}$

Table 1. Effects of saline, 5-FU, and 5-FU in combination with buprenorphine on disease activity index from days 0–5. Rats were administered with saline, 5-FU or 5-FU + buprenorphine on day 1 by intraperitoneal injection. Buprenorphine administration continued at 12 hourly intervals from days 1–5. Data expressed as mean ± SEM.Data are expressed as the mean disease activity index score. Data expressed on days 0–3 saline $n = 20$, 5-FU – 5-FU + Buprenorphine $n = 19$, days 4–5, saline $n = 10$, 5-FU – 5-FU + Buprenorphine $n = 9$. $^{*}p < 0.05$, $^{**}p < 0.001$ compared to saline, $^{\wedge}p < 0.001$, $^{\wedge\wedge}p < 0.05$ compared to 5-FU.

animal model refinement, and improve validity of novel therapeutic assessment. Consequently, this study aimed to validate a cognitive bias test through a judgement bias paradigm to measure affective state in a rat model of chemotherapy-induced intestinal mucositis. Further validation of the test was also performed through the addition of an opioid palliative treatment, buprenorphine, which was expected to improve wellbeing.

Results

Judgement Bias Test. The results from the judgement bias data indicated that rats administered 5-FU and buprenorphine exhibited significantly fewer optimistic decisions compared to saline alone treated rats (Fig. 1). There were no differences in optimistic decisions between treatment groups prior to administration of saline, 5-FU and buprenorphine (day 0: 100%, $p > 0.05$). 5-FU-injected rats expressed decreased optimistic decisions compared to animals administered 5-FU + buprenorphine (21%; 53% respectively, $p < 0.05$) and saline control animals (85%, $p < 0.001$) 72 hours post injection. There were no differences between treatment groups in the number of optimistic decisions made at the 120 hour time point (saline 90%, 5-FU 89%, 5-FU + buprenorphine 56%, $p > 0.05$). However, optimistic decisions increased between 72 hour and 120 hour time points for rats administered 5-FU alone ($p < 0.001$). No differences were detected for rats administered 5-FU in combination with buprenorphine or saline alone between the 72 hour and 120-hour time points ($p = 1.000$ respectively).

Daily bodyweight. There were no differences in bodyweight between groups prior to administration of saline, 5-FU and buprenorphine (day 0: $p = 0.49$; day 1: $p = 0.61$). Bodyweight change differed substantially between the groups post administration of agents (Fig. 2). Intergroup comparisons demonstrated that rats administered 5-FU alone had an increased bodyweight loss on days 2 to 5 compared to saline control (days 2 and 3: $p < 0.001$, days 4 and 5: $p < 0.05$). Furthermore, administration of buprenorphine in conjunction with 5-FU was associated with greater reductions in bodyweight compared to both 5-FU (day 2 $p < 0.05$, days 3, 4, and 5 $p < 0.001$) and saline controls (days 2–5 $p < 0.001$).

Disease activity index. There was no significant difference in disease activity index (DAI) prior to saline, 5-FU or buprenorphine injections ($p > 0.05$, Table 1). 5-FU administration increased DAI on days 2, 3 and 5 compared to saline controls ($p = 0.001$, $p = 0.003$, $p = 0.01$ respectively). Buprenorphine administration in 5-FU injected rats increased DAI on days 1–5 compared to both saline (day 1 $p < 0.05$, days 2–5 $p < 0.001$) and 5-FU controls (days 1 and 5 $p < 0.05$, days 2–4 $p < 0.001$).

Histological severity score. Histological gut architecture of the proximal jejunum and distal ileum was consistent with previous literature of mucositis on rats. 5-FU injected rats exhibited an increased histological severity score at 72 hours post injection compared to saline injected rats, with decreased goblet cells, crypt and enterocyte disruption, and shortening of villi present, and a decreased histological severity score at 120 hours post injection, with the presence of villi and crypt elongation (Fig. 3, data not shown for distal ileum). Animals administered 5-FU exhibited an increased disease severity score in the proximal jejunum and distal ileum at both 72 hour and 120 hour, compared to saline injected animals ($p < 0.001$, Fig. 4). Buprenorphine in conjunction with 5-FU had no effect on histological severity score at 72 hour and 120 hour time points for both ileum and jejunum compared to 5-FU alone treated rats ($p < 0.05$). Both 5-FU and buprenorphine showed a significant difference from 72 hour and 120 hour time points for jejunum and ileum (jejunum $p = 0.005$, $p < 0.001$; ileum $p = 0.03$, $p < 0.001$ respectively). This difference was not demonstrated in animals administered saline (jejunum $p = 0.90$, ileum $p = 0.71$).

Discussion

Mucositis is a common and serious side-effect following chemotherapy and radiotherapy. Despite the self-limiting nature of the condition and the negative impact on patient quality of life, cognitive parameters such as affective state are rarely studied in animal models. To our knowledge this is the first study to investigate affective state through judgement biases in a disease model. Importantly, this study represents the first to have demonstrated and validated a reliable judgement bias test to assess affective state exhibited by rats with chemotherapy-induced

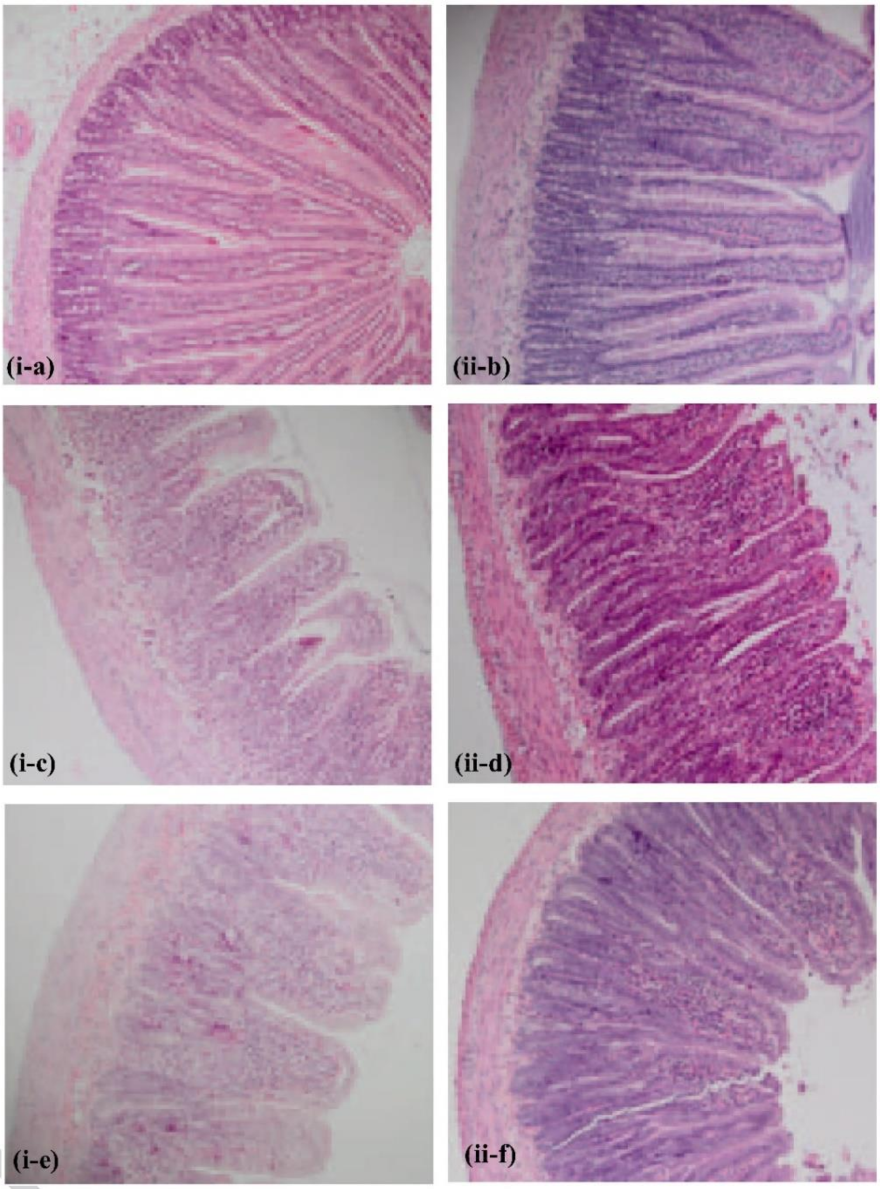

Figure 3. Representative photomicrographs of proximal jejunum at 72 hour (i) and 120 hour (ii) time points stained with haematoxylin and eosin (×40) in rats injected with saline (**a**,**b**), 5-FU (**c**,**d**) and 5-FU + buprenorphine (**e**,**f**).

mucositis. The results from this study indicated that the presence of intestinal mucositis caused a negative affective state, which was partially ameliorated by analgesic administration.

In the present study, judgement bias results followed a correlation with the pathophysiological progression of mucositis identified from previous studies which have evaluated histology and clinical score[6,17]. Healthy animals, prior to 5-FU and saline injections were in a positive affective state, evidenced by optimistic decision-making by all animals when exposed to the ambiguous probe. Administration of 5-FU significantly decreased optimistic decisions compared to healthy controls 72 hours post injection. This finding of negative affective state showed a correlation with the progression of pathological damage associated with mucositis development, where clinical scoring and histological assessment revealed increased histological severity score, decreased bodyweight and increased disease activity index parameters following 5-FU administration. This finding is consistent with previous literature in rats, in which 5-FU caused mucosal damage in the small intestine, villi blunting and fusion, intestinal inflammation characterized by infiltration of immune cells, and increased intestinal wall thickness, increased bodyweight change and clinical disease score at 72 hours post 5-FU injection[6,18].

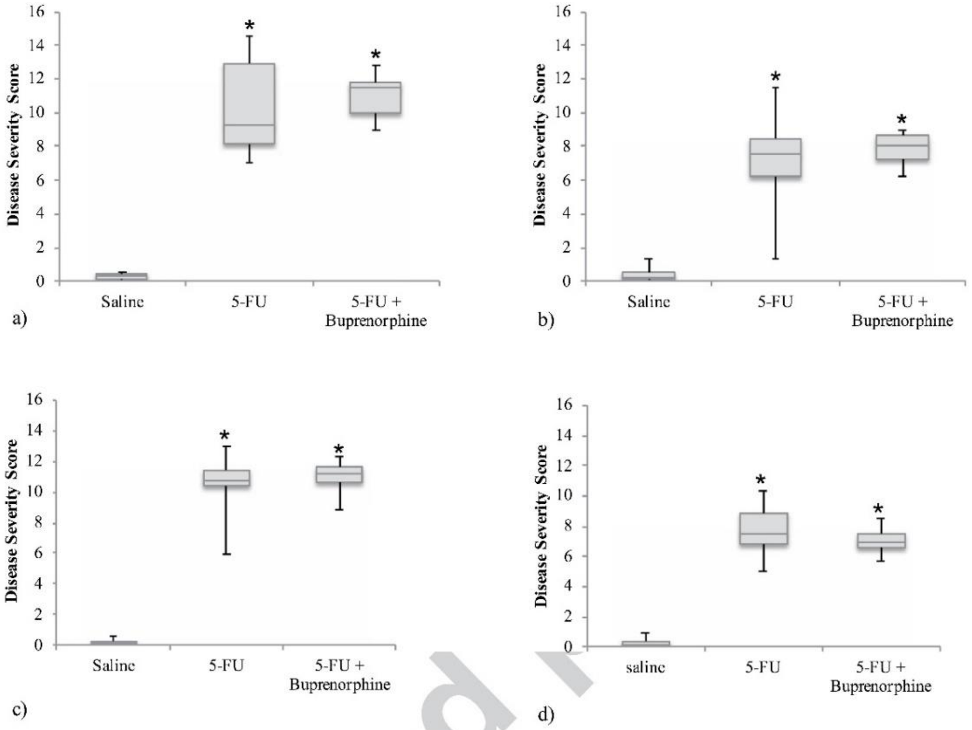

Figure 4. Disease severity score of distal ileum 72 hour (**a**) and 120 hour (**b**) time point and proximal jejunum at 72 hour (**c**) and 120 hour (**d**) time point of rats administered saline, 5-FU and 5-FU + buprenorphine. Data presented as the first and third quartiles, horizontal lines represent the median disease severity score and the whisker ends represent the maximum and minimum score. *Indicates $p < 0.001$ compared to saline.

Changes in affective state were observed at 120 hours post 5-FU administration. Optimistic decisions increased across time points in animals with mucositis, implying that the healing process of mucositis may have led to an increase in positive affective state. This was evident in the current study that showed histological severity score decreased with accelerated repair in the damaged intestine 120 hours post 5-FU injection. Histological and disease severity parameters in previous studies have shown evidence of a healing stage 120 hours post 5-FU administration in rats, as gastrointestinal tissue damage subsided, indicated by cell hypertrophy observed via compensatory crypt and villi elongation in the ileum and jejunum, myeloperoxidase activity normalization, and reduction of disease activity index parameters[6,19].

Interestingly, the analgesic agent, buprenorphine, failed to ameliorate the negative impact on affective state across the 72 hour and 120 hour time points. Differences over time were only present in rats treated with 5-FU alone. Whilst this result appears paradoxical given the commonplace use of opioids to treat severe pain, their use is hampered by side-effects including nausea, respiratory depression, constipation, and urinary retention[20,21]. The associated side-effects of buprenorphine exhibited in rodents are likely playing a part in this judgement bias observation. Side-effects of buprenorphine in rats include; appetite suppression, decreased body weight, and pica behaviour[21,22]. In the current study, buprenorphine had a striking effect on the daily disease activity parameters and bodyweight which is consistent with previous literature[17]. Rats administered 5-FU alone or in combination with buprenorphine showed a significant decrease in bodyweight compared to saline control groups. This decrease in bodyweight was further potentiated throughout the duration of the study in animals administered buprenorphine compared to 5-FU alone. The loss of bodyweight, and side-effects of buprenorphine, most likely had a substantial impact on affective state. These clinical signs are a potential cause of the decreased optimistic behaviour observed across time points when the recovery phase of mucositis was underway.

However, there is an alternative theory for the negative affective state recorded across the 72 hour and 120-hour time points. The repeated administration by injection, and the associated physical restraint used to administer buprenorphine may have had a substantial impact on affective state. A study by Stuart and Robinson[23] demonstrated that conventional animal restraint used during intraperitoneal substance administration provoked a negative affective state in male Lister Hooded rats. In the current study the route of administration was subcutaneous and the restraint method used was scruffing. Whilst restraint stress was likely present in our study, the subcutaneous restraint method is expected to have had minimal impact compared to restraint methods used during intraperitoneal administration. Nonetheless, repeated restraint stress has previously been applied to cause depressive-like phenotypes and induce negative affective state in rats[23–25]. This is a limitation of the current study, and further research is required to investigate the effects of analgesics, subcutaneous administration and restraint stress on affective state. Through addition of further groups to include an analgesic alone treatment group, saline injection group, and groups utilising alternative methods of analgesic administration such as oral delivery, it

Phase	Description
1	Rats were handled twice daily for a 10-minute duration to become acclimatised to handling. The duration of this phase was five days.
2	Each day rats were tested four times, with test duration of five-minute. Food bowls were located in the testing apparatus, with associated rewards for each individual rat positioned on top of the sand in each bowl. The PVC pipe did not contain any sandpaper. The duration of this phase was five days.
3	Sand paper was positioned in a PVC pipe between the two transparent perspex boxes in the testing apparatus. Each day rats were tested four times, comprised of two cheerio trials and two chocolate trials. The daily order of these trials was determined independently by randomisation. A reward item was positioned in the associated bowl with the associated sandpaper also present. A timer was started once rats were positioned in the start box, and times were recorded for latency to vacate start box, enter goal box, approach a reward bowl, approach the correct reward bowl, and begin to consume reward. Testing ceased once the rat started to consume the reward or when 5 minutes had lapsed. Cleaning of apparatus with seventy percent ethanol solution was performed at the completion of each test. Once rats successfully completed the training on five consecutive days, they were advanced to phase 4.
4	Duplicate protocol to phase 3, except reward items were positioned under the surface of the sand in reward bowls at various depths, and rats were required to unearth reward items. Following successful removal, burial depth of rewards was increased for each succeeding trial, until entirely below the surface. Conditions required for advancement to phase 5 were the same as phase 3.
5	Duplicate protocol to phase 4, except reward items were placed entirely below surface of sand for every trial, and one trial each day selected at random did not contain a reward item. If the first bowl the rat foraged in would normally have contained a reward, the trial was deemed a successful unrewarded trial. Conditions required for advancement to phase 6 were the same as phase 3 and 4.
6	Duplicate protocol to phase 5, except intermediate grade sandpaper was matched with unrewarded trial (P180). The duration of phase 6 was three days.
7	Each day rats were tested once. PVC pipe contained intermediate sandpaper (P180), and food bowls did not contain a reward. The time taken for rats to begin foraging in any bowl was recorded, and record of the first bowl approached and foraged in was taken. The duration of phase 7 was five days.

Table 2. Promotion criteria for each phase of the judgement bias test.

would be possible to tease apart the contribution of restraint and injection technique on affective state. Future study design would also benefit from inclusion of additional methods of assessing affective state such as the conditioned place preference test.

There is growing recognition of the limitations associated with animal models, and the challenges to translate outcomes from animal research to medical practice. In clinical studies changes in quality of life and affective state have been associated with cancer treatments[26]. Increase in negative affective state caused by chemotherapy has the potential to exacerbate treatment side-effects[11,27]. Whilst animal models in mucositis research are a valuable tool, they are not without limitations and complications and outcomes do not always translate to the clinic. Key limitations include; dose and scheduling issues (dose rates do not translate to humans), the absence of an emetogenic reflux in rats, impact on drug clearance and toxicities due to sex and strain differences in metabolic enzyme profiles, and difficulties assessing emotional states and other affective behaviours[14,28]. In previous studies the most common indicators used to assess disease progression and wellbeing in mucositis models have been non-specific clinical scoring, bodyweight change and histological analysis. These measures have drawbacks of being retrospective, and the possibility of not indicating true emotional experience[29]. Measuring judgement biases provides a clearer understanding of positive and negative emotional valence. Judgement bias assessment could also be utilised to improve humane endpoint implementation, thus improving animal welfare.

Whilst research into psychiatric disease has seen cognitive bias assessment utilised as an important tool, other biomedical areas of research are yet to incorporate this strategy into disease models. Results from this study demonstrate that the judgement bias test utilised was efficacious to evaluate the emotional state of rats with chemotherapy-induced mucositis. Furthermore, these findings provide a foundation for future biomedical research to incorporate cognitive bias methodologies such as a judgement bias test to determine effectiveness of novel therapeutics and the mechanisms by which emotion can influence cognitive processes in animal models. Refinement to the experimental design associated with use of these animal models will likely expedite successful transitioning of novel therapeutics to clinical practice.

Methods

Animals and Experimental design. Male Sprague Dawley rats (ArcCrl:CD(SD)IGS, n = 60) were acquired from a specific pathogen-free, barrier-maintained animal facility (Laboratory Animal Services, University of Adelaide, Adelaide, South Australia). This study selected male SD rats due to evidence from previous literature demonstrating successful training using cognitive bias methodologies[12,30,31]. Upon arrival rats were housed in standard open-top cages (415 mm × 260 mm × 145 mm, Tecniplast, Exton, PA, USA) in groups of three, maintained in a room temperature of 21–23 °C with a 12 hour reversed light/dark cycle. All cages were supplied with shredded paper and fibre cycle bedding (Animal Bedding, Fibrecycle Pty Ltd, Queensland, Australia). Food (standard rat chow, Rat and Mouse Cubes, Specialty Feeds, WA, Australia) and RO water was provided at *ad libitum*. All experimental protocols were performed during the dark phase, under red lighting.

Rats were randomly allocated into three experimental groups; saline ip (n = 20); 5-fluorouracil (5-FU) (n = 19) (150 mg/kg 5-FU ip;Mayne Pharma Pty, Ltd, Mulgrave, Vic, Australia); and 5-FU + buprenorphine (n = 19) (150 mg/kg 5-FU ip + 0.05 mg/kg buprenorphine q12hr sc). On day 0 all rats were injected intraperitoneally with 5-FU (150 mg/kg) or saline. Rats in 5-FU + buprenorphine treatment group were injected subcutaneously with 0.05 mg/kg buprenorphine. Buprenorphine was administered at 12-hour intervals for the duration of the study. Buprenorphine was chosen, as it is a commonly used analgesic in rodents. It is favoured due to its simple administration, extended action, partial agonist action at the μ-opioid receptor, and effectiveness in various pain models[22]. Judgment bias response, bodyweight and disease activity index data were collected as described below. Rats were humanely euthanised by CO_2 asphyxiation at two time points; either 72 hours or 120 hours post 5-FU or saline administration in order to assess gut architecture via histology.

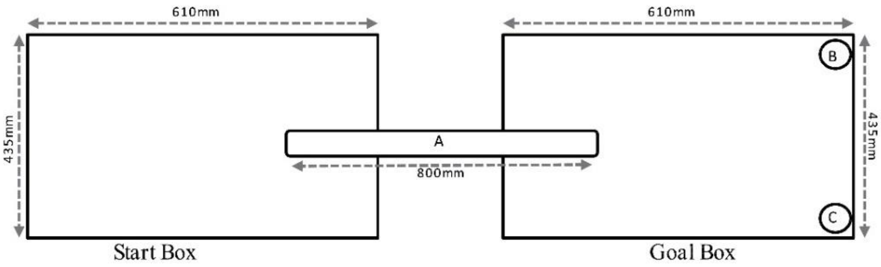

Figure 5. Schematic of the apparatus setup used in the judgement bias test. The apparatus consisted of a start and goal transparent perspex box. The start box was connected to the goal box by a PVC pipe (**a**) that was lined with sandpaper. A blue (**b**) and brown (**c**) reward bowl was placed at the end of the goal box.

Protocols were approved by the University of Adelaide Animal Ethics Committee and conducted in accordance with the Australian code for the care and use of animals for scientific purposes[32].

Judgement Bias Test. Rats were trained using a judgement bias paradigm to distinguish between two tactile stimuli associated with two rewards. The judgement bias paradigm used was based on a previous study by Barker, *et al.*[30], and consisted of seven phases that included a promotion criterion for each phase (Table 2). The training and testing apparatus consisted of two transparent perspex boxes (610 mm × 435 mm × 215 mm). The start box and goal box were connected with a PVC pipe (100 mm diameter). The inner surface of the PVC pipe was lined with either coarse (P80) or fine (P1200) sandpaper depending on the association. A blue and brown bowl was located at the end of the goal box and contained cinnamon and coriander-scented sand (1% by weight of spice) respectively (Fig. 5). The rewards consisted of a high-positive chocolate reward (Cadbury, London, England) or a low-positive cheerio reward (UncleToby's, Victoria, Australia). Each rat was randomly allocated a sandpaper association paired with a reward and bowl. During each trial the reward was placed in the bowl and paired with the associated sandpaper. During the training phase, each rat received two chocolate trials and cheerio trials per day. A trial commenced when the rat was placed in the start box and terminated when at the start to consume the reward or 5 minutes has lapsed. The daily order of these trials and associations was determined independently by randomisation. During the testing phase rats received one ambiguous test, which consisted of the PVC pipe, lined with intermediate sandpaper (P180) and no reward being present. Judgement bias was measured by investigating the foraging behaviour (bowl rat first foraged) in response to the ambiguous probe.

Once each training phase was successfully achieved, rats entered the test phase and were randomly assigned an experimental group. Judgement bias was measured 24 hours prior to 5-FU and saline injection to obtain a baseline (day 0), 72 hours post injection (day 3; saline n = 20, 5-FU n = 19, 5-FU + buprenorphine n = 19) and 120 hours post injection (day 5; saline n = 10, 5-FU n = 9, 5-FU + buprenorphine n = 9). Animals that were determined to be in a positive emotional state demonstrated foraging behaviours that would correspond with high-positive reward during the ambiguous trial compared to those in a negative emotional state.

Disease Activity Index and Bodyweight. Following 5-FU or saline injection disease activity index (DAI) and bodyweight were monitored and measured daily to determine the severity of mucositis. DAI was measured on a scale of 0–3 severity per parameter based on rectal bleeding, stool consistency, bodyweight loss, and overall general condition of the animals described by Mashtoub, *et al.*[6]. General condition was determined based on dull or ruffled coat, hunched, pale or sunken eyes, dehydration, squealing when handled and reluctance to move.

Histological Analysis. Sections (2 cm) of distal ileum and proximal jejunum were collected and fixed in 10% formalin buffer solution. Small intestinal sections were transferred to 70% ethanol 24 h post tissue collection. Tissue samples (4 μm) were processed and embedded in paraffin and stained with haematoxylin and eosin (H&E).

Histological analyses were conducted using a light microscope (Olympus Corporation CX-31, Tokyo, Japan). Histological severity was scored in the jejunal and ileal sections by grading eight histological criteria from zero (normal) to three (maximal damage) in a blinded fashion. This included: enterocyte disruption, reduction in goblet cells numbers, thickening of the submucosa and muscularis externa, Villus fusion and stunting, crypt cell disruption, lymphocytic infiltration and crypt disruption[33].

Statistical Analyses. Statistical analyses were performed using Megastat Excel Add-In (version 10.3 Release 3.1.6 Mac, McGraw-Hill Higher Education, New York, NY) and SPSS (SPSS Inc., Chicago, IL, USA). Judgement bias data were analysed using a generalised linear mixed model (binary logistic) with the logit link function, where the implicit residual variance is on the underlying scale $p^2/3$[34]. The fixed effects were day, treatment, and day x treatment interaction taking into account individual animal to allow for repeated measures. Pairwise comparisons of the estimated marginal means were performed with sequential Bonferroni adjustment. Bodyweight and DAI data were analysed using a repeated measures ANOVA with Tukey's post hoc test. Histological disease severity score was analysed using a Kruskal-Wallis test with Mann-Whitney U-test. Data were deemed significant at $p < 0.05$. All data were expressed as means ± standard error of the mean.

Q2 **Data availability.** All datasets generated and analysed during this study are included in supplementary information files.

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Author Contributions

R.P.G. conducted experimental work, data analysis and prepared the manuscript. T.H.B., K.A.L. and D.A.B. contributed in experimental work and data analysis. G.S.H. assisted in data interpretation and manuscript preparation. A.L.W. was involved in experimental design and work, data interpretation and manuscript preparation.

Additional Information

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