A COMPARATIVE ANALYSIS OF THE BEHAVIOURAL AND COGNITIVE EFFECTS OF TOXIN-INDUCED SICKNESS IN THE RAT *(RATTUS NORVEGICUS)* AND THE HONEYBEE *(APIS MELLIFERA)*

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Tasks designed to measure affective state in animals are valuable in researching the aetiology and treatment of affective disorders in humans. However, many traditional measures are ineffective in assessing affective valence, and judgement bias tasks were designed to overcome this problem. In this thesis I aimed to identify whether rats displayed a judgement bias during sickness, and also whether the task outcomes were translatable to an invertebrate species.

A comparative study was performed with a vertebrate (rat) and an invertebrate (honeybee) model exposed to toxin-induced sickness. Behavioural indicators of sickness were assessed following toxin administration, and the animals' expectations of reward and punishment were measured on a judgement bias paradigm.

This thesis includes the first behavioural characterisation of sickness in honeybees. Quinine-induced sickness in the honeybee was accompanied by a biasing of ambiguous information consistent with a negative affective state. A judgement bias was also observed in rats treated with lithium chloride, but this finding was not repeated on replication of the experiment. Methodological problems were identified and the training protocol was revised to accelerate learning of the task and to reduce extinction of responding.

In conclusion, evidence of a sickness-induced negative affect in animals was identified in this thesis. This correlates with sickness in humans, thus reinforcing the argument that negative affective states associated with sickness may have an evolutionary basis. In addition, the honeybees' performance on the task was similar to that seen in vertebrate animals, showing the potential for the honeybee model to be used in investigations of emotion. However, alterations need to be made to the specific protocols to improve the methodology for measuring judgement bias in both honeybees and rats, and recommendations are made for future experimental designs.

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Chapter 1 - Affect and biased judgement

1.I Introduction

It was once the consensus of scientific communities that animals other than humans (henceforth referred to as 'animals') were not capable of higher cognitive processing and were therefore devoid of emotion or the capacity to suffer. In recent years, opinions have moved on and our approaches to scientific research involving animals are changing accordingly. One milestone that is still to be reached is the answer to the 'big question' as to what extent animals are capable of subjectively experiencing emotions. The idea that animals may actively experience emotions, rather than display automatic, mechanistic responses to stimuli is one that is regularly discussed. To our current knowledge, humans are perhaps unique in their abilities to subjectively experience pleasantness and unpleasantness, and have conscious experiences. The debate is still open as to whether animals can experience a form of 'consciousness' (see Mendl et al. (2009)), but overall there is a growing willingness to accept that animals are capable of experiencing affective and emotion-like states, where their exhibition combines many features of physiology, cognition and behaviour in common with those seen in humans experiencing emotions (Harding et al., 2004, Brydges and Braithwaite, 2008). I do not attempt to confirm or deny the notion of animal consciousness in this thesis, although it is discussed further in section 1.I.i. Instead, the direction of current research serves to measure the emotional responses of animals without implying a capacity for subjective experience. In this manner, we can continue to work towards improved welfare and understanding of animals without having to make too great a leap of faith. This is achieved by concentrating on two aspects of emotion that can be measured objectively, namely cognition and behaviour.

Box 1.1: A note on definitions: Moods, emotions and affective states

Emotions are typically defined as acute, and resulting from specific stimuli (e.g. being frightened or scared by something), whereas moods are long-lasting and do not necessarily result from an immediate cause (e.g. anxiety). Emotions and moods can be classified in terms of arousal (e.g. calm/ excitable), and in terms of valence (e.g. positive/negative). Affective state is a term often used to refer to both mood and emotions. (See Paul et al. (2005)).

The experiments in this thesis were undertaken to determine whether an emotional component accompanied toxin-induced sickness with the view that this would expand our current knowledge of the set of biological syndromes that alter affective states in animals. Furthermore, I compare experimental outcomes from rats – a species commonly used in mood and emotion research and the third most commonly used species in scientific research overall (Home Office, 2013) - and the honeybee - an invertebrate model currently almost absent from the field of emotion research. It is an aim of this thesis to determine whether honeybees are potential candidates for replacement of other mammals in emotion research. The major technique that I used to study emotion in these two species was the judgement bias task. The task has already been demonstrated as being capable of detecting symptoms of negative mood in vertebrate and invertebrate species subjected to a range of different manipulations of affective state.

1.I.i Sentience and consciousness

The existence of the field of animal welfare primarily arises from the assumption that animals may be sentient, which refers to their ability to experience pleasurable states such as joy, and aversive states including fear and pain (Broom, 2007). We are not able to assess these abilities directly, but rather by investigating whether some animals are capable of complex processing of information in a manner that reflects emotional processing in humans.

When we talk about affective states in animals, we do so without implying consciousness, which in this review is defined as the awareness of the experience of an affective state. While emotions and moods likely exist in animals, there is not necessarily any meta-cognition involved (Shettleworth, 2009). A well-developed example of the distinction between an emotion and its subjective experience is that comparing nociception and pain. Most animals are equipped with neural mechanisms that cause them to withdraw from or avoid nociceptive stimuli, and to a layman, that might be enough to deduce that they are experiencing pain when in fact they are observing a reflex. Pain is the feeling that nociception has occurred, and is

characterised by an awareness of its unpleasant nature. Overt behaviour does not necessarily reflect conscious awareness. For example nociceptive reflexes are observed in unconscious, anaesthetised or brain-dead humans who are presumably unable to experience affective state or emotion, indicating that a conscious awareness of pain is not necessary for these reflexes to occur. Similarly, this line of thinking can be applied to the display of other emotions, where emotional responses and subjective awareness can be dissociated.

1.I.ii How do humans experience mood and emotion?

As humans, our moods and emotions guide our behaviour and decision-making in order to maximise Darwinian fitness (Bateson et al., 2011a). We can consider emotions as a system of ascribing value to events, whether desirable or undesirable, which in turn influences future behaviour and decision-making in pursuit of experiencing more of the desirable and less of the undesirable events. Emotion-inducing events are thus afforded a greater significance in terms of attention and memory (Rolls, 2005). For example, if we recount the events of say, the previous week, we will tend to notice those that elicited feelings of joy or fear or anger or pleasure etc. and dismiss the more ordinary and mundane moments.

However, our affective states do not always reflect the valence of stimuli in the immediate environment, and this is typical of more enduring moods. Underlying mood states can affect logical reasoning of and the perception of our surroundings. For example, during periods of sadness normally pleasurable activities may not provoke as great a hedonic response, food many not be so appealing, and one may have less of an desire to socialise (Treadway and Zald, 2011). We therefore differentiate emotion and mood in terms of their immediate and 'free-floating effects', respectively (Box 1.1).

It is thought that the range of emotional states that an organism can experience is indicative of the complexity of its adaptive niche, which in humans involves sociocultural and interpersonal contexts as well as those that are physical (Dolan, 2002). With many animals, we possess a more simplistic view of their emotional experience with less consideration of these contexts; however, this does not prevent

many people from assigning anthropomorphic constructs to them (Shettleworth, 2010).

1.I.iii A translational framework for emotion

Unsurprisingly, as our only subjective measure of emotions arises from linguistic self-report in humans, there is an inherent problem with emotion research in animals that manifests as their inability to simply tell us how they are feeling. To this end, Mendl et al. (2010) presented a model that can be translated to non-human species that is based on an established functional framework of human emotion (e.g. see Russell and Barrett (1999) and Burgdorf and Panksepp (2006)). The model outlined in Figure 1-1 encompasses a two dimensional framework correlating certain emotions with an associated degree of arousal and emotional valence. It circumvents many of the limitations of physiological and behavioural observations (see 1.I.v) by allowing the distinction to be made between oppositely valenced emotions with similar levels of arousal (such as fear and excitement), or similarly valenced emotions that are difficult to distinguish behaviourally (such as anxiety and depression). In principle this model can be applied to any species regardless of the degree of cognitive ability or the capacity to experience subjective feelings as it relies solely on instinctive responses of animals to achieve survival goals (Nettle and Bateson, 2012).This model is developed from well-established versions of a framework that covers the principle variations in moods and emotions in humans, albeit it does not span the entire extent of emotional complexity (Mendl et al., 2010).

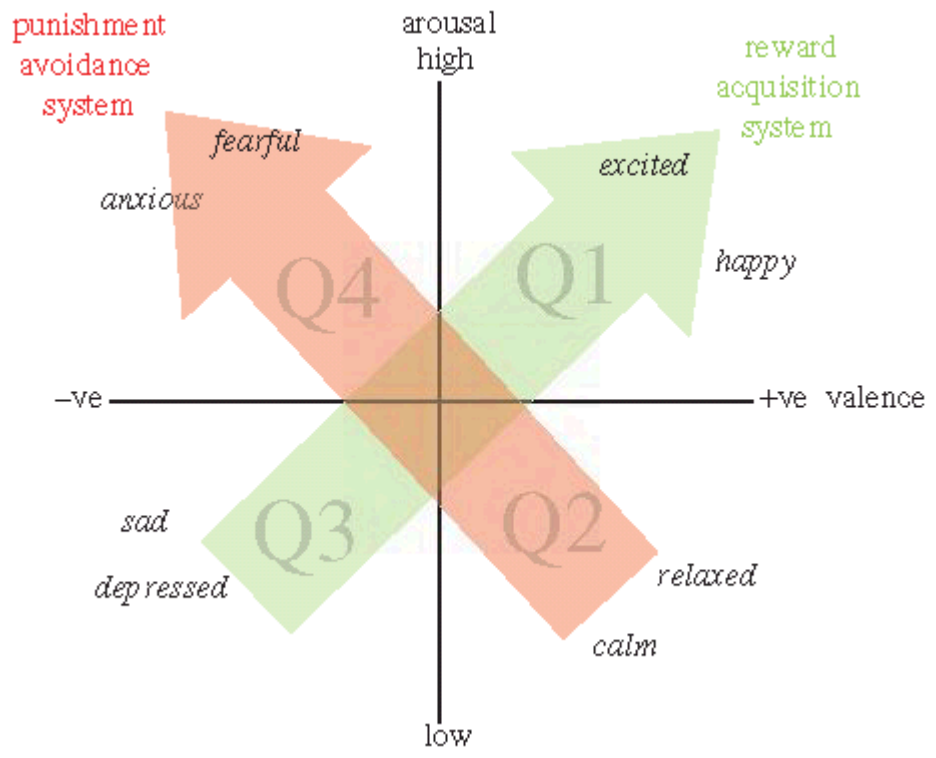


Figure 1-1 ***Core affect represented in two-dimensional space*** *. Words in italics indicate possible locations of specific reported affective states (including discrete/basic emotions.)Positive affective states are in quadrants Q1 and Q2, and negative states in quadrants Q3 and Q4. Arrows indicate putative biobehavioural systems associated with reward acquisition and the Q3–Q1 axis of core affect (green), and punishment avoidance and the Q2–Q4 axis of core affect (red). Adapted from Russell (e.g. Russell & Barrett 1999) and Panksepp (e.g. Burgdorf & Panksepp 2006).Figure reproduced from Mendl et al 2010.*

In this model, Q1 represents moods and emotions of high arousal and positive valence such as happiness and excitement; Q2 shows those of high arousal and low valence, such as calmness and contentment; Q3 emotions and moods have low arousal and are negatively valenced, and so represent depression and sadness; and finally Q4 represents those with high arousal and high valence, such as fear and anxiety.

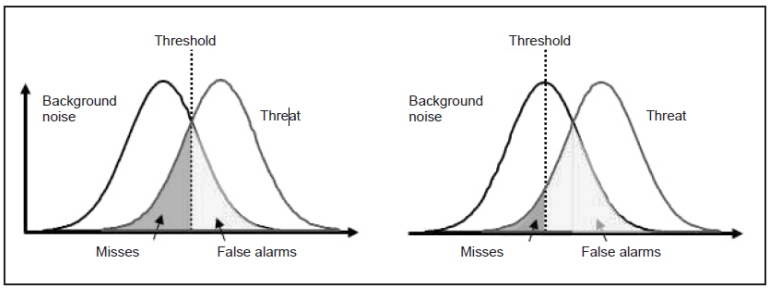
Each of the four quadrants (Q1-4) can be classified in terms of reward acquisition and threat avoidance, and an animal's position in the core affect space reflects the relative success or failure of an animal's achievement of either of these outcomes. Q1 and Q3 consist of affective states associated with the acquisition or loss of reward (or other positive reinforcers) respectively, and Q2 and Q4 represent the affective states following the avoidance or receipt of punishers respectively.

Emotions are transient and an animal's position in the core affect space is modulated by sensations and motivations. This can be illustrated by an example of a stressed animal hunting for food. With their emotion initiating in the Q4 quadrant due to frustration, they might obtain prey, leading to temporary elation, which lies in the Q1 quadrant. As they have consumed the food and are contented by the consummatory pleasure, their emotional state would lie in the Q2 quadrant (Mendl et al., 2010).

Moods similarly also occupy this two-dimensional model and reflect a cumulative function of discrete emotions with similar valence that become longer-term states. Whereas emotions are induced by appraisals of the immediate environment and invoke instant actions and are by that nature short-term, moods are long-lasting and can exist in the absence of these immediate emotion-inducing stimuli (Nettle and Bateson, 2012). We can interpret the outcome of reward acquisition as not only inducing a positively valenced emotion, but also increasing the animal's expectation of further attainment of reward. With an increased expectation of reward, weaker environmental cues are more likely to be interpreted as predicting fitness-related events, provoking a behavioural response (see Box 1.2). In humans we refer to this phenomenon as optimism (Nettle and Bateson, 2012). On the contrary, a number of failed attempts to achieve reward leads to a more negative affective state which correlates with an increase of the signal detection threshold, where animals require stronger or more numerous reward-predicting stimuli in order to initiate reward-approach behaviours (Nettle and Bateson, 2012). We might interpret this as pessimism. In summary, the past experience of rewards and punishers raises or lowers the threshold of signal presentation required to provoke animals to actively respond to cues predicting rewards or punishers. This underlines the dynamic nature of emotional responses, and their effects on longer-lasting, but flexible mood states. In this sense moods also provide an adaptive function, for example individuals exposed to acute stress from predatory attack may become fearful short-term, and with repeated exposure develop an anxiety-induced and long-lasting increase in vigilance towards threat. Periods of happiness resulting from repeated bouts of excitement related to capture of prey or exposure to a sexual mate in a certain environment may cause the animal to spend more time in that location to serve survival and reproductive goals.

Box 1.2 Signal detection thresholds: A summary

It is typical for the fitness-related stimuli in an animal's environment to be obscured by background noise overlapping the signal. For example the sound of a predator stalking in a bush might be obscured by the sound of the wind passing through it.



The horizontal axes represent the strength of sensory information reaching the subject, while the 2 normal distributions represent the probability of different amounts of evidence generated under the presence of a threat, and under background noise only. Crucially, the 2 distributions overlap. If the subject sets a high threshold for mobilizing a threat response (left panel), there will be a fair number of real threats missed as well as a fair number of false alarms. If the subject sets a lower threshold (right panel), the subject will miss many fewer real threats, but only at the cost of much more frequent false alarms. We argue that anxiety disorders represent unusually low personal thresholds for threat response.

Thus, animals possess a system whereby a threshold of the signal strength must be met in order for them to mobilise the physiological and behavioural systems needed to respond to the signal. Falsely interpreting the signal can be costly to the animal, as is described below in the context of incorrectly responding to signals for threat and food events:

Response outcome	Threat	Food
False negative – behavioural response not made when threat/food was present	Predation – potential injury or death	Hunger
False positive – behavioural response made when threat/food was not present	▪ Cessation of current activities that have fitness costs (e.g. feeding, sleeping etc.) ▪ Caloric value of mobilising behavioural response	

The likelihood of an outcome occurring as well as the potential costs and benefits of responding to a signal contribute to the decision-making process of an animal to perform a response. If the likelihood of the event occurring is low, a high threshold is set (i.e. animals will respond when there is little or no ambiguity regarding the environmental stimuli to avoid false positives), whereas if the event is very likely to occur, a low threshold is set.

Typically the costs of false negatives tend to be higher than false positives, but can vary depending on the physical state of the animal. For example, the cost of not obtaining a prey item is higher to a hungry animal than a satiated animal.

Summarised from Nettle and Bateson (2012) and Bateson et al (2011a)

The cost to an animal of performing a behavioural response also contributes to the position of its affective mood in this framework; for example it is more costly to an animal with compromised fitness than a fit animal to chase prey or escape attack, and thus their levels of responding will be reduced accordingly (Nettle and Bateson, 2012). A reduction in activity is compatible with the definition of a negative mood. It is, however, important to note that although the emotional responses of an animal might reflect underlying mood states, this is not always the case e.g. a happy mood might be interrupted by the threat of predation, or a depressed mood might be temporarily lifted by the discovery of prey.

Finally, the model can be used to explain core affective state, which reflects the combination of an animal's long-term mood state and any reactions to immediate emotion-inducing events. For example, animals with Q1 affective states (happiness, excitement) might be more expectant of reward, whereas in Q3 (depression, sadness) they would be less expectant of reward. In Q4 states (anxiety, fear) they might have a greater anticipation of negative events occurring, whereas in Q3 states (calm/contentment) this anticipation would be reduced. That an animal's affective state, and particularly its valence, can be identified by their anticipation of rewards or punishers is of considerable value in emotion research, particularly as there are many pitfalls associated with other measures which are briefly outlined in section 1.I.v.

1.I.iv The necessity of measuring mood and emotion in animals

The assumption that animals can experience affective states is of interest to researchers in many different fields where the cause, effect or merely the presence of an altered affective state needs to be correctly identified. This includes a wide range of disciplines, from psychopharmacology and neuroscience, to zoology and comparative psychology (Paul et al., 2005). In addition, we must also consider the welfare of animals that we use in our research as well as those kept domestically, on farms, and in zoos, and many who research emotion in animals do so to provide guidelines that influence their husbandry and treatment to maximise their wellbeing. Researching

emotions in animals can also provide a functional perspective of emotion and their evolutionary basis, which may inform us about the origins of human emotion.

1.I.iv.1 Animal welfare

There is concern regarding the extent of the use of animals in scientific research. According to the Home Office, in 2012 alone over 4 million animals were used, and 3.3 million of these were rodents. There tends to be a hierarchy regarding the treatment of animals, where animals that show evidence of suffering or self-awareness that is reflective of that observed in humans are afforded greater protection. For example, the rights and protection of great apes were influenced by research implicating higher levels of cognitive function, including intelligence, self-concept and theory of mind (Cavalieri and Singer, 1993). Legislation controlling animal research in the UK also provides additional protection for primates, cats, dogs and horses over other vertebrate animals, where our assumption is that species with less-developed neural systems lack complex cognitive abilities and thus the ability to suffer, and as such there are fewer restrictions regarding their use in research.

(Russell and Burch, 1959) introduced the concept of the 3 R's (Reduction, Refinement and Replacement) to encourage better scientific practice in regards to the use of laboratory animals in scientific research, and it is the responsibility of all researchers working with animals to adhere to these principles. Reduction seeks to minimise the number of animals required to obtain the necessary data, which can be achieved by sharing resources and developing more effective means of data collection and analysis; Refinement refers to the improvement of the conditions of the animals used in scientific procedures, with a particular focus on the reduction of pain, stress and suffering. The drive to improve animal welfare has traditionally focused on biological functioning, such as good general physical health and growth, but more contemporary thinking has led to including considerations of mental health in welfare assessments (Harding et al., 2004, Broom, 2007, Boissy et al., 2007b, Dawkins, 2008) and promoting positive welfare in captive animals (Boissy et al., 2007b). These considerations of welfare are by no means limited to animals kept for scientific research, but are

extended to animals reared for farming, kept domestically or in other captive environments. The development of the judgement bias task introduced in 1.I.iii (and further discussed in 1.III) has improved our assessment of captive animal welfare and may lead to improvements in animal husbandry.

The final 'R'- replacement - involves using methods that avoid the use of animals defined as protected under the Animals (Scientific Procedures) Act 1986 - which includes all living vertebrates - or to replace these animals with ones that are not protected under the act. Replacement of protected animals with invertebrates in research is becoming more prevalent, and not surprisingly, we are learning more about the capacity of invertebrates to suffer. In 1993 the octopus was the first invertebrate species to be added to the list of animals protected in an amendment to the Animal (Scientific Procedures) Act 1986 due to the discovery of more complex neural systems and cognitive capabilities. This was followed in 2012 with the inclusion of all cephalopods. Whether this will be followed with the addition of other invertebrate animals is not yet known, however evidence is building in favour of protecting more species, particularly when questioning whether invertebrates can experience emotion or feel pain.

1.I.iv.2 Perspectives on the function and evolution of emotion

As humans, it is abundantly clear that emotions are an essential part of survival. Repair of emotional disorders is a primary concern of current medicine, where the quality of life of many is affected by the increased morbidity of these disorders. The World Health Organisation lists depression as the leading cause of disability worldwide, and estimates that it is the third largest cause of morbidity (WHO, 2008). By learning more about the evolution of emotional states and their functions, we might gain a better understanding of their biological bases.

Arnold (1960) described emotional experience as a three-step process. The first is appraisal of external stimuli, the second is the physiological effects preparing the body for action and the concurrent changes in internal states of arousal (which may or may

not involve subjective awareness), and the third is the appropriate behavioural response (e.g. approach, flee etc.). The term 'emotion' may be used as a single, general term to describe this group of phenomena that serve to increase the evolutionary fitness of an individual (Plutchik, 2001). Appraisal of environmental stimuli occurs in terms of familiarity, predictability, and whether they have positive or negative associations. For example, an animal encountering something in their environment that is unpredictable, unfamiliar and unpleasant may induce the emotion of fear, resulting in an urge to flee and the physiological changes necessary to facilitate this movement (e.g. increased heart rate). Consequently, emotions are viewed as event-focused, and transient.

In many ways, emotions act as a feedback loop that restores equilibrium towards the very stimulus that induced it. For example a threatening stimulus may invoke behavioural impulses to flee that stimulus, thus re-establishing the condition that existed before that threat (Plutchik, 2001). A more relevant example to humans may be a display of emotional distress that signals the attention and support of others, which will in turn help re-establish the emotional equilibrium. Emotions arise in response to anticipation of positive and negative events, and serve to alter the behaviour and physiology of an animal in order to achieve survival or reproductive goals. Emotions help animals organise information in their environments, by distinguishing predator from prey, and a potential mate or enemy (Scott, 1980).

It is important to note that even single celled organisms that lack cognitive complexity (such as bacteria) are capable of these approach and avoidance responses when exposed to rewards and punishers (Macnab and Koshland, 1972). When we also consider that the mechanisms by which these behaviours are regulated in invertebrate animals are functionally homologous to those present in more complex vertebrate animals (LeDoux, 2012), it can be surmised that the origins of emotions have an evolutionary basis. This functional perspective that emotions are adaptive would suggest that some degree of emotionality is present throughout phyla with less complex neural systems, with the most sophisticated version present in humans. Changes in behaviour and physiology in animals that match patterns associated with

emotions reported by humans provide an estimation of the emotional state experienced by the animal, which we can then study more explicitly.

1.I.iv.3 The use of animals in research

Research animals are used to model emotional symptoms of mood and psychiatric disorders in humans in order to identify the cause of emotional symptoms, or to determine whether they are sensitive to treatment. It is of importance to establish that the physiology and behaviour of the animal model is at least in part related to the emotion intended to be measured. This can only be achieved by the development of more sophisticated measures of emotion and enhanced understanding of the function and valence of emotional responses.

In addition to this, in fields of scientific research that may or may not be interested in animal psychology, the emotional state of the animal can still contribute to research outcomes. As emotions inherently signify changes in an animal's physiology and behaviour, they have the potential to impact the data generated. If data are obtained from animals with compromised affective states it may be abnormal, leading to false positive or false negative results, essentially wasting the animals and resources used in the study itself and any replication of the study (Garner, 2005). There is therefore a strong argument for maintaining a consistent affective state in lab animals in order to eliminate variability in research.

1.I.v Modelling and measuring emotion in animals

As stated earlier, to measure emotion in animals, comparisons have to be drawn from humans. In animal emotion research, physiological, behavioural and cognitive markers are mapped out and compared with the subjective component of human emotions to approximate the emotional state of the animal. This can be difficult as many of the behavioural and physiological markers are not unique to specific emotions and can be observed during emotional responses of differing valences (For examples see Table 1-1).

Emotion	Physiological response	Behavioural response	Subjective feeling
Fear	Increased heart rate, increased cortisol	Freeze/Flee	Dread
Anger	Increased heart rate, increased blood pressure	Attack	Anger
Elation	Increased breathing rate, increased cortisol	Active	Happiness

Table 1-1 ***Physiological, behavioural and subjective components of emotion.*** *The table describes changes in physiology and behaviour related to the emotions of fear, anger, sadness and happiness, and also the subjective feeling as reported by humans. Summarised from Paul et al 2005.*

1.I.v.1 Physiology and behaviour as proxy measures of emotion

Many of the physiological markers used by emotion researchers indicate emotional arousal (e.g. levels of circulating stress hormones (cortisol, adrenaline), changes in heart rate and changes in blood pressure (Bradley and Lang, 2000, Boissy, 1995)), but are limited in assessment of emotional valence. Many of the physiological indicators measured in highly aroused animals in a positive state (e.g. sexual arousal, play etc.) are similar to those found in animals highly aroused negative states (e.g. stress), so this approach offers a limited opportunity to differentiate positive and negative stressors. For example an increased heart rate could equally indicate anticipation of reward, fear, or simply a non-emotional increase in activity. Invasive procedures necessary to obtain this data, such as blood sampling, may also affect the animal's emotional state and subsequently skew the interpretation of study outcomes (Broom and Johnson, 1993). Normally, to sidestep this problem, several physiological indicators would be coupled with behavioural observations to produce a more rounded view of the animal's affective state (Broom and Johnson, 1993).

Behavioural proxy measures of emotion in animals include observing spontaneous behaviour and assessing learned responses. The most commonly used behavioural tests measure unconditioned responses in animals and tend to exploit the conflict of natural behaviours (e.g. exploratory behaviour versus fear of open spaces) to judge whether animals are experiencing emotion-like states (fear or frustration in this

example). However, when an animal exhibits a response to a behavioural test, it can be difficult to interpret the emotional state contributing to their actions - there is ambiguity in assessing whether this is a positive or negative response. For example, in the forced swim test animals are forced to swim in an inescapable cylinder; when the animals 'give up' (become immobile), they are said to be experiencing emotional despair (Porsolt et al., 1978, Porsolt et al., 1977). On the other hand, immobility in this test can be interpreted as an adaptive response, allowing them to survive and conserve energy until they are removed from the apparatus by the researcher (Abel and Bilitzke, 1990). Another example of a behavioural test with controversial interpretations is approach behaviour which is observed as a response to both rewarding stimuli (Tanimoto et al., 2004), as well as towards presumably threatening stimuli such as predators (FitzGibbon, 1994, Krams and Krama, 2002). Table 1-2 gives an overview and critique of these and other techniques used to measure anxiety- and depression-like states in rodent models.

It is difficult to make *a priori* predictions of how animals act when experiencing particular emotions as they cannot subjectively validate their behaviours verbally. Interpretation of the behaviours observed in laboratory settings is typically dependent on the experimental set-up itself, which may or may not contain the necessary features for the animal to engage in instinctual emotional behaviours, giving an incomplete picture of particular emotional responses. In summary, there are limitations with current methods for measuring emotional states. Behavioural and physiological measures can indicate whether an animal is emotionally aroused by a stimulus, but they are limited in the identification of the valence of an emotion (i.e. whether it is positive or negative), which is of our primary concern. There is, however, a cohort of studies that utilise a non-verbal indicator of 'subjective' experience in animals and humans based on the model of affective state described further in section 1.III. These measure a bias in cognitive processes that occurs with changes in affective valence, and is reflected in discrete emotional responses. The application of this judgement bias task is continuing to broaden and become an extremely useful resource in determining animal emotion.

Affective state	Measurement	Test	Observations	Criticisms	References
Depression	Behavioural despair	Forced Swim Test	↑ Immobility - reversed by antidepressants	• Immobility reflects adaptation to the test • Confounds of hypothermic exposure • Reliant on locomotor capability • Sensitive to acute treatments only • Poor face and construct validity	(Porsolt et al., 1977), (Abel and Bilitzke, 1990), (Lucki et al., 2001), (Petit-Demouliere et al., 2005)
		Tail Suspension Test	↑ Immobility and ↓ escape-related behaviours - reversed by antidepressants	• Restricted to mouse models • Sensitive to acute treatments only • Poor face and construct validity	(Steru et al., 1985), (Cryan et al., 2005)
	Anhedonia	Sucrose Preference	↓ Sucrose consumption	• Disagreement in interpretation – we observe changes in consummatory processes rather than preference	(Willner et al., 1987), (Weiss, 1997)
		Intracranial Self-Stimulation	↓ Self-stimulation for reward	• Confounded by changed activity levels and response deficits	(Olds and Milner, 1954), (Liebman, 1983)

Affective state	Measurement	Test	Observations	Criticisms	References
Anxiety	Conflict – exploratory urge vs. avoidance of brightly-lit areas	Elevated Plus Maze	↓Exploration of open spaces	• Lack of replicability between labs - differences in experimental set-up and data analysis • Sensitive to uncontrolled, experimentally-induced changes in state anxiety • Confounded by changed activity levels	(Pellow et al., 1985), (Hogg, 1996),
		Open Field Test	↓Exploration of open spaces ↑ defecation		(Hall, 1934), (Prut and Belzung, 2003)
		Light/Dark Box	↓Exploration of light compartment		(Crawley and Goodwin, 1980), (Bourin and Hascoët, 2003)

Table 1-2 ***An overview of behavioural tests used to measure anxiety- and depression- like states in rodents.***

1.II The relationship between affective valence and cognition

Cognition has been defined as "the mechanisms by which animals acquire, process, store and act on information from the environment" (Shettleworth, 1998), and this appraisal plays a role in the generation of emotional states (as outlined previously in 1.I.iii). Conversely, mood states have been shown to influence decision-making and other cognitive processes that underlie this appraisal. Mood states influence these cognitive processes in the form of attentional, memory and judgement biases, with perception and risk assessment also affected (Paul et al., 2005). In human psychology it has been clearly demonstrated that these cognitive processes are influenced by emotional states, where negative affective states in individuals induce greater attention towards threats, an increased recollection of negative memories and negative biasing of judgement when compared to those with positive affective states (Keogh et al., 2001, Koster et al., 2004, Fox et al., 2009, Caseras et al., 2007, Mendl et al., 2009). These biases in cognitive processes are explored in the subsequent sections of this thesis.

1.II.i The processing of reward and punishment in anxiety and depression

The theory introduced in section 1.I.iii explained the influence of discrete emotional information on the valence of a free-floating mood state. This affective process is impaired in subjects with psychological disorders (Eshel and Roiser, 2010). A hallmark of these disorders is an inability to exploit affective information in order to guide future behaviour, where abnormalities in the cognitive processing of reward and punishment are implicated in the aetiology and symptomatology of depression and anxiety (Eshel and Roiser, 2010).

The dysfunctions in cognitive processes observed in patients suffering from depression include indecisiveness and reductions in concentration (American Psychiatric, 2013), and attention and memory deficits (Roiser et al., 2009). A summary of cognitive tasks related to these processes is outlined in Table 1-4. These disruptions in the processing of affective stimuli are thought to cause the symptoms of depression (Eshel and Roiser,

2010), and are accompanied by function abnormalities in brain regions that are critical for information processing such as the amygdala and hippocampus (Clark et al., 2009, Ebmeier et al., 2006, Schultz and Dickinson, 2000). These dysfunctions translate cognitively, for example patients with MDD are less able to modulate their behaviour based on previous reinforcement (Eshel and Roiser, 2010), and are less likely to alter strategies in task performance in order to optimise reward (Henriques and Davidson, 2000). Patients with MDD are hypersensitive to negative feedback, which might represent a lack of - or loss of - reward. These patients show an increased probability to continue to make errors on tasks if an error was made on a previous trial (Beats et al., 1996, Elliott et al., 1996), reflecting a sensitivity to the lack of rewarding feedback. This hypersensitivity is congruent to a perceived lack of control by the individual and this in turn biases future actions leading to a cycle of learned helplessness (Seligman, 1972).

When we consider the aetiology of anxiety, we similarly see the abnormal processing of information in an individual's environment in terms of attention (Bar-Haim et al., 2007) and decision-making (Hartley and Phelps (2012); also see Table 1-4). Oppositely to depression, these dysfunctions tend to be related to the processing of punishing stimuli. Vigilance towards perceptual cues associated with threat needs to be rapid, and often occurs in preference to identification of competing cues in order for appropriate responses to be initiated (Mathews and Mackintosh, 1998). Processing of these threat-related cues can occur before individuals become aware of them, for example the speed of affective judgements of words is markedly faster when the word is preceded by a masked word (a word that cannot be consciously detected) which possesses the same affective valence (Greenwald et al., 1995, Greenwald et al., 1989). Similarly, pictures are also less likely to be given a positive rating if subliminally paired with an unpleasant image such as that of an angry face (Murphy and Zajonc, 1993, Niedenthal, 1990, Winkielman et al., 1997). Importantly, this emotional interference of information processing of threatening or punishing stimuli is exaggerated in anxious people (Fox, 1996, MacLeod and Rutherford, 1992, Mogg et al., 1993), and when subjects are administered drugs with anxiolytic actions such as diazepam (Murphy et

al., 2008) and citalopram (Harmer et al., 2006), we see diminished attentional vigilance.

It is interesting to note that when cognitive deficits are restored by pharmacological treatment in both anxious and depressed individuals (as well as in healthy individuals) this can occur without accompanying changes in self-reported affective states (Harmer et al., 2009b). This phenomenon implies that improvements in emotional processing precede observable effects on mood. For example, immediate changes in cognition have been identified with administration of antidepressant and anxiolytic drugs, whereas reported changes of mood state occur following longer-term administration (Harmer et al., 2003, Harmer et al., 2006, Murphy et al., 2008, Harmer et al., 2009b). This has led to the theory that the normalisation of affective information processing is required in order to normalise an individual's perception of their environment, which in turn improves affective states (Harmer, 2008). The argument is that deficits in cognitive processing leads to the symptoms of depression, and when these deficits are reduced, these symptoms are attenuated (Eshel and Roiser, 2010). It has been hypothesised that this improvement in cognitive processing is the underlying mechanism by which antidepressant and anxiolytic treatments exert their therapeutic effects (Murphy et al., 2008).

The following sections further describe how cognitive processes are biased by affective states, and ends with a summary of tasks that are used to measure these biases in humans (see Table 1-4).

1.II.ii Biasing of attention

Stressful conditions can induce anxiety in most individuals; this is an evolutionary advantage whereby increased vigilance improves identification and processing and subsequent avoidance of threatening stimuli. Anxious individuals bias their attention towards threatening stimuli more than non-anxious individuals (Bar-Haim et al., 2007) and when this presents as a clinical disorder, such as Generalised Anxiety Disorder this is amplified. These individuals award a disproportional level of anxiety to neutral

events and display an even greater attentional bias towards threatening stimuli and information.

A visual dot probe task was developed by MacLeod et al. (1986), to identify the biasing of attention towards threatening information. In this task, participants were presented with two words on a screen, one threat word and one neutral word. Both of the words disappeared and one was replaced by a dot. The participant indicated the location of the dot as quickly as possible, and the latency to respond was measured. Participants with anxious states were found to respond to the dot replacing a threat word faster than a dot replacing a neutral word, indicating an increased vigilance for threat (also see Mogg et al. (1992) and Keogh et al. (2001) for further examples of attentional bias tasks). Some groups have successfully replicated this task in a non-verbal manner, where subjects show an attentional bias towards fear-relevant pictures such as animals, spiders and snakes instead of words. In addition, these tasks have been shown to identify positive vigilance in humans possessing a genotype linked to reduced susceptibility to mood disorders (Fox et al., 2009, Pérez-Edgar et al., 2010). In humans with clinical anxiety, avoidance of negative stimuli is thought to be a coping strategy to avoid aggravating an already aversive state of fear, which is reflected in this task by diverting attention away from emotional faces (Mathews, 1990).

To date, only two dot probe touchscreen tasks have been developed for animals, specifically for rhesus macaques (King et al., 2012, Parr et al., 2013), however a number of researchers have adopted a task where gaze is measured in animals in order to assess attentional bias. A study of rhesus monkeys measured the latency to the first gaze and the duration of the gaze towards either an emotional or neutral picture of a conspecific face (Bethell et al., 2012). They found that all of the monkeys showed an attentional bias to emotional faces, as they were quicker to look at them, which was interpreted as vigilance. However, monkeys with negatively manipulated affective states (via restraint and ketamine sulphate injections routinely carried out during health checks) were quicker to divert their gaze from emotional faces compared to neutral faces. They were also found to look away more quickly than monkeys with positively manipulated affective states (via environmental enrichment), suggesting that emotional states mediate attention both towards and away from emotive stimuli.

Levels of vigilance behaviour in animals may be a useful indicator of anxiety-like states. Visual scanning is an example of this behaviour, and it increases when circumstances are more threatening, for example when the distance to safe cover is increased, or when views are obstructed making potential threats harder to identify (Lazarus and Symonds, 1992). Equally, visual scanning decreases when threat is reduced, for example when in a larger group where more animals are able to alert others to the danger of a predator, and also where the individual threat of attack is diluted (Elgar, 1989).

A task that could exploit this natural behaviour to identify attentional biases towards threatening or rewarding stimuli would be a useful addition to the battery of tests used in animal emotion research. However, the lack of development in this area suggests that it may not be possible to translate this to a greater range of species, presumably due to operational difficulties in measuring gaze or operation of touch screens in laboratory species.

1.II.iii Biased memory formation and retrieval

Situations which induce emotional arousal are associated with enhanced memory performance in both humans and animals (Hamann et al., 1999, Cahill and McGaugh, 1995). This is an adaptive response, whereby processing and storage of memories of both positive and negative events (e.g. food related, predation threat) are of a greater contribution to survival than of memories of neutral stimuli (Cahill and McGaugh, 1998). It has been shown that individuals in anxious or depressed states have a bias towards retrieval of negative memories, whereas happier people are more likely to recall more positive ones (Clark and Teasdale, 1982, Burke and Mathews, 1992, Mineka et al., 1998) and so identification of these biases could be indicative of affective states.

It is likely that these memory biases also exist in animals, but the linguistic nature of the research again presents a challenge in translating these studies into experimental paradigms that can be reliably used with animals.

1.II.iv Biases in interpretation of ambiguous information

Judgement making is complex, and combines many aspects of appraisal of a situation or stimulus. These include risk-taking, expectations of the future and interpretation of ambiguous stimuli (Loewenstein et al., 2001, MacLeod and Byrne, 1996, Wright and Bower, 1992). Anxious individuals tend to bias their interpretation of ambiguous information in a negative manner, whereas happy individuals tend to bias this in a positive manner. As with the identification of other cognitive biases in humans, biasing of judgement tends to be measured via linguistic tasks. For example, humans suffering from anxiety are more likely to interpret ambiguous homophones (pain/pane, die/dye), and sentences like 'the doctor examined little Emma's growth' negatively (Eysenck et al., 1991, Mathews et al., 1989). It is difficult, however, to translate a task that identifies biases via this method for use in animals.

	Reward	Punisher
Signalled	↑expectation of +ve events (Q1)	↑expectation of –ve events (Q4)
Removed	↓ expectation of +ve events	↓ expectation of –ve events (Q2)

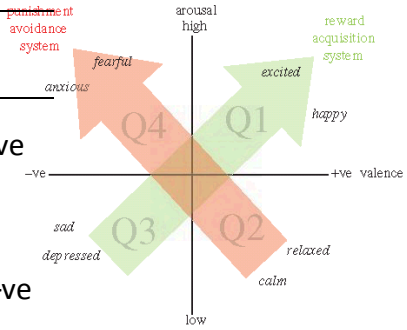


Table 1-3 ***Emotion represented by expectation of reward and punishment****. The table summarises the relative expectation of rewarding (+ve) events and punishing (-ve) events occurring in relation to the signalling or removal of a reward or punisher, and the correlating quadrant location in Figure 1-1(Mendl et al 2010).*

With reference to the discussion of Figure 1-1 in section 1.I.iii, it was explained that mood states exist in the absence of emotion-inducing stimuli, and can influence appraisal of the environment in a biased manner. For example, happy individuals are more likely to have an optimistic outlook and have a greater expectation of positive events occurring than a depressed individual, and similarly anxious individuals are more likely to have a pessimistic outlook and have a greater expectation of negative events occurring. These biases in judgement of future events (hereby referred to as

judgement biases) correlate with affective state, and, when measured, reflect those states. It is therefore possible to generate affective states via manipulating the presence or omission of rewards and punishers in an animal's environment (see Table 1-3), where the corresponding behaviours and physiology produced can subsequently be used as indicators of particular affective states. However, in the presence of emotion-inducing stimuli, an animal's behaviour will be modified accordingly and may or may not reflect their underlying mood state, so generation of these states in this manner would have to be under sufficient control to eliminate the chance of transient emotions being recorded rather than indicators of longer-lasting moods. To overcome this issue Harding et al. (2004) developed a paradigm where biased expectations of positive and negative events could be objectively measured via the presentation of ambiguous stimuli that the animal had not before encountered, and therefore had no predetermined emotional valence attached. Their task provided a unique opportunity to more reliably identify affective states in a non-linguistic manner and is discussed further in the subsequent section.

Bias	Test	Observations in depressed patients	References
Attention	**Emotional Stroop** (Identify the colour of emotional words whilst ignoring the meaning)	↑ latency for negative words ↑ perigenual ACC response to negative words	Gotlib and McCann (1984); Segal et al. (1995); Broomfield et al. (2007) McCabe and Gotlib (1995); Mitterschiffthaler et al. (2003)
	Dot probe task (Respond to the location of a dot that replaces an emotional stimulus)	↑ response latency for positive vs. negative stimuli	Mathews et al. (1996); Gotlib et al. (2004); Joormann and Gotlib (2007)
	Affective go/no-go (Respond/withhold response to emotional stimuli)	↑omission errors to positive stimuli ↑ subgenual cingulate response to negative stimuli	Murphy et al. (1999); Elliott et al. (2002); Erickson et al. (2005); Kyte et al. (2005); Kaplan et al. (2006)
Perception	**Emotional categorisation** (categorising the valence of affective stimuli e.g. self-referent phrases or facial expressions	↓ response latency to negative vs. positive faces ↑amygdala response to negative faces	Gilboa-Schechtman et al. (2002); Joormann and Gotlib (2006); Harmer et al. (2009a); Murphy et al. (2009); Yoon et al. (2009),Sheline et al. (2001); Fales et al. (2009)

Bias	Test	Observations in depressed patients	References
Memory	**Emotional recall** (recall of emotionally valenced words)	↓ recall of positive vs. negative stimuli	Gilboa-Schechtman et al. (2002); Ellwart et al. (2003); Harmer et al. (2009b)
		↑ amygdala response to recalled negative stimuli	Hamilton and Gotlib (2008)
Feedback sensitivity	**Probabilistic reversal learning**	↑ reversal following negative feedback	Elliott et al. (1997); Murphy et al. (2003)
		↓dorsal ACC response to negative feedback	Steele et al. (2007)
		↑ amygdala response to negative feedback compared to controls	Taylor Tavares et al. (2008)

Table 1-4 ***Summary of findings from human neuropsychological tests of cognitive affective biases in MDD.*** *Figure reproduced from Hales et al (2014).*

Box 1.3: Discriminated operant learning and reinforcers

Operant learning involves the modification of an individual's behaviour by manipulating the consequences of its performance. For example, the delivery of food following a lever press will result in an increased frequency of lever pressing behaviour. Discrimination learning occurs when a response is reinforced only by the presence of a specific stimulus. For example, the food may be delivered only by pressing the left lever and not the right, so as a consequence, only the left lever is pressed.

Positive reinforcers are favourable outcomes presented following the desired behaviour and are commonly referred to as 'rewards'. Negative reinforcers are unfavourable outcomes (punishers) that are avoided by performing the desired behaviour. Note that both positive and negative reinforcers *increase* the occurrence of the desired behaviour.

Conversely to reinforcers, punishment *decreases* the occurrence of an undesired behaviour. With positive punishment, a punisher is received if the undesired behaviour is performed. Negative punishment involves the removal of reward if the behaviour is performed.

1.III The judgement bias task paradigm

If we revisit the model in Figure 1-1 and Table 1-3, we can see that affective states correlate with the anticipation of rewards or punishers. An animal with a high expectation of reward can be described as having a positive affective state, whereas an animal with a low expectation of rewards has a negative affective state (the reverse applies to expectation of punishers). Therefore, if we measure animals' anticipation of positive or negative events we can identify whether animals are in a putatively positive or negative affective states after exposure to affective manipulations (e.g. drug treatments, painful stimuli, different husbandry practices etc.). For example, captive animals provided with environmental enrichment in their housing show a greater expectation of rewarding events occurring than animals without enrichment, and are subsequently deemed to be in a more positive state (or 'optimistic'; e.g. Burman et al. (2008), Brydges et al. (2011), Bateson and Matheson (2007), Matheson et al. (2008), but see Parker (2008), Brilot et al. (2010)).

There are a variety of methods that can be employed in order to measure the anticipation of positive and /or negative events, which are discussed in the remainder of this section, but they all follow the same principle initially developed by Harding et al. (2004), referred to as the 'judgement bias paradigm'. In Harding's experiment, rats were trained to press a lever to gain a food reward and another lever to avoid an aversive white noise. Rats differentiated the two response outcomes by the presentation of two different tone frequencies that cued for the positive and negative trial outcomes. These auditory cues were at two ends of a continuous stimulus dimension - 2 kHz and 4 kHz respectively. Here a response on the lever associated with food reward was assigned a positive value whereas the lever associated with white noise was deemed negative. The authors then induced a negative affective state in rats by introducing stress-inducing unpredictable housing environments to one of two groups, and assessed both group's responding to three ambiguous-cues that lay between these two endpoint auditory cues – 2.5 kHz, 3 kHz and 3.5 kHz (Figure 1-2).

Their hypothesis was that the animals subjected to stress would less frequently judge an ambiguous-cue as being positive than those animals that had not been subjected to the manipulations in affective state, and therefore perform fewer responses on the positive lever. In other words, the animals housed in the stressful environment would display a negative affective state (or 'pessimism'). Those that were subjected to unpredictable housing made fewer and slower responses to the rewarded tone and the probe tones closest to it (Figure 1-3) which supported their hypothesis that stressed rats had a reduced anticipation of a positive event occurring. Response latencies also reflect the salience of cues, and animals tend to respond much quicker to cues that they interpret to be positive. This mirrors findings that humans in depressed states interpret ambiguous stimuli negatively, and also have a reduced expectation of positive events occurring (MacLeod and Byrne, 1996, Eysenck et al., 1991).

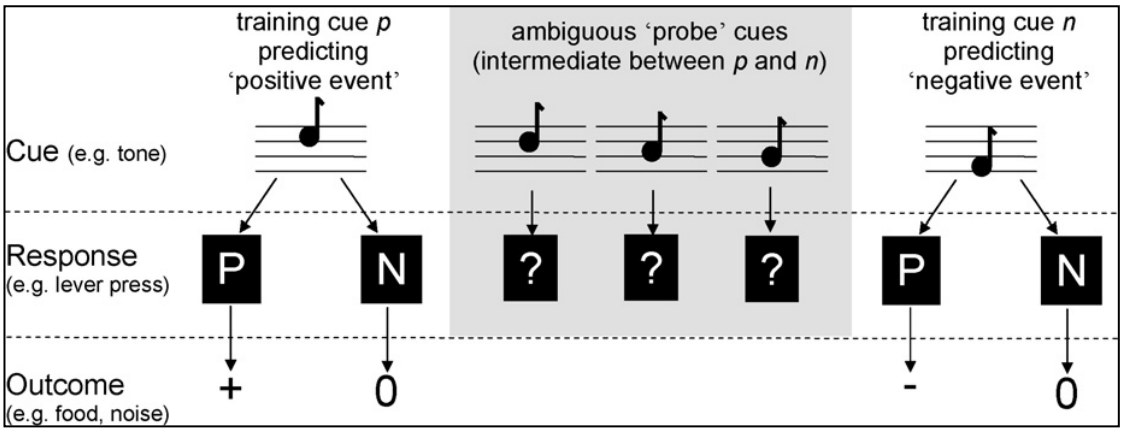


Figure 1-2 ***Experimental paradigm for the original judgement bias task.*** *The training cues were auditory, where a lever press on 'P' resulted in a food reward if preceded by training cue p, whereas refraining from pressing a lever resulted in avoidance of a period of white noise if preceded by training cue n. After the animals reached criterion of responding correctly to each tone more than 50% of the time, they were subjected to unpredictable housing conditions and then entered the testing phase. The rats were played tones intermediate of the two learned tones that lay on a continuous scale, and the proportion of these responded to and the latencies to respond were recorded. (Reproduced from Harding et al 2004).*

In this study, the animals generalised the ambiguous-cues in accordance with how closely the cue resembled the stimulus associated with food reward (positive cue). Responding to the ambiguous-cues decreased as a function of this resemblance (Figure 1-3). Stimulus generalisation was first reported by Pavlov (1927) who determined that once a response had been established to one stimulus, a response could be elicited by another similar stimulus when itself had not been associated with reinforcement. It was also observed that there was a decline in the effectiveness of a stimulus to elicit a conditioned response that was proportional to the distance from the training stimulus on the stimulus dimension (e.g. light intensity, audio frequency etc.). This sloping curve of stimulus-response outcomes was defined as stimulus generalisation. The overlap of elements of the test stimuli and the training stimuli contributes to the extent that a subject will respond to them, and the slope of the generalisation gradient correlates to the discriminability of the learned cues (Ganz, 1962). This phenomenon is a primary factor of judgement bias paradigms, where the effectiveness of an unlearned stimulus to be generalised to learned stimuli relates to whether it is interpreted by the animal to have salience either alike to the positive or negative stimulus. For example, when an ambiguous stimulus elicits responding to a similar extent as the positive conditioned stimulus, this represents a transferred positive associative salience. If an affective

manipulation alters the extent to which an ambiguous stimulus is responded to, it can be said to reflect an animal's altered expectation of salient outcomes as signalled by the stimulus. For example, if an animal performs more behaviours associated with a rewarding outcome, they can be thought to have a greater expectation of reward, and equally, expressing more behaviours that are associated with the avoidance of a punisher indicates a greater expectation of negative events occurring. The expectation of rewarding or punishing events can be related back to the framework of affective state that was described in 1.I.iii.

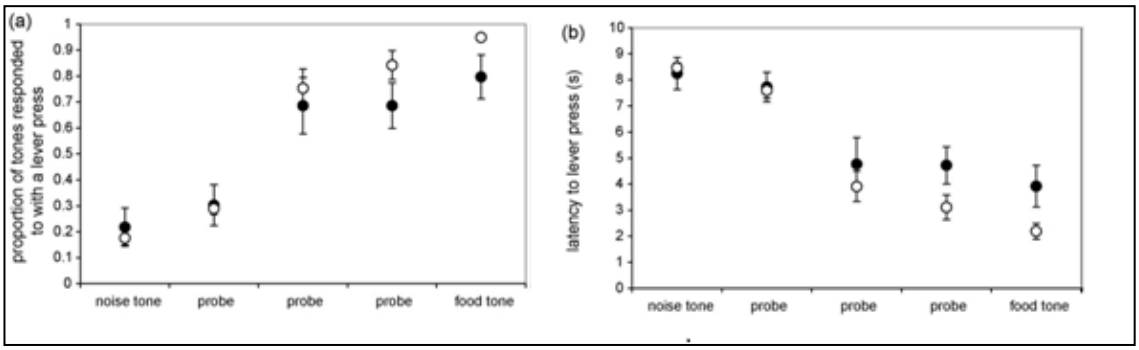


Figure 1-3 ***Experimental outcomes of the original judgement bias task .*** *Graphs show the proportion of cues responded to with a lever press (a), and latency to lever press (b) during test sessions in which rats were presented with either training cues ('food tone' = tone predicting positive event (food); 'noise tone' = tone predicting negative event (white noise)) or ambiguous probe cues. During this phase of the study, subjects were kept either in unpredictable (filled circles) or predictable (open circles) housing conditions. (Reproduced from Harding et al 2004)*

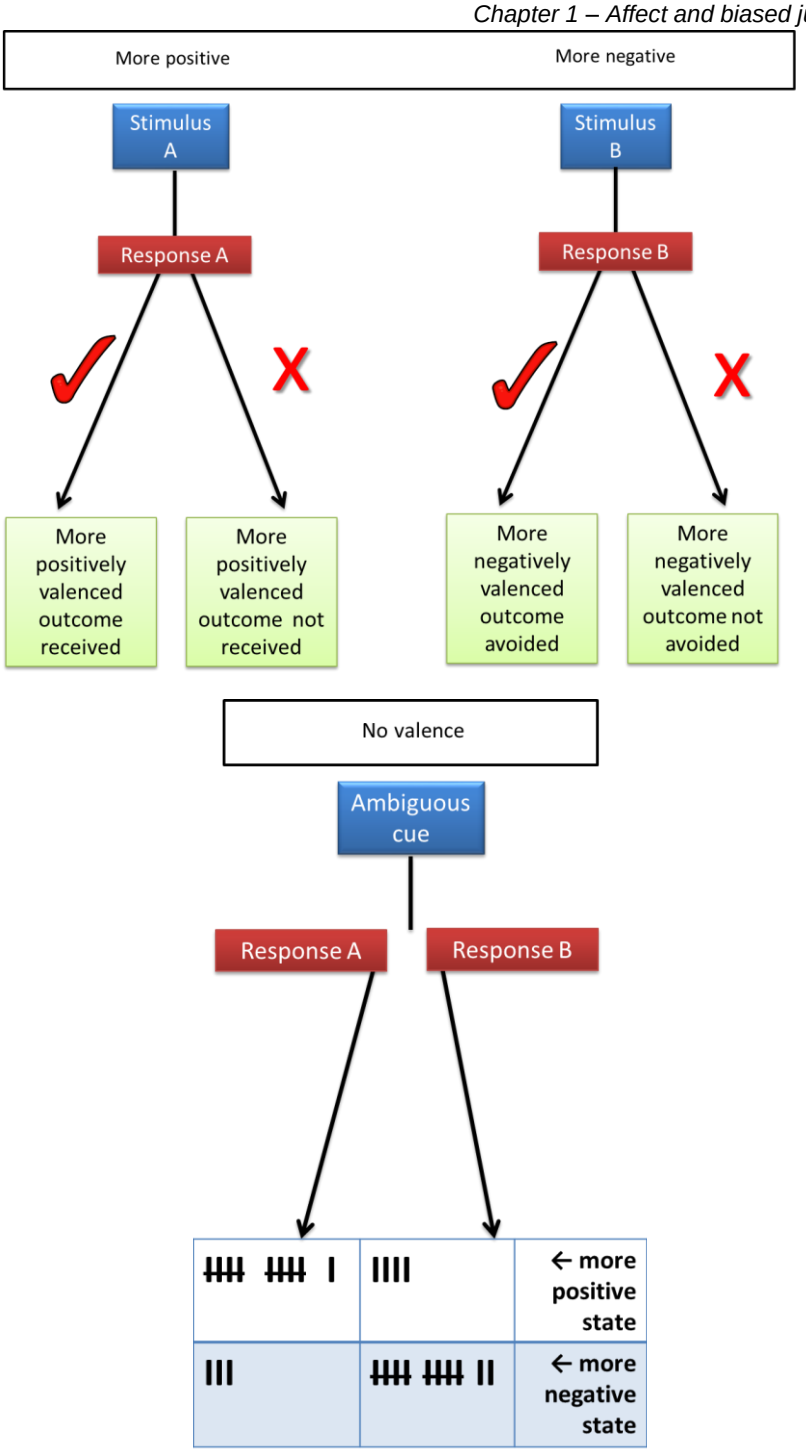


Figure 1-4 ***Schematic of the judgement bias task and interpretation of its outcomes.*** *The panels show a) discrimination task and b) ambiguous-cue testing outcomes of the judgement bias paradigm. a) Individuals are trained to expect a positive event in response to one cue and a less positive or a negative event in response to another. b) They are then presented with ambiguous-cues that lay on a continuous scale intermediate between the two learned cues and data is retrieved from their responses .If the ambiguous-cue is interpreted to represent the positive cue, this could be said to represent a more positive affective state, and if it is interpreted as the negative cue, this could be signifying a more negative affective state.*

When we strip the judgement bias paradigm of its specific cues, response mechanisms and outcomes, we are left with a basic framework that can be manipulated (Figure 1-4). The test setting can be designed to best match the locomotive or cognitive abilities of the species being used, and by varying the stimulus type and presentation and the type of response required, it has the potential to be truly translational. Versions of this paradigm have been adapted for 15 different species, spanning laboratory (rats and mice), farm (sheep, cows, pigs, goats and chickens), domestic (cats and dogs), and captive wild animals (starlings, monkeys, grizzly bears), insects (honeybees) as well as humans (Figure 1-5), the latter providing face-validity support for this paradigm.

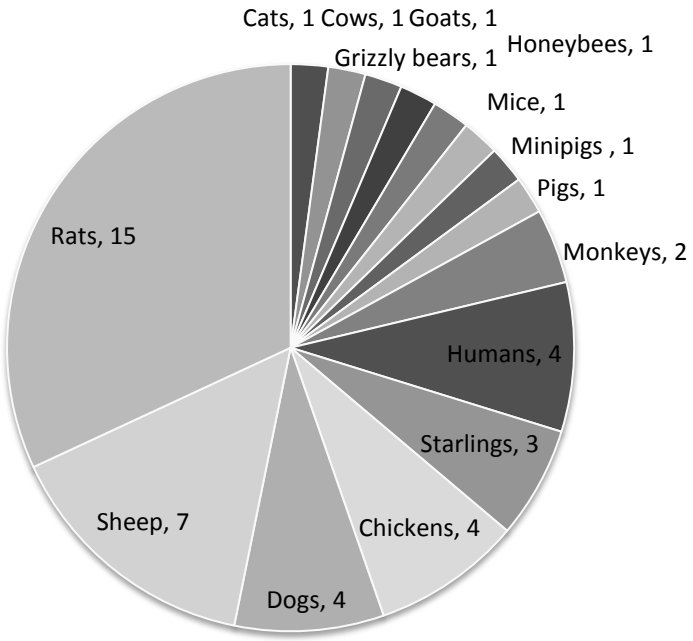


Figure 1-5 ***Species used in judgement bias tasks****. Summarised from Table 1-5.*

The task can be designed to measure particular hypotheses, i.e. if investigating manipulations with *a priori* predictions associated with happiness or depression, a task that focuses on reward loss and acquisition would be appropriate. Alternatively, when measuring anxiety or contentment the task can be designed to focus on punishment avoidance. It is also possible to combine both the reward acquisition and the punishment avoidance systems to create a task that is sensitive to affective changes in any direction.

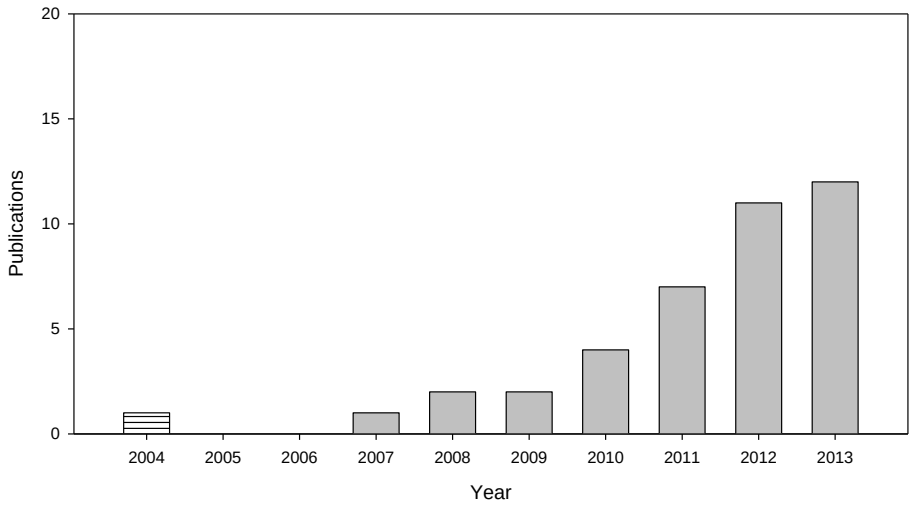


Figure 1-6 ***The number of judgement bias tasks published per year*** *(grey bars) since the initial publication of Harding et al, 2004 (striped bar).*

In this section I will discuss and critique the various interpretations of the judgement bias paradigm. This analysis includes all judgement bias tasks in publication as identified by searching for papers that cited Harding et al. (2004) using the Web of Knowledge database. The abstracts were reviewed to select papers by identifying those that included a judgement bias task. Also included is one conference abstract (Mendl et al., 2006) and one PhD thesis (Parker, 2008) in which the experiments were not published in journals. If the studies included multiple experiments (where experiments are defined as separate questions asked using separate cohorts of animals), these were assessed as standalone experiments. In total 42 published studies and 46 separate experiments were identified. Following publication of the initial task (Harding et al., 2004), other versions of the judgement bias task did not appear in journals until 2007, and since 2009 there has been year-on-year increases in the number of publications (Figure 1-6) indicating a greater acceptance of the task into mainstream research.

The data extracted from these studies were the number, strain and species of the subjects used, the specific parameters of the task design, the affective manipulation employed and the authors' predicted outcomes of the task. The affective manipulations were categorised in accordance with whether they are specific in

inducing a particular emotion or whether they produce a more general change in affective valence (i.e. where anxiety is specific and negative and 'a more positive state' is general and positive) and is explained in 1.III.vi.1. The authors' predictions of the task outcomes were also categorised in this manner. Where no predictions were made, this was categorised as a general and bidirectional. A tabulated review of these studies is presented below (Table 1-5) and is followed by a critique of the design parameters.

1.III.i An overview of judgement bias tasks

Author(s)	Year	Species	Cue type	Response	Positive reinforcer	Negative reinforcer	Affect manipulation	Expected outcome?
Harding et al	2004	Rats	Auditory (tone frequency)	Go/no-go (lever press)	Food	Noise	Unpredictable housing	Yes
Mendl et al	2006	Humans	Visual (location on screen)	Active choice (key press)	Nice image and gain points	Nasty image and lose points	Individual variation in mood	Yes
Mendl et al	2006	Humans	Visual (location on screen)	Active choice (key press)	Nice image and gain points	Nasty image and lose points	Music mood manipulation (happy music v sad music)	Yes
Bateson and Matheson	2007	Starlings	Visual (colour cues)	Go/no-go (flipping lid)	Food	Unpalatable food	Enrichment	Yes
Burman et al	2008	Rats	Spatial location	Active choice (latency to approach)	Food	No food	Enrichment	Yes

Author(s)	Year	Species	Cue type	Response	Positive reinforcer	Negative reinforcer	Affect manipulation	Expected outcome?
Matheson et al	2008	Starlings	Visual (key illumination duration)	Active choice (coloured key peck)	Instant food (after 1s)	Delayed food (after 15s)	Enrichment	Yes
Parker	2008	Rats	Auditory (tone frequency)	Active choice (lever press)	2 food pellets	1 food pellet	Addition/removal of enrichment	No - opposite
Parker	2008	Rats	Auditory (tone frequency)	Active choice (lever press)	2 food pellets	1 food pellet	Unpredictable housing	No bias
Burman et al	2009	Rats	Spatial location	Active choice (latency to approach)	Food	Unpalatable food	Bright light vs. dim light	Yes - anxiety indicated but depression also recorded
Enkel et al	2009	Rats	Auditory (tone frequency)	Active choice (lever press)	Food	Foot-shock	Depression-like phenotype	Yes - depression indicated but anxiety also recorded
Enkel et al	2009	Rats	Auditory (tone frequency)	Active choice (lever press)	Food	Foot-shock	Pharmacological induction of stress	Yes -but reference cue responding altered

Author(s)	Year	Species	Cue type	Response	Positive reinforcer	Negative reinforcer	Affect manipulation	Expected outcome?
Brilot et al	2010	Starlings	Visual (colour cues)	Active choice (lid flipping)	3 mealworms	1 mealworm	Enrichment and poor welfare measured by stereotypy scores	Yes
Doyle et al	2010	Sheep	Spatial location	Go/no-go (locomotion)	Food	Presence of dog	Release from restraint	No - opposite
Mendl et al	2010	Dogs	Spatial location	Active choice (latency to approach)	Food	No Food	Separation-related behaviour	Yes - but task measured depression when anxiety was induced
Bateson et al	2011	Honeybees	Olfactory (scent cues)	Go/no-go (proboscis extension reflex)	Food	Unpalatable food	Predator-like threat	Yes -but reference cue responding altered
Brydges et al	2011	Rats	Tactile (sandpaper grade)	Active choice (bowl selection)	Chocolate	Cheerio	Enrichment	Yes - valence only
Burman et al	2011	Dogs	Visual (colour cues)	Active choice (latency to approach)	food	No food	Exposure to a rewarding event	No - opposite

Author(s)	Year	Species	Cue type	Response	Positive reinforcer	Negative reinforcer	Affect manipulation	Expected outcome?
Doyle et al	2011	Sheep	Spatial location	Go/no-go (locomotion)	Food	Fan blower	Chronic mild stress	Yes - but task measured depression when anxiety was induced
Doyle et al	2011	Sheep	Spatial location	Go/no-go (locomotion)	Food	Presence of dog	Pharmacological depletion of brain serotonin	Yes
Salmeto et al	2011	Chicks	Visual (conspecific -> predator morphed images)	Active choice (latency to approach)	Chick image	Owl image	Isolation	Yes
Sanger	2011	Sheep	Spatial location	Go/no-go (locomotion)	Food	Dog	Shearing	No - opposite
Anderson et al	2012	Humans	Auditory (tone frequency)	Active choice (key press)	Monetary reward	Aversive noise	Individual differences in emotional state and trait variables	Yes
Bethell et al	2012	Rhesus macaques	Visual (line length)	Go/no-go (touch screen)	Food	Noise	Vetinary inspection vs. enrichment	Yes

Author(s)	Year	Species	Cue type	Response	Positive reinforcer	Negative reinforcer	Affect manipulation	Expected outcome?
Boleij et al	2012	Mice	Olfactory (scent cues)	Active choice (latency to approach)	Food	Unpalatable food	Light conditions	Yes -but reference cue responding altered
Brydges et al	2012	Rats	Tactile (sandpaper grade)	Active choice (bowl selection)	Food	Less rewarding food	Juvenile stress	No - opposite
Destrez et al	2012	Sheep	Spatial location	Go/no-go (locomotion)	Food	Air blower	Pharmacological reduction of anxiety	Yes
Douglas et al	2012	Pigs	Auditory (glockenspiel, clicker, squeak toy)	Go/no-go (locomotion)	Food	Aversive noise	Environmental enrichment	Yes
Hymel and Sufka	2012	Chicks	Visual (conspecific -> predator morphed images)	Active choice (latency to approach)	Chick image	Owl image	Isolation and antidepressant drugs	Yes -but reference cue responding altered
Mueller et al	2012	Dogs	Spatial location	Go/no-go (locomotion)	Food	No food	Owner absence	No bias

Author(s)	Year	Species	Cue type	Response	Positive reinforcer	Negative reinforcer	Affect manipulation	Expected outcome?
Pomerantz et al	2012	Monkeys	Visual (line length)	Active choice (lid flipping)	Food	Less food	Presence of stereotypies and presence of faecal corticosterone	Yes - valence only
Richter et al	2012	Rats	Spatial location	Active choice (latency to approach)	Food	Unpalatable food	Depressive strain and enrichment	Yes -but reference cue responding altered
Rygula et al	2012	Rats	Auditory (tone frequency)	Active choice (lever press)	Food	0.5mA foot shock	Tickling	Yes
Wichman et al	2012	Chickens	Spatial location	Active choice (latency to approach)	Food	No food	Environmental enrichment	No - opposite
Anderson et al	2013	Rats	Auditory (tone frequency)	Active choice (lever press)	Food	Foot-shock	Pharmacological reduction in depression or anxiety	Yes -but reference cue responding altered
Briefer and McElligott	2013	Goats	Spatial location	Active choice (latency to approach)	Food	No food	Poor welfare vs. good welfare	No - opposite

Author(s)	Year	Species	Cue type	Response	Positive reinforcer	Negative reinforcer	Affect manipulation	Expected outcome?
Chaby et al	2013	Rats	Tactile (sandpaper grade)	Active choice (bowl selection)	Food	Less food	Early-life stress	Yes
Destrez et al	2013	Sheep	Spatial location	Go/no-go (locomotion)	Food	Fan blower	Chronic stress	Yes
Keen et al	2013	Grizzly bears	Visual (colour cues)	Active choice (touch with nose or paw)	Food	Less food	Enrichment	No bias
Murphy et al	2013	Minipigs and also pigs	Auditory (tone frequency)	Active choice (number of responses)	Food	Less food	Restraint stress	Yes
Neave et al	2013	Cows	Visual (colour cues)	Go/no-go (nose press on screen)	Food	No food	Pain	Yes
Papciak et al	2013	Rats	Auditory (tone frequency)	Active choice (lever press)	Food	Foot-shock	Chronic psychosocial stress	Yes
Rygula et al	2013	Rats	Auditory (tone frequency)	Active choice (lever press)	Food	Foot-shock	Restraint	Yes

Author(s)	Year	Species	Cue type	Response	Positive reinforcer	Negative reinforcer	Affect manipulation	Expected outcome?
Schick et al	2013	Humans	Auditory (tone frequency)	Active choice (key press)	Smiley face + monetary reward	Frowning face + monetary loss	Individual differences in emotional state and trait variables	Yes
Seehus et al	2013	Chicks	Spatial location	Active choice (latency to approach)	Palatable food	Unpalatable food	Interruption of consummatory processes	No - opposite
Titulaer et al	2013	Dogs	Spatial location	Active choice (latency to approach)	Food	No food	Short term vs. long term kennelled dogs	No bias
Verbeek et al	2014	Sheep	Spatial location	Go/no go (locomotion)	Food	Dog	Hunger	No bias

Table 1-5 ***An overview of judgement bias tasks .*** *Tasks were identified via a search on the Web of Knowledge database of studies citing Harding et al 2004. The abstracts were reviewed to select papers by identifying those that included a judgement bias task. Also included is one conference abstract (Mendl et al, 2006) and one PhD thesis (Parker, 2008).*

1.III.ii Cues and task design

Since the introduction of this paradigm, an abundance of tasks have been developed utilising a variety of sensory stimuli. The tasks have been developed to match the locomotor, sensory, behavioural and cognitive capacities of the species used. Tasks for rodents utilise their sensitivities in distinguishing auditory cues (e.g. Harding et al. (2004), Enkel et al. (2009), Schick et al. (2013)) and spatial cues (e.g. Richter et al. (2012), Burman et al. (2008), Burman et al. (2009)), whereas in larger animals such as domestic and farm animals, larger arena settings are needed and tend to involve exclusively spatial discriminations (e.g. Doyle et al. (2010a), Briefer and McElligott (2013)). In order to create ambiguity these cues must exist on a continuous scale (see 1.III). Animals are presented with two cues that are discriminable on this scale and learn that one is associated with a positive event and the other a negative or less positive event. When testing judgement of ambiguous-cues, the animals must not be able to easily identify that they are not the reference cues predictive of rewards or punishers, and are consequently ambiguous. The ambiguous-cues are therefore selected to lie at intermediary points between the reference cues. Animals tend to generalise the ambiguous-cues, assigning them a more positive or more negative valence depending on which of the reference cues they most closely resemble (see Figure 1-7). One of the studies analysed did not adhere to this methodology and instead used audio cues of different types (a glockenspiel and a clicker); in this sense it could be argued that their test was not one that measured judgements of ambiguity, but judgement of novelty (Douglas et al., 2012).

Task design also varied in the manner that animals were required to respond to the cues. Some species, including primates have been trained to discriminate spatial or pictorial cues via responding on sophisticated touch-screens (Bethell et al., 2012). Approach behaviour tends to be the response measured in larger animals, such as cows (Neave et al., 2013), goats (Briefer and McElligott, 2013), sheep (Doyle et al., 2010a), pigs (Douglas et al., 2012), chickens (Wichman et al., 2012) and dogs (Burman et al., 2011), as well as rodents (Burman et al., 2008). Lever pressing is also used in rodent choice tasks (Enkel et al., 2009). The judgement bias tasks performed with starlings utilise key pecking and the removal of lids covering food rewards (Matheson

et al., 2008, Brilot et al., 2010); and more recently, this task has been adapted for the honeybee, where their well-developed olfaction allowed for the discrimination of scent cues, and a distinct behavioural reflex (the proboscis extension reflex) was used as an observable response (Bateson et al., 2011b).

This scope of applications of the task for a wide range of species highlights the translatability of this paradigm. It has also been modified for use in humans, where the researchers validated that the outcomes of the task matched *a priori* predictions of response outcomes. Mendl et al. (2010) developed a non-verbal judgement bias task designed to be homologous with the tasks they were using in other species. They induced positive associations with an on-screen stimulus (a cross positioned at one end of a horizontal line) reinforced with points and a pleasant image when the appropriate response was made, and negative associations when the cross was located at the opposite end of the line, which was reinforced with the loss of points and the display on an unpleasant image if the incorrect response was made. In the test phase, the crosses were presented in an ambiguous position (mid-point of the line). Those who made more of the responses relevant to the positive stimuli when faced with this ambiguous stimulus were classed as more positive than those who performed more negative responses. This correlated significantly with the moods states of the participants, which were established via self-report on questionnaires. Mendl and his group showed that this non-linguistic measure of cognitive bias revealed the same emotion-cognition link as linguistic measures in humans, and argued that these studies could provide the link between linguistic reports of subjective emotion and non-linguistic indicators that can be used in animals.

1.III.ii.1 Design of test sessions

Variations of the test sessions include the number of ambiguous stimuli presented to the animal and the method by which the reference and ambiguous trials are reinforced. The majority of studies employed the use of either a single ambiguous-cue equidistant and central between the two reference cues (‘mid’), or, in addition, cues equidistant between this central-cue and the positive cue (‘near-positive’) and

negative cue ('near-negative'). It is not recommended to expose animals to a vast number of non-reinforced ambiguous-cues as this can lead to the extinction of responding, where animals learn that the ambiguous cues have no outcome and thus refrain from performing a response. The single-cue approach is therefore advantageous to the multiple-cue approach in terms of statistical power as it can be presented to the animal a greater number of times and more data can be retrieved. However, what is gained in statistical power is lost in sensitivity; single-cue tasks are limited to identifying changes in valence (i.e. whether the animal's affective state is more positive or more negative), but do not provide information regarding whether an animal has a changed perception of the more positive or more negative events occurring. Using the multiple-cue approach, deviations in responding to cues that are 'near-positive' or 'near-negative' can give this information. Ambiguous probes can also be more 'reward-like' or more 'threat-like' depending on the response outcomes used (see1.III.v) and can therefore indicate changes in anticipation of reward or threat if multiple ambiguous-cues are used. For example greater responding to the 'near-negative' cue indicates a greater expectation of the negative event occurring, whereas lesser responding to the 'near-positive' cue is indicative of a reduced anticipation of positive events occurring (Figure 1-7).

To reduce the risk of response extinction when multiple-cues were used, six of the experiments employed a schedule of partial reinforcement of the reference cues prior to testing or in the test sessions themselves (Matheson et al., 2008, Bateson and Matheson, 2007, Schick et al., 2013, Neave et al., 2013, Richter et al., 2012, Brilot et al., 2010, Brydges et al., 2011). In these studies the authors hypothesised that if animals were less expectant of a response outcome on all of the reference trials, they would not learn as quickly that the ambiguous trials were not reinforced. The relative success of this approach can be identified by reviewing whether animals' responding dropped below criterion during the testing sessions and to what extent. Of the experiments utilising partial reinforcement, only Brilot et al reported a reduction in responding to ambiguous-cues over the testing sessions, and explicitly stated that they believed that a subset of their animals had learned that ambiguous-cue trials were not rewarded. A point to consider is that Brilot et al tested their animals over the course of

three weeks whereas the testing periods of the other three studies were in the range of one-three days, suggesting that this method of partial reinforcement may not be efficient for conserving responding over longer durations. Doyle et al. (2010b) specifically investigated the hypothesis that learning of non-reinforced ambiguous-cues occurred over prolonged periods of repeated testing. They exposed trained sheep to ambiguous-cue testing sessions over a period of three weeks, and found that week-by-week, the sheep displayed an increasing negative bias. This was of concern as no alterations had been made to the animals' environment which would explain a negative bias. The authors explicitly claimed that their animals learned that the ambiguous-cue trials were not reinforced, and reduced their responding accordingly. However, Doyle et al did not employ the use of partial reinforcement or additional reinforced training between testing sessions, both of which are methods employed to slow the speed of learning of non-reinforced trials. So far no direct comparison has been made between animals that are given extra training or partial reinforcement in a judgement bias task with those that are not, and also there has been no assessment of the effect of partial reinforcement over short-term periods of testing. The optimal protocol in order to preserve the ambiguous nature of the task is therefore unclear. In light of these potential effects of time on repeated task performance, many authors of the judgement bias tasks attempt to control for any effects that repeated testing may have on response extinction by counterbalancing treatments over the entire testing period.

In three of the experiments the authors took the opposite approach and reinforced the ambiguous trials in order to prevent response extinction. In Anderson et al. (2012) and Anderson et al. (2013) the authors reinforced responding on the ambiguous-cue trials. In the earlier study multiple ambiguous-cues were used and were reinforced to match the reference cue they most closely resembled, and in the later study they reinforced the single ambiguous-cue so that half of the time the outcome would match that of the positive trial and the other half it would match the outcome of the negative trial. It could be argued that receipt of rewards or punishers might produce a transient emotional response and therefore overshadow the underlying affective state (see 1.II.iv). These animals may have also assigned affective value to the ambiguous-cues

after encountering the outcomes, biasing their decision-making on subsequent ambiguous-cue trials. The authors of the study consequently do not refer to their task as a measurement of judgement bias, and instead call it an 'affective tone discrimination task'. Similarly, Chaby et al. (2013) baited the goal pots in their experiments with food reinforcers of higher and lower quantity in an identical manner to the discrimination training, again providing the potential for the animals to learn about the reinforcement of ambiguous cues. They did, however, state that the animals visited the 'less food' goal pot even if they had chosen the 'more food' goal pot in earlier ambiguous trials and argued that this confirmed that animals did not exhibit learning of the reinforcement. There are similarities between these tasks and the 'response bias probabilistic reward task' which focus on the assessment of implicit learning of reward contingencies during testing (Der-Avakian et al., 2013) and the biasing of subsequent responding. In summary, there is an overt chance that the responding of the animals in these three tasks was influenced by learning of the response outcomes rather than an underlying affective state.

In Bateson et al. (2011b) neither the ambiguous trials nor the reference trials were reinforced in the testing session as the test odours were presented in quick succession (30 seconds apart), where stimulation of the mouthparts with sucrose would potentially produce response artefacts to the subsequent odours.

1.III.iii Choice vs. 'go/no-go' tasks

The design of Harding's judgement bias task is described as 'go/no-go', where animals respond to one stimulus ('go') and refrain from responding to another ('no-go') in order to achieve the preferable outcomes. Fourteen of the 46 judgement bias tasks analysed (30%) were of the go/no-go discrimination variety. An advantage of this design is the speed in which animals can be trained to perform the discrimination. It is considerably easier for animals to discriminate response outcomes which can be assigned opposite valences (rewarding and aversive), and to follow from that, training a 'passive avoidance' response to aversive stimuli is also less time consuming (this phenomenon is discussed further in Chapter 3). In twelve of the fourteen experiments

training passive behaviours, it was in response to an aversive stimulus. There are fewer welfare issues surrounding avoidance of aversive stimuli when passively avoided, as animals are exposed to the stimuli far less than if animals must actively perform a response to avoid them. It can also be argued that repeat exposure to an aversive event can affect the underlying affective state of the animal, biasing the experimental outcomes, hence why passive avoidance was selected in these paradigms. However, there can be ambiguity when interpreting the response outcomes of 'go/no-go' tasks. For example, we cannot reliably differentiate between a 'no-go response' where animals actively refrain from lever pressing, or a response omission (Figure 1-7). Response omissions could result from a number of non-emotion-related circumstances, for example a simple reduction in activity or of hunger. The display of either of these may be confused with an anhedonic state associated with depression (Willner et al., 1987). A reduced motivation to respond to ambiguous trials due to learning that these are not reinforced could also be confused with a pessimistic bias.

Although this version of a judgement bias task inevitably leads to setbacks in interpretation, they are the task of choice for some researchers. 'Go/no-go' tasks tend to be employed in larger farm animals such as sheep, pigs and cows (e.g. Verbeek et al. (2014), Douglas et al. (2012), Neave et al. (2013)) and domestic animals separated from their owners (Mueller et al., 2012) where there may have been time restrictions for training and testing, and in these cases a trade-off in the sensitivity of the task was acceptable. 'Go/no-go' tasks have not been widely used in research animals such as rodents, where presumably time and resources available for these studies are less constrained.

The alternative design of a judgement bias task requires both the positive and negative stimuli to be responded to in order to gain the preferred outcomes. These tasks are referred to as 'choice tasks', and in many, but not all of these tasks, differential responses must be made in response to the positive and negative cues. Of the 32 tasks that were categorised as choice tasks, there were 13 where positive and negative responding were not differentiated by two distinct response mechanisms (e.g. right and left lever presses), but instead by the latency by which the animal took to respond to the positive or negative cue (Figure 1-7). This is based on evidence that animals will

actively approach more rewarding stimuli quicker than less rewarding stimuli (e.g. Burman et al. (2011), Brydges et al. (2011), Richter et al. (2012)). One such study is the 'Spatial Judgement Task' developed by Burman et al. (2008). Burman et al aimed to determine whether changes in background emotional state, here induced by removal of environmental enrichment, could be assessed using a choice judgement bias task. Rats were trained to discriminate between two goal locations, one of which was rewarded and the other unrewarded, and the rats had to approach a pot to complete a trial. The researchers then attempted to induce a putatively more negative affective state in half of the rats by removing environmental enrichment from their home cages. The rats were then presented with pots in locations intermediate between the two learned locations, with the hypothesis that rats with more negative affective states would display slower running times to the ambiguous pot locations indicating that they interpreted these cues more negatively. The rats with environmental enrichment removed showed increased latencies to approach the pot in one of the intermediate positions – the ambiguous pot closest to the unrewarded location. They suggested that this was because the rats with more negative affective states were more likely to anticipate a lack of reward. That when the unrewarded pots were in the negative position they were still approached indicates that there were no additional factors inhibiting responding such as a general reduction in locomotor activity. Thus it can be argued more confidently that the increased latency to reach the pot after ambiguous stimuli was due to a pessimistic judgement bias.

Versions of the judgement bias task that measure the latency to approach goal locations are somewhat limited as they cannot measure approach latency towards overtly punishing stimuli (for example the presence of a dog in tasks designed for sheep). Instead, most will use the absence of food reward or the presence of unpalatable food as the negative/less positive consequence. The implications of this limitation are discussed further in section 1.III.v.

The use of overtly punishing stimuli in active avoidance pathways in judgement bias tasks is quite rare. In non-human animals this has been limited so far to the use of rats in paradigms where rats are trained to lever press in order to avoid a mild foot-shock (Rygula et al., 2013, Rygula et al., 2012, Papciak et al., 2013, Enkel et al., 2009,

Anderson et al., 2013). For example, Enkel et al. (2009) described a task with a similar design to Harding's task with lever presses in response to two auditory reference cues with ambiguous tones that were intermediate between these on a continuous auditory scale. Contrary to the 'go/no-go' task design, the rats had to actively respond in order to avoid the negative consequences predicted by the negative cue, rather than refrain from responding. In this manner it could be determine whether a reduction in positive responding was accompanied by an increase in negative responding, indicating an increase in negative affect, or whether it was accompanied by an increase in response omissions, which would be interpreted as a reduction in activity.

The requirement to respond actively to both positive and negative stimuli eliminates the ambiguity in interpretation of response outcomes, as we can measure response omissions to identify non-emotional changes in activity. This is particularly relevant when we identify changes in responding following ambiguous-cues as we can additionally assess responding following the reference cues (those that signal the presence of reward or a punisher/lesser reward). If discrimination of the reference cues falls below the response criteria set during training, we again cannot rule out changes in activity or retention of the discrimination as the reasons for altered responding to the ambiguous-cues like with go/no-go tasks.

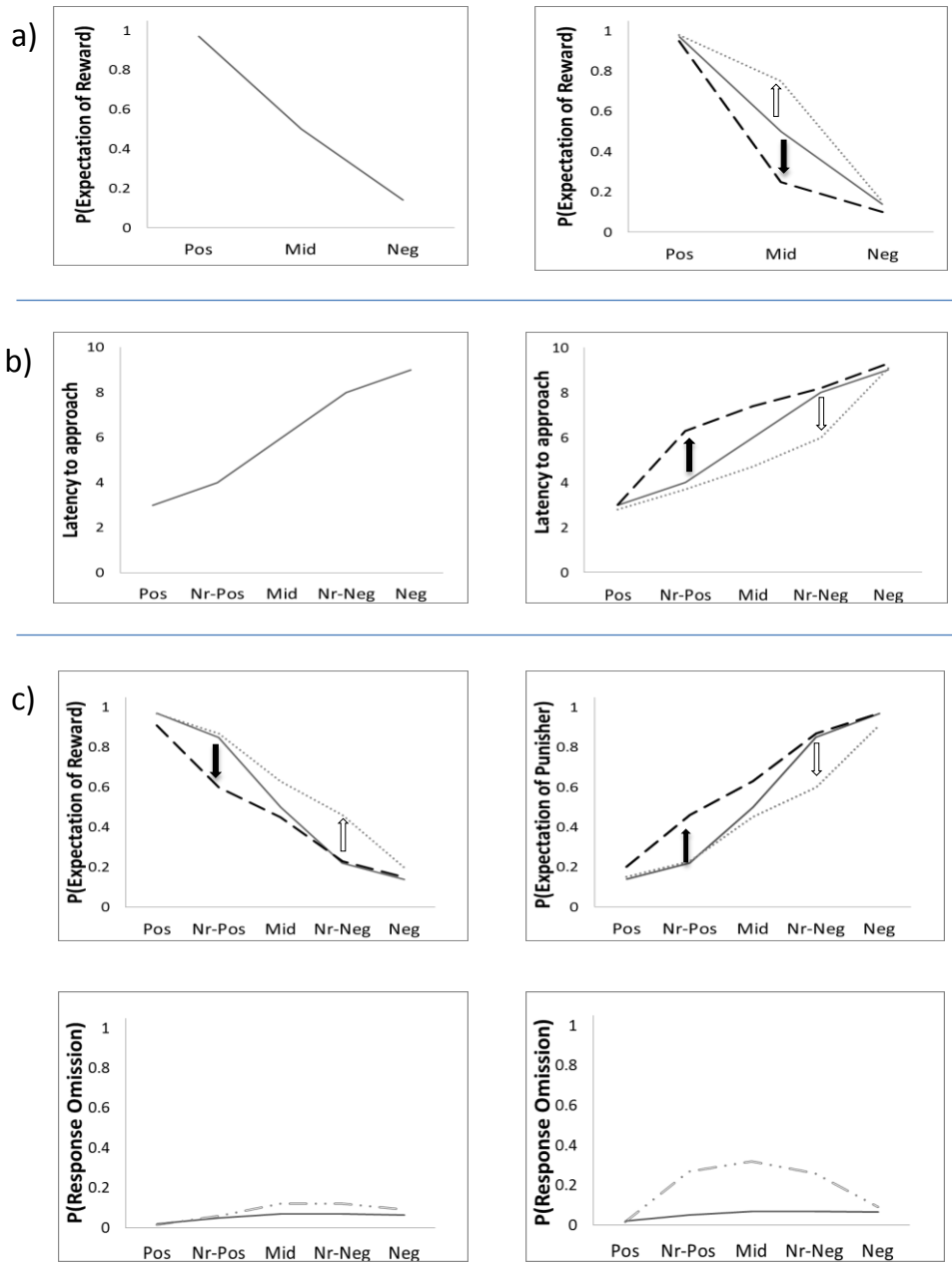


Figure 1-7 ***A diagrammatic representation of judgement bias task outcomes.*** *Panels show a) a 'go/no-go' task with one ambiguous-cue; b) an active choice task measuring latency to approach with multiple cues and c) an active choice task with differential responding and multiple cues. Pos and Neg represent the reference stimuli with positive and negative (or less positive) outcomes. Near-positive (Nr Pos), middle (Mid) and near-negative (Nr Neg) represent the ambiguous cues and their respective resemblance to the reference cues. Black arrows and dashed lines represent a negative biasing of the ambiguous cues and white arrows and dotted lines represent a positive bias. a) The left panel shows the generalisation to the ambiguous-cue. The right panel shows that a single ambiguous-cue can detect positive and negative changes reflect changes in valence. b) The left panel shows generalisation to multiple ambiguous-cues. The right shows an increased latency to respond to the Nr Pos cue representative of a decreased expectancy of reward. Decreased latency to respond to the Nr Neg cue represents a decreased expectancy of negative outcomes. c) With differential responding a decreased positive responding to Nr Pos represents a decreased expectancy of reward, with concurrent increased negative responding. Decreased negative responding to Nr Neg cue represents decreased expectancy of negative outcomes with a concurrent increase in positive responding to this cue. Response omissions are also measured in this task, where the left hand panel shows no increase in omitted responses and the right hand panel shows a decrease in responding to ambiguous-cues, which may signify that ambiguous-cues were learned to be unreinforced.*

1.III.ivReinforcer salience

When training a discrimination task the response outcomes must vary in their associated valences. Their discriminability relies on the capability of reinforcing or punishing an animal's behaviour. 'Go/no-go' judgement bias tasks typically include positive reinforcers (rewards that increase the performance of a behaviour) and either a positive or negative punishment (see Box 1.3). Positive punishment reduces an animal's performance of a behaviour by the occurrence of a negative event if the behaviour is performed, whereas negative punishment reduces the performance of a behaviour by the removal of a positive event if the behaviour is performed. In choice tasks, positive reinforcement and either less positive or negative reinforcement is used. Less positive reinforcers (i.e. smaller rewards) induce a response to obtain a reward, but are less attractive than a more positive reinforcer, whereas negative reinforcement causes a response to be performed in order to avoid an unwanted outcome. I will go on to discuss the rewards and punishers used in the judgement bias tasks reviewed and the relative strengths and weaknesses of their combinations.

1.III.iv.1 Same valence, different strengths

22% of tasks were designed where both stimuli result in rewarding events, but had higher and lower values and as such differed in their associated valences. These can be reflected in a different quantity of reward, the delay to obtain the reward, or rewards of different types. It is hypothesised that if an animal is optimistic it will interpret ambiguous stimuli positively and attempt to retrieve the higher value reward, and if pessimistic will interpret the stimuli negatively and attempt to retrieve the lower value reward. In order for discriminations to be learned, the differences between the reward size and type must be distinguishable, and both outcomes must be sufficiently salient to induce animals to actively respond to the cues.

Two rewarding foodstuffs with detectable differences in caloric value and taste can also be used, where preference tests often precede these studies in order to establish their relative attractiveness and hence associative valence. In Brydges et al. (2011), the authors used two different rewards in a choice task to measure an increase in positive

affect related to enrichment of the housing of rats. The rewards were demonstrated prior to the experiment to have a higher and lower value (chocolate buttons and 'Cheerio' breakfast cereal respectively). Using a judgement bias protocol originally developed in the University of Newcastle, Brydges utilised tactile cues for the discrimination. The apparatus was lined with either coarse or fine sandpaper, both of which were associated with a specific reward, or with an intermediate grade of sandpaper acting as ambiguous-cues. Their study was successful in measuring an increase in positive affect associated with environmental enrichment.

There is also evidence that manipulating the delay to reward using a choice task, where the positive outcome is an immediate food reward and the less positive outcome is a delayed food reward, has suitably discriminable saliences for a judgement bias task. This is based on studies demonstrating a preference in animals for the immediate delivery of food vs. a delay. Matheson et al (2008) showed that starlings displayed optimism in a cognitive bias task, where the probability of classifying ambiguous cues as those associated with an instant food outcome were significantly increased when enrichment was added to their housing.

A criticism of using the combination of reward vs. no reward for the response outcomes is that there is no discernable consequence whether or not a response is made to the negative stimulus. A salient event occurs only when a correct response is performed when animals are presented with a positive stimulus, whereas no reward is obtained regardless of the response made to the negative stimulus. Without punishment of performing an incorrect response, or reinforcement when performing the correct response, it can be argued that the outcome of the negative stimulus does not hold sufficient salience to induce responding. This means that there may be a skew in the judgement of ambiguity in favour of a positive outcome when rats are faced with ambiguous stimuli, as there is no overt consequence involved in making an incorrect positive choice. It is therefore not surprising that of eight experiments that adopted food vs. no food as the response outcomes, less than half the biases measured matched the *a priori* predictions set out.

1.III.iv.2 Oppositely valenced outcomes

Avoidance of an aversive outcome provides a contrasting event that balances the positive in appeal or repulsion, reducing the potential for the rats to have a skewed judgement of the probe (Doyle et al., 2010a, Burman et al., 2008). In continuing the discussion of consummatory outcomes, there are a number of studies that utilised palatable vs. unpalatable response outcomes as the reinforcers. For example, the compound quinine is often added to palatable foods, so that they differ only in the sensation of taste, but produce a negative association. However, it has been found that hungry animals may not refrain from consuming the unpalatable food, particularly if they are maintained on a restricted diet (Barnett et al., 2012).

There are also reinforcer combinations that include the element of risk in order to improve motivation to respond to negative cues. Here the avoidance of an aversive outcome or punisher by the response to a negative cue contrasts the presence of a reward associated with a response to a positive cue. In earlier unpublished work, Harding attempted a reinforcer combination of a food reward for the positive cue, and the avoidance of white noise for the negative cue, both of which required an active lever press response (Harding, 2002). The rats did not respond when subjected to the stimulus paired with the negative event, and instead refrained from responding during a 30 s period of white noise. This explains their subsequent use of a 'go/no-go' paradigm rather than an active choice paradigm. White noise is known to be an aversive stimulus for the rat, however this is at volumes greater than 90db (Campeau and Watson, 1997) which is perhaps why it was apparently not aversive at the volume of 70db used in Harding's study. For this reinforcer combination to be successful the outcome must be more strongly aversive, enough to warrant a response from the animal in order to avoid it. More recently, Enkel et al. (2009) attempted a similar paradigm where the aversive outcome was a mild foot-shock, and found that animals would actively respond in order to avoid the shock. They used this paradigm to determine whether a negative affective state was produced by a genetic model (congenitally helpless rats) which displays depression-like symptoms. In their task, rats were trained to expect a sucrose reward following a lever press in response to one tone (referred to as the positive tone), and to press another lever to avoid foot-shock

in response to another tone (referred to as the negative tone). Presentation of the negative tone was followed by shock unless the rat performed in an active lever press avoidance schedule, which prevented the shock (recorded as an 'avoidance response'). They were then tested for their responses to ambiguous probe tones of intermediate frequencies, showing their expectations of a positive or negative event. The rats with the depression-like phenotype made fewer positive lever presses to the central and near-negative tones, and more negative lever presses to the central tone than those not displaying this phenotype, suggesting the presence of a negative response bias in rats congenitally presenting a depressive-like state. They also confirmed the use of a foot-shock as a sufficiently salient outcome to induce avoidance responding to the negative stimulus. The threat of predation can also be used as a negative reinforcer or punisher, for example Verbeek et al. (2014), Sanger et al. (2011), Doyle et al. (2010a) and Doyle et al. (2011) used the presence of a dog as a positive punisher that sheep learned not to approach.

Choice tasks that utilise aversive outcomes may therefore be a more effective method in terms of maintaining a discrimination, however there is as of yet few direct comparison in the literature assessing the outcomes of tasks that use more positive and less positive reinforcers and those using overtly positive and negative reinforcers (but see Bateson et al. (2011b)). It can, however, be argued that the use of such aversive outcomes is less attractive from a welfare perspective, and these tasks can also be critiqued in terms of learning theory. Responses to ambiguous-cues are typically not reinforced (i.e. there is no response outcome) and this lack of an outcome is equivalent to a correct response to the negative stimulus. After the first presentation of an ambiguous-cue where the animal responds negatively and no outcome is received, they may 'learn' that it is the correct negative response and subsequently treat ambiguous-cues as the negative cue. Animals learning about non-reinforced probes has been highlighted as an issue in some recently published studies (Doyle et al., 2010a, Brilot et al., 2010).

1.III.viReinforcer suitability

As was discussed in 1.I.iii and 1.III, the measurement of emotion via judgement biases is related to the anticipation of punishment avoidance or reward acquisition. Thus, if we wish to measure an affective change that we predict *a priori* to be akin to depression or happiness, we must include reinforcers that stimulate the reward acquisition pathway in the task in order to observe changes in its anticipation. Equally, if we wish to measure changes in affect that we would describe as anxious or calm, reinforcers that stimulate the punishment avoidance system must be used. As can be seen in Figure 1-8, all of the judgement bias tasks contained an element of reward as the positive response outcome, and to this end, affective changes from depression to happiness can be measured. In contrast, only 54% of the tasks include negative response outcome that stimulate the punishment avoidance pathway (Figure 1-9) which include aversive sensory events (unpalatable food, mild pain, noise or air puffs) or predation-like threats. The negative response outcomes in the remaining 46% of the tasks are based on rewards, where animals experience reward loss, lower quality or quantity of reward, delay to reward or the absence of reward. When we are solely assessing an animal's expectation of reward loss or acquisition, we can only observe changes in affect that occur in this system. Measuring expectation of reward will not detect anxious or calm states that may also be present. If we are to assess changes in affect alike to induction or relief from anxiety-like states, we must review the animal's expectation of punishers.

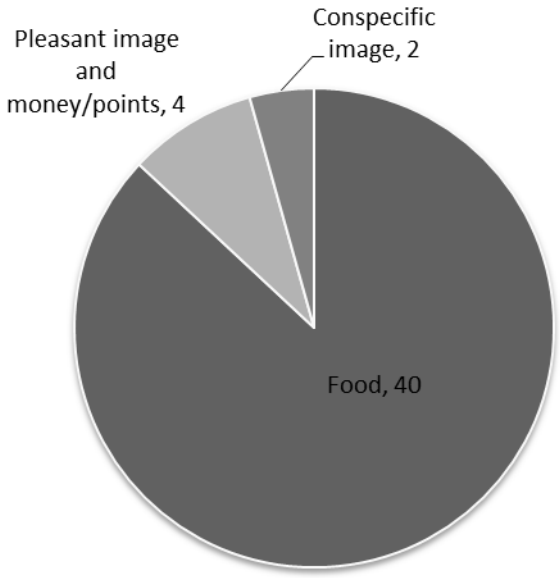


Figure 1-8 ***A summary of positive reinforcers used in judgement bias tasks*** *. Apart from chick and human tasks which used images and/or monetary reward, all tasks had positive consummatory response outcomes. Summarised from Table 1-5.*

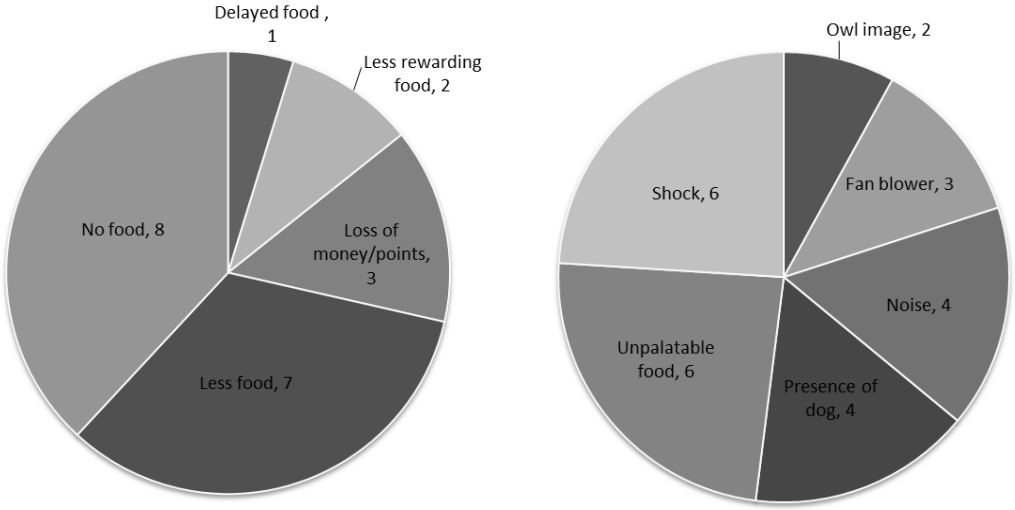


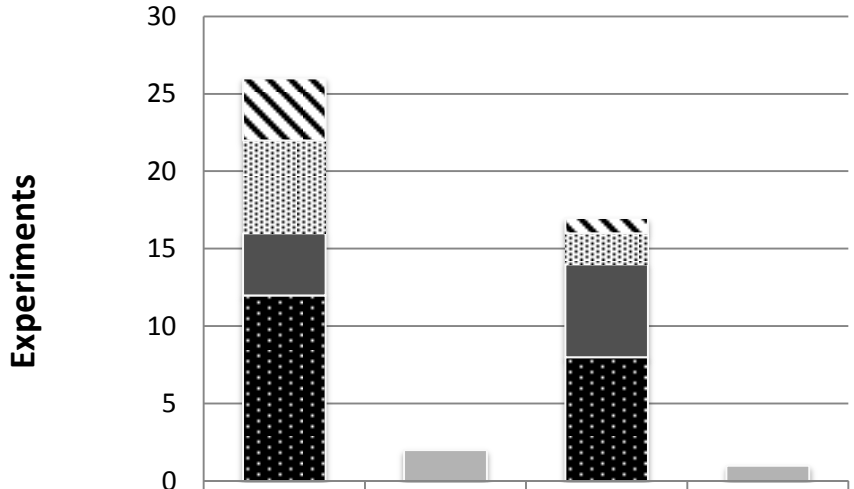
Figure 1-9 ***A summary of negative reinforcers used in judgement bias tasks .*** *The graphs represent less rewarding outcomes (left) and aversive outcomes (right.) Summarised from Table 1-5.*

1.III.vi.1 Do the reinforcers match the manipulations used?

The experiments were evaluated in terms of whether the task design suited the manipulations in affective state that it was intended to measure. First the reinforcers were categorised as belonging to the reward acquisition pathway or the punishment avoidance pathway (see 1.I.iii). Next, the studies were then reviewed and any *a priori* predictions related to the affective manipulations employed were also categorised, and finally affective manipulations were allocated to categories of anxiety, depression etc. (Table 1-6). These were compared to assess whether the reinforcer combination used matched the affective pathway they intended to observe. As all of the studies included some form of reward, this analysis was performed in terms of whether or not a punisher was needed, and whether or not one was used (Figure 1-10).

Affective pathway	Affective manipulation	Examples	References
Punishment	Anxiogenic/ anxiolytic	• 5 minutes isolation • Bright light • Predator-like threat • Anxiolytic drugs • Veterinary inspection	• Salmeto et al 2011 • Burman et al 2011 • Bateson et al 2011 • Anderson et al 2013 • Bethell et al 2012
Reward	Depressive/ antidepressant	• 60 minutes isolation • Depressive phenotypes • Antidepressant drugs • Brain serotonin depletion	• Salmeto et al 2011 • Enkel et al 2009 • Anderson et al 2013 • Doyle et al 2011
Punishment & Reward	Unspecified negative affect	• Unpredictable housing • Chronic mild stress • Poor welfare • Early life stress • Restraint stress • Food restriction	• Harding et al 2004 • Doyle et al 2011 • Brilot et al 2013 • Brydges et al 2012 • Doyle et al 2010 • Verbeek et al 2014
Punishment & Reward	Unspecified positive affect	• Enrichment	• Matheson et al 2008
Punishment & Reward	Unspecified affect	• Personality trait scores • 'Mood- manipulating' music	• Anderson et al 2012 • Mendl et al 2006

Table 1-6 ***A summary of affective manipulations used in judgement bias task.*** *The affective manipulations were characterised as to whether they stimulated changes on the spectrums of anxiety or depression, or both types in a negative or positive manner, or were indiscriminate in their predictions of affective change. The table also references the pathways necessary for inclusion in a judgement bias task to identify these changes in affective states. Summarised from Table 1-5.*



	Used, necessary	Used, not necessary	Not used, necessary	Not used, not necessary
Anxiety	4	0	1	0
All affect	6	0	2	0
Positive affect	4	0	6	0
Negative affect	12	0	8	0
Depression	0	2	0	1

Figure 1-10 ***A summary of judgement bias tasks where reinforcement correlated with a priori predictions .*** *The graph shows the number of judgement bias experiments where the use of a punisher was necessary (i.e. if the authors intended to measure anxiogenic/anxiolytic changes in affect), and the number of experiments where a punisher was used. The patterned bars represent the classification of affective manipulations. Summarised from Table 1-5.*

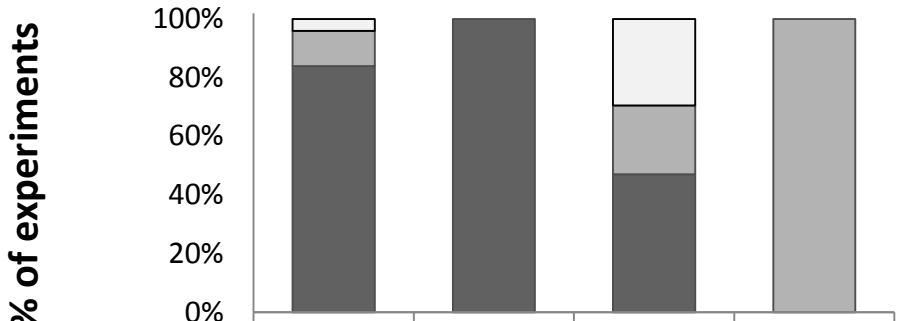
There can be indistinctness in categorising some of the affective manipulations. This is especially true when considering anxiogenic and depressive manipulations, but this is unsurprising when we consider the overlap in the morbidity and symptomology of the two disorders in humans. Stress-inducing paradigms such as unpredictable housing and chronic mild stress tend to produce depression-like symptoms with (Bondi et al., 2007) or without (Willner et al., 1987) concurrent presentation of anxiety-like symptoms. In this instance, it would be advisory to include both reward and punishment pathways in a judgement bias task. Indeed, when considering the opposite manipulation, environmental enrichment, most of the authors of judgement bias studies refer to this as a manipulation that increases positive affect, without distinguishing between 'happy' or 'calming' effects. The choice of reinforcers used would however indicate

that they perceive enrichment to be an inherently rewarding experience as they do not include response outcomes associated with punishment avoidance (Brydges et al., 2011, Matheson et al., 2008, Keen et al., 2013, Burman et al., 2008, Brilot et al., 2010, Wichman et al., 2012, Parker, 2008). There is, however evidence at least with mice (Roy et al., 2001, Benaroya-Milshtein et al., 2004) and rats (Klein et al., 1994), that environmental enrichment also has anxiolytic properties. Therefore, when assessing the impact of environmental enrichment on affective state, I would also recommend that both the reward acquisition and punishment avoidance pathways are included in the study design. The majority of judgement bias tasks measuring affective states following environmental enrichment and/or unpredictable housing conditions do not utilise both pathways and as such may not provide a complete overview. The exceptions are Harding et al. (2004), Douglas et al. (2012), Bateson and Matheson (2007), Bethell et al. (2012) and Richter et al. (2012), who all observed a change in affective valence in line with *a priori* predictions. There were, however, no indications that this was specifically related to anxiety where Bateson and Matheson (2007) and Douglas et al. (2012) used tasks with a single ambiguous-cue and so were unable to specifically differentiate between anticipation of rewards or punishers (see Figure 1-7); Richter et al. (2012) observed a reduction in latency to choose all goal pots in a choice trial, but there was no specific mention of whether the latencies to choose the reference pots was altered or not; Bethell et al. (2012) did not use a non-stressed control group; and the bias that Harding et al. (2004) identified in their seminal paper with rats exposed to unpredictable housing was of a reduction of reward anticipation.

It is also especially relevant to utilise both the reward and punishment pathways when investigating affective changes with no *a priori* predictions, or when assessing individual personality traits. In fact, only 15% of the experiments had clear *a priori* predictions where the affective manipulations could be classified solely as being on either the reward acquisition pathway or punishment avoidance pathway; the remaining studies should therefore have had both of these included in the task design. This was the case in only 54% of these studies.

1.III.vii Were predicted biases observed?

In addition, it was of interest to determine whether the bias that was predicted by the authors was observed, and to identify potential patterns when this was not the case.



	Used, necessary	Used, not necessary	Not used, necessary	Not used, not necessary
no bias	1	0	5	0
opposite direction	3	0	4	1
predicted direction	21	3	8	0

Figure 1-11 ***The relationship between task reinforcement and whether biases occurred in predicted directions.*** *The graph shows the percentage of the 46 judgement bias experiments reviewed in this thesis where the valence of the experimental outcomes matched the predictions; were opposite to the predictions; or where no bias was identified. The table shows the number of studies. The experiments were classified by their inclusion of a punisher in the task, and whether or not the affective manipulation used required it. Summarised from Table 1-5.*

Of the experiments reviewed, 32 (70%) showed a biased judgement in animals that was compatible with *a priori* predictions of its valence as stated by the authors. However, of these, in five of the experiments it was also recorded that responding to the reference cues was also altered, so it could be argued that there were non-emotional influences on responding (see 1.III.iii). Four of the 32 experiments also indicated the presence of affective changes that were anxiety-like or depression-like, but where the other had been predicted. In nine of the experiments, the investigators used only one central probe, which meant that only information about the valence of the affective change could be measured (see Figure 1-7).

In six (13%) of the experiments no judgement bias effect was recorded. In five of these experiments, the experimenters used only the anticipation of reward to assess affective state, but used affective manipulations that could be argued to require the

additional assessment of anticipation of punishers. In two of these studies where no bias was recorded from the experimental manipulations, the data was further analysed with a consideration of stereotypy behaviour that was observed during the study. In both cases the experimenters detected a judgement bias. When assessing somersaulting behaviour in starlings a negative bias was indicated which the authors interpreted as a behaviour associated with negative valence (Brilot et al., 2010), but when grizzly bears were classified as demonstrating a stereotypy also associated with negative valence - pacing- the authors found a positive bias. It is quite possible that some stereotypies are associated with more positive affect and some with more negative, and there is debate, as highlighted by Brilot et al. (2010), over what is predicted by a stereotypy.

Eight of the total 46 experiments (17%) detected a significant change in the biasing of ambiguous information with a valence opposite of that predicted. There does not appear to be any common features of the experimental design that may explain this phenomenon – this occurred with a variety of species (rats, dogs, goats, sheep, chickens), in active choice tasks and go/no-go tasks, in tasks assessing positive and negative changes in affect, and tasks that did and did not use punishers. Half of the experiments exposed animals to long-term affective manipulations, the other half exposed animals to acute affective manipulations. Where animals exposed to stressful or negative conditions acted more positively during the task it was hypothesised that, in these cases, engaging in the judgement bias task was inherently enriching in itself and was thus rewarding. The difference in affective state prior to the task compared to during task performance is argued to be more pronounced in animals with underlying negative affect, which is why they might appear to be more positively biased during the task. This is the line of reasoning adopted by a number of the authors of these papers (Sanger et al., 2011, Doyle et al., 2010a), including that of the grizzly bear study outlined previously (Keen et al., 2013). It is hard, however, to accept this argument when we examine more closely some of the stressors used in these experiments. Some of the affective manipulations have been used in tasks elsewhere, and the experimental outcomes conflict with those outlined above. The first experiment published that reported this phenomenon was a paper by Doyle et al. (2010a) who

identified a positive bias in sheep that had undergone restraint stress, and attributed this to a relief experienced once the restraint stress was removed. Subsequently, restraint stress has been shown to instead induce a negative bias in the rat and the pig (Rygula et al., 2013, Murphy et al., 2013). Similarly, conflicts in the outcomes of judgement bias tests have been uncovered in the review with other similar affective manipulations. Brydges et al. (2012) reported that rats that had undergone juvenile stress responded more positively than control rats, whereas Chaby et al. (2013) reported that rats stressed in early-life responded more negatively than control rats. However, Chaby et al used male Long Evans rats with a relatively short period of exposure to the stressors (three days), whereas Brydges et al used male and female Lister Hooded rats and employed unpredictable stressors over a period of 40 days, so arguably the affective manipulations were not comparable. The review of studies also showed that of a total of ten experiments that were performed that investigated environmental enrichment, two showed biases opposite to those predicted, whereas six showed biases in the predicted direction and another two reporting no bias. Therefore the two studies reporting biases in the opposite direction to predicted are in the minority. The shearing of sheep (Sanger et al., 2011), the interruption of consummatory processes of chicks (Seehuus et al., 2013) and dogs' previous exposure to a rewarding event (Burman et al., 2011) are the remaining affective manipulations that caused changes in judgement biases opposite to that predicted. In light of the contradictory outcomes of the affective manipulations mentioned previously, it would be recommended that these experiments with experimental outcomes conflicting with *a priori* predictions are repeated before conclusions are made regarding their interpretation.

It is also important to remember that although use of this task is increasing, compared to many other established paradigms it is in its infancy, so it proves difficult at present to deduce as to whether these inconsistent outcomes are the exception or the rule. We must also consider the unknown number of failed or contradictory experiments that remain unpublished. It is also noteworthy that due to the extensive training requirements of bias tasks, the sample sizes in many of these experiments are relatively small compared to more established behavioural tasks. 21 (45%) of the

studies used 20 or fewer subjects and as such may have been inherently underpowered, thus increasing the probability of type I and type II errors occurring.

Box 1.4: How should I design my judgement bias task?

To adequately interpret the outcomes of a judgement bias task with clear *a priori* predictions regarding altered affective states, care must be taken in its design.

The following questions must be considered involving the direction of valence associated with the emotional responses that is expected; whether this change is evident on the reward or punishment pathway; whether responding may be altered by changes in activity levels; and how many test sessions are necessary to obtain sufficient data.

Do we need to eliminate changes in activity levels?

In most cases, the responses made by the animal to the cues will be active (e.g. approach behaviour, lever pressing etc.) so it is important to measure whether activity levels are sustained in a judgement bias test. To this end, go/no-go tasks should be avoided where possible, as 'no-go' responses can be indistinguishable from a lack of activity.

Is the affective manipulation associated with reward or punishment?

If anticipation of a loss or gain of reward is of interest (e.g. if the manipulation induces or relieves depression-like states), then this is what the task should measure. If the anticipation of a punisher or its avoidance is of interest (e.g. the manipulation is anxiogenic or anxiolytic), then there must be a punisher of which the animal can avoid. Where there are no clear *a priori* predictions, a combination of rewards and punishers should be used.

Are we interested in identifying a particular emotion or just emotional valence?

Tasks using one ambiguous-cue are very effective tools to measure positive or negative valence, but to identify the specific emotional quadrant in which an animal's affective state lies, multiple cues must be used.

Are repeated testing sessions necessary to obtain data?

Precautions should be taken to minimise the risk of response extinction which include limiting the repetitions of test sessions and the number of exposures of animals to non-reinforced ambiguous trials. Partial reinforcement of reference trials during testing should also be considered.

1.III.viii Applications of the judgement bias paradigm

The judgement bias task is preferred to other traditional measures of affect as it has the potential to identify changes in affective valence induced by a range of manipulations, and can be applied in the study of many research questions. In the field of animal welfare, there are concerns related to the husbandry of lab animals, farm animals and captive wild animals, as well as the care of domesticated animals. For example studies using rats, starlings, sheep and dogs suggest that variations in housing conditions such as environmental enrichment, lighting conditions or unpredictable housing affect the valence of emotional responses made by the animal (Burman et al., 2008, Doyle et al., 2010a, Matheson et al., 2008, Casey et al., 2008). Also, veterinary inspection of rhesus macaques induced a negative bias as indicated by a judgement bias task (Bethell et al., 2012). Similarly, common farming procedures have been shown to induce negative biases, such as calf dehorning (Neave et al., 2013), shearing of sheep (Sanger et al., 2011) and restraint stress (Doyle et al., 2010a). Separation anxiety – a phenomenon induced by long periods of no contact with owners – has also been shown to induce a negative bias in domestic dogs (Casey et al., 2008). It is therefore of importance to assess if there are significant changes in affective state as a result of husbandry, handling and care, so that steps can be taken to improve practices. This evidence that husbandry and welfare standards are variable factors that potentially alter data output of behavioural tasks also highlights the need for them to be both improved and standardised to improve reproducibility in studies.

Furthermore, in scientific research, judgement bias tasks have been used to identify changes in affective state associated with neurobiological stressors and drug treatments. Enkel et al. (2009) used a judgement bias task to identify a negative bias in animals genetically bred to display depressive-like characteristics, and they also went further to demonstrate that a negative state was caused by pharmacological induction (reboxetine and corticosterone, the rodent analogue to cortisol), an analogous neurobiological stressor to that which induces stress in humans (Kukolja et al., 2008). A similar judgement bias task was adopted by Anderson et al. (2013) to assess the affective changes produced by long- and short-term administration of antidepressant and anxiolytic drugs, who found them to both alleviate and potentiate negative

affective states. Sheep have also been demonstrated to show biasing of ambiguous information in line with *a priori* predictions of a negative change in affective state when brain serotonin levels were depleted by administration of P-Chlorophenylalanine (Doyle et al., 2011).

One question yet unanswered is whether this task has the potential to be developed further to assess sickness in animals. The latter is of great interest as there is an absence of a vomiting reflex in one of the most commonly used research species, the rat, so there is a necessity for establishing reliable indicators of nausea in animals. This is also particularly relevant to humans who can't communicate with language (e.g. infants, the demented and brain damaged).

1.IV Research questions tackled in this thesis

The review of affective manipulations of existing judgement bias experiments revealed a gap in the study of affective state associated with sickness. Sickness, in humans, is accompanied by low moods and it would be plausible that this has an evolutionary basis, as is the argument for a variety of other emotional responses such as fear and anxiety (see 1.I.iv.2). The first research question addressed in this thesis was whether modified affective states existed in a rat model of sickness. Initially a judgement bias paradigm measuring both the expectation of reward and punishment was trialled to detect anxiety following ethanol hangover (Chapter 2). I then ran a set of experiments to establish a dose of a sickness-inducing agent (lithium chloride) that would be suitable to use in a modified version of this task. The presence of a judgement bias in sickness and its reversal with an anti-sickness drug was then investigated (Chapter 3).

Another highlight of the judgement bias experiment review was that there have been few studies that directly compare identical affective manipulations between species to determine whether experimental outcomes are universal or species-specific. In addition, there has also been a surprising lack of development of the use of invertebrate animals in the measurement of judgement biases following from Bateson et al. (2011b), essentially overlooking a potential replacement for mammals in emotion

research. To this end I investigated the behavioural effects of sickness-inducing toxins in honeybees, establishing whether injection or ingestion was responsible for toxic effects (Chapter 4), and then measured judgement biases in these animals administered the agents (Chapter 5). The comparisons of the outcomes of these tasks, and of the tasks themselves, are discussed in Chapter 6.

Chapter 2 - The affective component of ethanol hangover in rats

Summary: In this chapter I explored the affective component of ethanol hangover in the rat. Hangover presents with anxiety and depression in humans, and there is a body of evidence that it is also anxiogenic for the rat. A judgement bias task was used to identify negative affective states in hungover rats, as were more established measures of anxiety: the elevated plus maze and open field test.

The judgement bias paradigm utilised the anticipation of a negative reinforcer (foot-shock) along with anticipation of a food reward in order to ensure shifts in affect on the anxiety pathway could be identified. A total of 9 rats of 24 were successfully trained on the task, but only 3 maintained task performance to criterion over repeated testing sessions. No effect of ethanol hangover was observed on the judgement bias task, nor was any effect seen using the more established measures of affect.

2.1 Introduction

However trivial the hangover may seem, its prevalence has substantial social and economic consequences. Alcohol-related mortality contributes to over 8,000 deaths per year in the UK alone according to the Office for National Statistics (2014), and alcohol has been indicated in over 60 different disease states (Rehm et al., 2002). The hangover would appear to be a universal problem amongst drinkers, and possibly the greatest cost associated with hangover is decreased productivity in the workplace in the form of increased absenteeism and impaired work performance (Crofton, 1987).

Hangover in humans is characterised by symptoms like headache, nausea and fatigue, and it also presents with a psychological component. Depression, dysphoria and guilt (Bogin et al., 1987, Smith and Barnes, 1983) as well as anxiety and irritability (Mossberg et al., 1985, Roelofs, 1985) are mood states associated with a hangover in humans. It seems counterintuitive that although hangover presents with aversive symptoms, people continue to drink on a regular basis. Surprisingly, hangover rarely

acts to effectively deter future alcohol consumption (Earleywine, 1993a) and in some cases, excessive drinking may be maintained in order to alleviate the negative consequences of hangover and alcohol withdrawal (Earleywine, 1993b). It has been found that subjects dependant on alcohol are over 3 times as likely to drink in order to terminate these adverse psychological effects, rather than to alleviate the physiological symptoms (Hershon, 1977).

Typically, the hangover begins several hours after an individual has ceased drinking and blood ethanol levels (BEL) decrease, and peaks when they have returned to zero (Prat et al., 2009). The hangover can subsist for up to 24 h after this (Swift and Davidson, 1998). The physiological component of hangover is attributed to a number of different effects of alcohol, whether caused by alcohol directly or by its metabolites, by beverage congener effects, or an effect of mild withdrawal (Wiese et al., 2000). Following intoxication, we might observe a range of physiological effects such as a drop in blood sugars, dehydration, increased acetaldehyde (a metabolite of ethanol), and disruption of sleep and other biological rhythms (Swift and Davidson, 1998). There is no agreement as to which of these is directly responsible for the hangover; in fact the general consensus is that any combination of these effects might produce symptoms. The causes and effects of hangover may also vary between individuals, and between drinking bouts. Although the causes of hangover present as an interesting and varied topic, I will not be focusing on the physiology of hangover in this chapter and instead investigating the psychological symptoms.

2.I.i Ethanol hangover in the rat

Anxiety-like behaviour has also been observed in the rat after ethanol hangover in a time- and dose-dependent manner. The hangover is again said to begin when BEL return to zero, and markers of anxiety peak within 3 h after this point (Doremus et al., 2003, Varlinskaya and Spear, 2004) with only limited evidence of anxiogenesis 7.5 – 9 h post-clearance (Varlinskaya and Spear, 2004). Analyses of the ethanol content of tail blood sampled hourly showed in that adult male Wistar and Sprague Dawley rats BEL reached zero at 6 h following 2g/kg ethanol, between 9 and 10 h following a 3g/kg

dose, and 13 – 14 h for a 4g/kg dose (Schulteis and Liu, 2006, Morse et al., 2000, Gauvin et al., 1993). Adolescent rats are also shown to clear ethanol from their blood faster than adult rats, which correlates with reduced behavioural symptoms of hangover (Doremus et al., 2003).

Typically, large doses (greater than 1g/kg i.p.) are required to produce acute withdrawal presenting with anxiety-like effects. Acute bolus doses of 2g/kg and 3g/kg ethanol produce acute and prolonged anxiogenic response on the elevated plus maze (EPM) without reductions in locomotor activity at 6 h and 9 h post-injection respectively (Zhang et al., 2007). This method of administration is said to increase the anxiogenic 'load' in the rat by applying greater physiological stress. In addition, acute binge patterns of intoxication followed by daily abstinence periods can lead to potentiation of negative emotional states (Zhang et al., 2007). Schulteis and Liu (2006) identified transient but significant anxiogenic behaviours with acute doses (2g/kg) that were accompanied by elevations in brain reward thresholds, where repeated bouts of intoxication resulted in a significant extension of the duration of this effect. As repeated dosing was adopted in the judgement bias study in this chapter to allow for multiple testing sessions, a strengthening of a negative bias with repeated exposure to ethanol might be predicted.

2.I.ii Pentylenetetrazol and ethanol hangover

The $GABA_A$ receptor antagonist pentylenetetrazol (PTZ) was used during the 1930's as a convulsant therapy in humans suffering from schizophrenia (Fink, 2001). Although the drug was therapeutically beneficial in treating this disorder, a high incidence of panic attacks were recorded and patients were reluctant to receive the multiple doses required (Fink, 2001). Further investigation also showed that anxiety was induced by this drug at subconvulsant doses in a normal population (Rodin, 1958). The popularity of this drug declined later in the 1930's when electroconvulsive therapy was introduced as a treatment for schizophrenia (Fink, 2001), but its anxiogenic properties were not unnoticed. PTZ is now a prototypical anxiety drug extensively utilized in animal models of anxiety, increasing anxiogenic effects in animals exposed to the

elevated plus maze (EPM; Garcia et al. (2011)), and producing place aversion in conditioned place-preference test (Garcia et al., 2011, Gauvin et al., 1991, Gauvin et al., 1996). PTZ is also used widely in drug discrimination paradigms, which are used to index anxiety as a symptom of withdrawal from drugs including nicotine, morphine and cocaine (Prather and Lal, 1992, Harris et al., 1986, Emmett-Oglesby et al., 1984). The mechanism of action of this drug is still incompletely understood, but its properties as a discriminative stimulus are known to be mediated via the $GABA_A$ receptor (Shearman and Lal, 1980). The effects of PTZ are blocked by benzodiazepines (Shearman and Lal, 1980) which produce anxiolytic effects via allosteric modulation of these receptors (Sigel and Buhr, 1997).

In these drug discrimination paradigms animals are trained to discriminate between two levers in an operant chamber; one that is positively reinforced when the animal is treated with saline and another that is reinforced when the animal is given the anxiogenic treatment, PTZ. Following this, they are administered other treatments, and whether they generalise the treatment as anxiogenic (as measured by pressing the PTZ-lever), or not (by pressing the saline-lever) is assessed. Withdrawal from the abovementioned drugs causes animals to respond in a PTZ-appropriate manner. Ethanol hangover also precipitates PTZ-appropriate responding in drug discrimination studies (Lal et al., 1988, Gauvin et al., 1993) and as such PTZ would be a suitable drug for comparison of the anxiogenic properties of ethanol hangover in this study.

2.I.iii Hypothesis

Due to the similarities between PTZ and ethanol hangover in drug discriminability studies, and the anxiogenic properties of ethanol hangover observed on the EPM, it was hypothesised that we would observe anxiogenic psychological effects of both of these treatments on a judgement bias task. An anxiety-like bias is reflected by an increased expectation of punishment, as indicated by more 'negative' responding on the judgement bias task (Figure 2-1). It was also predicted that ethanol hangover would produce anxiety-like symptoms in the rat in the more established measures of affect: the elevated plus maze and open field tests.

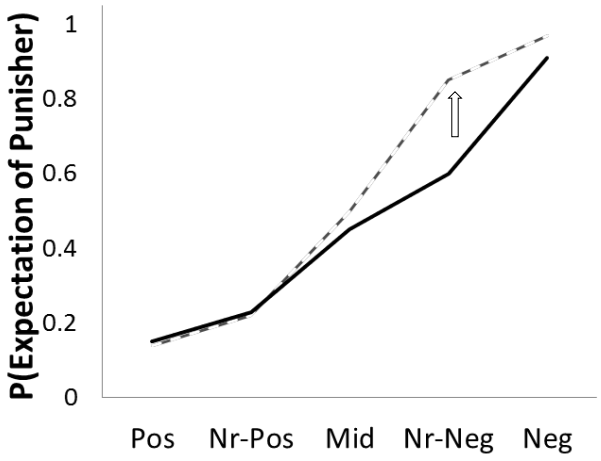


Figure 2-1 ***A diagrammatic representation of an anxiety-like bias on the judgement bias task.*** *Interpretation of ambiguous information as predicting negative outcomes is reflective of an anxious state in individuals.*

2.II Judgement bias in the hungover rat

2.II.i Rationale

As extensively discussed in Chapter 1, judgement bias tasks are used to identify particular affective states in animals. They have identified biasing of information in an anxiety-like manner in a number of studies (e.g. Destrez et al. (2012), Burman et al. (2009)), but as yet have not been used to identify psychological effects of hangover. We expected to observe an anxiogenic effect of ethanol hangover, with biasing of ambiguous information occurring in a similar manner to that seen with PTZ treatment.

2.II.i.1 The rat judgement bias task

In this judgement task, rats were trained to discriminate between two audio tones on a continuous stimulus-dimension. One of the tones predicted a food reward, and the other a punishing foot-shock, and these tones were referred to as the reference tones. Both of the reference tones were reinforced (i.e. the rats had to actively respond via lever presses to obtain reward or avoid the punisher). Both rewards and punishers were used, as although hangover is likely to present with anxiety, it might also present

with depression-like symptoms, like dysphoria (Swift and Davidson, 1998). In these circumstances it is important we are able to detect the presence of either psychological symptom (Chapter 1). Rats that met the criterion during the discrimination testing phase were treated with either ethanol hangover, PTZ or a saline control, and then underwent test sessions. In the test sessions their responses to ambiguous tones intermediate of the reference tones were compared.

The detectable audio range of the rat is ~200 Hz to 90 kHz, just overlapping that of humans which ranges from 16Hz to 20 kHz (Warfield, 1973). An audio range that was audible to both rats and the experimenter was used for the experiment. 2 kHz and 4 kHz cued for the reinforced outcomes in the initial discrimination task, and intermediate frequencies of 2.18 kHz, 2.58 kHz, 2.82 kHz, 3.08 kHz and 3.67 kHz as the ambiguous tones[1]. The tone volumes were also adjusted according to the audiogram of the Hooded rat (Heffner et al., 1994).

Foot-shock has been employed successfully in a number of judgement bias studies as an aversive outcome (Enkel et al 2009, Anderson et al 2013, Rygula et al 2012, etc.), and allows researchers to measure the expectation of punishment. It was necessary to measure expectation of punishment, as this represents a key hallmark of anxiety (Bateson et al., 2011a). A relatively mild foot-shock of 0.25mA was delivered to the animals; higher amperes provoke freezing behaviour in animals (e.g. 0.5mA, Conti et al. (1990)), which is undesirable in a task where animals are required to make active responses. Also, repeated exposure to more painful foot-shocks can produce learned helplessness when inescapable (e.g. multiple 0.8mA shocks over a 40 min period, Vollmayr and Henn (2001)). Learned helplessness is used as a model of 'despair' as part of a cohort of models of depressive symptoms, and we did not wish to modify the affective state of the animals in this study before we applied our own affective manipulations. Consequently, we aimed to keep the number of and degree of foot-shocks that the rats were exposed to at a minimum.

[1] Note that the kHz scale is non-linear, and these frequencies were selected by calculating the centre frequencies on the Hertz (log) scale using the following equation $\frac{f1}{f0} = \sqrt{\frac{f1}{f2}} = \frac{f0}{f2}$, where f_1 and f_2 represent the outermost frequencies and f_0 represents the midpoint frequency.

2.II.ii Methods

2.II.ii.1

2.II.ii.2 Ethical approval

All procedures conformed to the Association for the Study of Animal Behaviour's 'Guidelines for the use of animals in research' (Animal Behaviour, 1991) and were approved by Newcastle University's local ethical review committee. When procedures were regulated, they were carried out under Project Licence 60/3793.

2.II.ii.3 Animal Husbandry

24 experimentally naive male Lister Hooded rats (Charles River, Margate, Kent, UK) aged 11 weeks and weighed 298g ± 16g, were housed in groups of 4 in standard RC2 cages with sawdust as bedding ('Aspen', BS and S Ltd, Edinburgh, UK), a chew block and *ad libitum* access to water. Standard rat diet (RM3, Special Diet Services, Essex, UK) was fed at a restricted rate to maintain rats at no less than 85% of their free-feeding body weight with allowance for normal growth. The rats were housed with a 12:12 h light cycle (lights on at 0700) in a temperature and humidity controlled room. The animals were free from any common pathogens according to the FELASA Health Monitoring Recommendations. On completion of all experiments, rats were humanely euthanised with slow rising concentration of CO2.

2.II.ii.4 Apparatus and software

Eight conditioning chambers (measuring 300mm x 245cm x 200cm) constructed from clear Plexiglass were housed in sound attenuating boxes (Med Associates, Sandown Scientific, Middlesex, UK). An automatic feeder at the top of the unit fed down into a magazine where food was dispensed (45mg dustless precision pellets; Bio-Serv, LBS, Surrey, UK). The levers were 115mm apart and 55mm above the floor of the chamber, with a light above each. The levers extended 20mm into the chamber. A speaker and house light were located on the back wall of the chamber (Figure 2-2). A tone generator, speaker and attenuator were used to produce and control the volumes of

the training and testing tones. The floor of each chamber comprised of metal bars (0.2 cm diameter) spaced 1 cm apart and connected to a shock generator and scrambler (model ENV-412, Med Associates, St. Albans, VT). A PC running MED-PC IV software (Med Associates, St. Albans, VT) was used to control the operation of the levers, tone generators, speakers, shockers, attenuators and feeders.

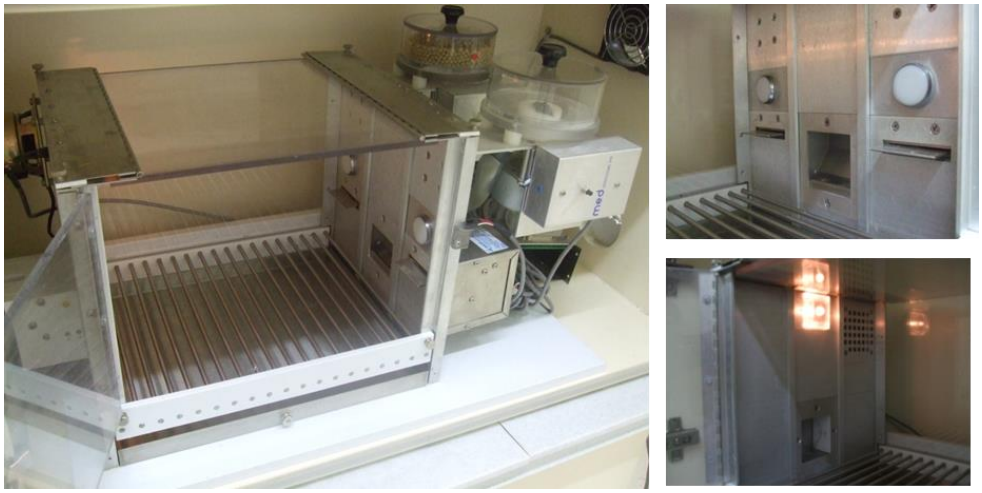


*Figure 2-2**Photographs of the operant chamber.** The photographs show the operant box (left), the levers and magazine (top right) and the speaker and house light (bottom right).*

2.II.ii.5 Training paradigm

24 rats were trained to respond on a two-choice discrimination task with differential reinforcement (Enkel et al., 2009). The rats were trained using a positive and a negative reinforcer to perform different responses when exposed to two different stimuli, which in this task was exposure to 10 s auditory tones of either 2 kHz or 4 kHz frequency. Responding to these tones involved pressing one of two levers, one located on the left of the front wall (the left lever) and one on the right of the front wall (right lever). Each of these stimuli cued for either the positive or negative reinforcer, and represented positive and negative trials respectively. The positive reinforcer was the delivery of one food pellet to a food hopper central to the two levers, and the negative reinforcer was the avoidance of a shock, which in the failure of a negative trial was delivered through steel bars on the floor of the operant chamber (Enkel et al. (2009);

Figure 2-3). For example the 4 kHz tone stimuli would lead to a food reward when the left lever was pressed, whereas the 2 kHz tone stimuli would lead to avoidance of shock when the right lever was pressed. This combination of tones, levers and reinforcers was consistent for each rat throughout the duration of the study and was counterbalanced between subjects.

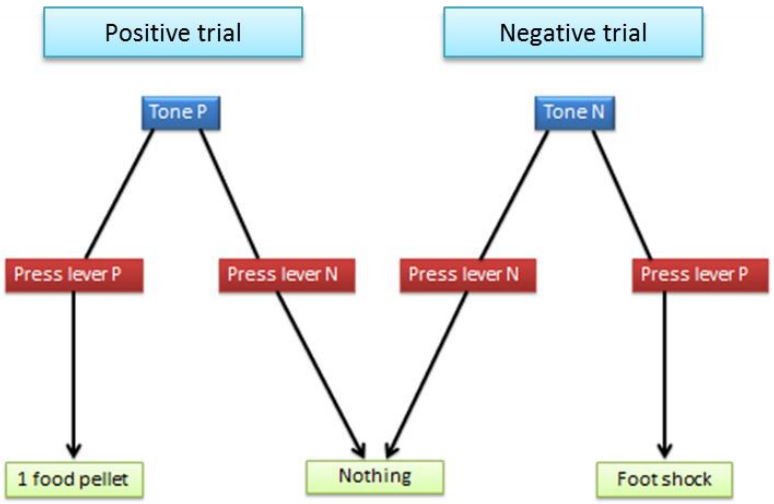


Figure 2-3 ***Schematic of the training protocol.*** *The figure shows the response outcomes of the positive and negative trials. P and N refer to positive and negative respectively. Tones P and N were either 2 kHz or 4 kHz and were counterbalanced between animals. Levers P and N refer to the right or left levers, which were also assigned in a counterbalanced manner between animals. Correctly responding on Lever P on a positive trial resulted in a food reward of one pellet dispensed to the central magazine, and a correct response on Lever N on negative trials resulted in the avoidance of an aversive outcome (1 s of 0.25mA foot-shock). The rats could also perform an escape response where pressing Lever N after the onset of shock caused its cessation.*

Positive lever training During the first training session the association between the positive tone and a food reward was introduced without a response contingency (i.e. the levers were not extended during this session). The tone predicting a food reward (Tone P) was played for up to 10 s, followed immediately by delivery of a food pellet into the magazine. 30 trials were presented in this session with a 70 s interval between trials.

In subsequent sessions, rats were required to depress Lever P in order to gain the immediate delivery of food. Tone P was played for 10 s following which the positive lever was extended for 10 s. A lever press within this time resulted in the immediate

delivery of a food pellet to the magazine. If a lever press was not made during this time the trial was terminated and a food pellet was delivered after a further 8 s. The lever was retracted on delivery of a food pellet. A 70 s ITI followed. The session ended either when 50 rewards had been received or 50 min had elapsed, whichever was soonest. Rats were required to meet a criterion of responding on ≥90% of trials over three consecutive sessions in order to move to the negative lever training phase.

Negative lever training In the first of these sessions trials consisted of a 10 s presentation of Tone N followed by 1 s of inescapable foot-shock (0.25mA). Only six of these trials were presented to avoid exposing the animals to excessive inescapable shocks and to prevent the development of a learned helplessness. A 6 min intertrial interval separated the trials.

In the subsequent sessions, the tone was presented for 10 s and was followed by the extension of the negative lever (the opposite to those which were presented in the positive lever training sessions, Lever N). If the rat pressed the lever during this time the trial would cease and an avoidance response was recorded. If the rat did not press the lever during this time a mild foot-shock (0.25mA) was delivered through the grid floors for 1 s. This was recorded as a response omission. In these sessions there were a maximum of 8 trials per session with a 4 min intertrial interval, again to minimise the number of shocks rats received while learning. Sessions ended when the maximum number of trials had been reached, or when 50 min had elapsed, whichever was soonest.

Following 16 sessions of training on this schedule with no indication of learning taking place, it was amended. Here, the duration of the tone was extended to overlap with the lever extension and shock to improve their association, and the duration of the shock was extended with additional escape responses permitted to provoke the rats to associate pressing the lever with the cessation of shock. A 10 s tone was followed by the consecutive presentation of both Lever N and the tone. If the lever was depressed within 10 s the trial ended and an avoidance response was recorded. If the lever was not pressed, the rats received 10 s foot-shock until the lever was pressed (which was recorded as an escape response). Escape responses are more easily learned by rats

than avoidance responses (Meyer et al 1960) and it was thought that these escape responses, where shock cessation is paired simultaneously with lever pressing, would transfer to avoidance responding (Rakover, 1980). The criterion for progressing to the next stage of training was to perform ≥ 75% avoidance responses over three consecutive sessions.

Discrimination training – forced choice Once rats were reliably performing on the positive and negative trials, the two trial types were combined within a session. 15 positive and 15 negative trials were presented in a pseudo-randomised order where no more than two of each trial type was given in sequence. In these trials, a 10 s positive or negative tone was played followed by a 10 s extension of the associated lever. Only one lever was extended during a trial and was retracted on completion of the trial. A 50 s interval separated the trials. Positive trials were as above, where correct responses during positive trials resulted in immediate food delivery but here response omissions were not reinforced. Correct responses during negative trials were reinforced with the avoidance of foot-shock, which was delivered for 10 s when responses were omitted. The foot-shock was escapable via a lever press in this 10 s period. Rats were required to perform ≥70% correct responses on both trial types for 1 session before beginning discrimination training.

Discrimination training – active choice Discrimination training sessions were as the combination training sessions, but here both the positive and negative levers were extended during a single trial and the rats were required to choose the relevant lever. Incorrect responses during trials had the same outcomes as response omissions. The criterion for this session was ≥66% responding on each trial type over 3 consecutive sessions. Nine rats met this criterion and continued onto the testing phase.

Ambiguous-cue test sessions Ambiguous-cue testing sessions were as the discrimination sessions but 5 of each positive and negative trial were replaced by ambiguous-cue trials. The 5 ambiguous-cues were played twice each in the testing sessions. The tones were equally spaced on the logarithmic Hz scale between the two training tones and served as ambiguous stimuli. During ambiguous-cue trials the rats were presented with both the positive and negative levers after 10 s. There was no

outcome when either lever was pressed, or if no lever was pressed (response omission). Responding on the reward-associated lever was classed as a positive response, whereas a response on the lever associated with foot-shock was classed as a negative response. Their choice of lever pressed (or response omission) was recorded. Rats underwent 3 ambiguous testing sessions over 3 consecutive days in the testing period. During the testing periods animals were administered one of three treatments - ethanol withdrawal, PTZ or saline – which were given in a pseudorandomised order to control for order effects. There was a 14 day washout between treatments (Figure 2-5). Animals were retrained with discrimination testing sessions daily for 3 days prior to each testing period. Rats were required to meet a criterion of ≥60% correct responses on both trial types on the last discrimination session before retesting.

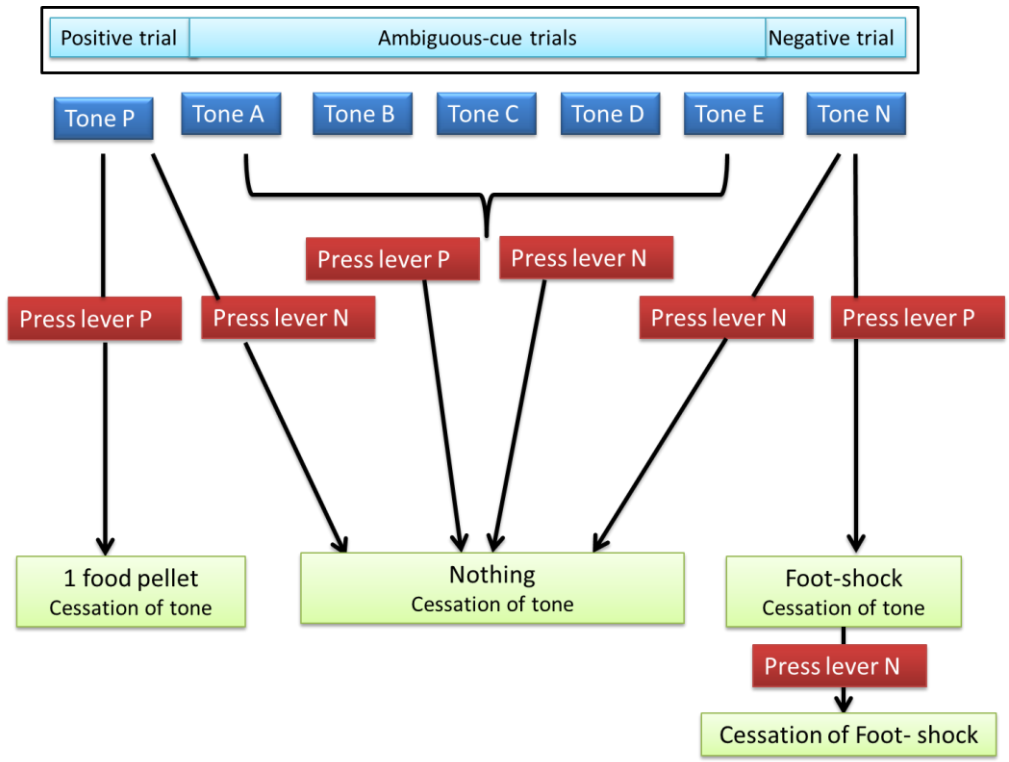


Figure 2-4 ***Schematic of the ambiguous-cue testing sessions.*** *The rats were trained to press Lever P in response to the positive tone to receive food, and to press Lever N to avoid foot-shock. Once this training was completed the rats were tested with five ambiguous, intermediate tones (Tone A – E). There was no response outcome on ambiguous-cue trials.*

Training phase	Performance criterion	Sessions to criterion ± s.d.	N° rats trained	N° rats met criterion
Positive training	≥90%, 3 consecutive sessions	6.0 ± 2.5	24	24
Negative training	≥ 75%, three consecutive sessions	14.3 ± 5.0	24	23
Forced choice	≥70%, 1 session	3.5 ± 2.8	23	20
Active choice	≥60%, 3 consecutive sessions	9.5 ± 4.4	20	9
Retraining	≥60%, last discrimination session before retesting	n/a	9	3

Table 2-1 ***Summary of the time taken to reach performance criteria of training sessions.*** *The table shows the performance criteria for each stage of testing, the number of sessions for criteria to be met, and the number of rats that were trained during each phase and how many succeeded in meeting these criteria.*

2.II.ii.6 Ethanol dose selection

Constraints with the use of the operant chambers meant it was necessary to leave an 18 h gap between injection and testing. Doremus-Fitzwater and Spear (2007) reported that adult rats administered 4g/kg ethanol (20% w/v) showed anxiety-like behaviour following an 18 h period. However, in-line with good practice guidelines, we were not able to administer this dose to our rats, and instead they received a 3.3g/kg ethanol injection. This arose from two considerations of the methods; firstly, the concentration of ethanol in the injection solution was limited to 20% (w/v) as it is reported that concentrations higher than 15-20% are irritating to the animal when injected i.p. (Schulteis and Liu, 2006); Secondly, to administer a 4g/kg dose of ethanol at this concentration, we would have to inject animals at a volume of 24.2ml/kg. Guidelines for the administration of substances recommend that, in good practice, injections of no more than 10ml/kg should be given in the peritoneal cavity (Diehl et al 2001, Morton et al 2001). As a maximal volume, 20ml/kg has been recommended (Diehl et al

2001). As dosing was repeated daily for three daily testing periods, it was decided that exceeding this proposed maximum volume would compromise the welfare of the animals in the study. As such, an injection volume of 20ml/kg, of ethanol (20% w/v) was used, which effectively administered a dose of 3.3g/kg ethanol.

2.II.ii.7 Ethanol and PTZ administration

Ethanol (20%w/v) was prepared by diluting a 95% stock of ethanol with distilled water. Animals received intraperitoneal injections at doses of 3.3 g/kg in a volume of 20ml/kg body weight. Rats were anaesthetised prior to ethanol administration to reduce the irritation experienced from ethanol injection. Rats were placed into a chamber and anaesthetised for 30 s with 8% sevofluorane at a rate of 1L/min. Once anaesthesia was achieved (which was confirmed by the loss of righting reflex) animals were transferred to a mask providing 4% sevofluorane to sustain anaesthesia for the injection. Following completion of the injection the mask was removed, and the rats were transferred to an incubator (27^{o}C – 34^{o}C) for a period of 2 h or until full movement was restored.

15 min before testing, animals in the PTZ group were administered 20mg/kg PTZ dissolved in isotonic saline for injection administered i.p. 1ml/kg (Gauvin et al., 1996, Lal et al., 1988). Animals in the saline and ethanol withdrawal groups were administered saline (0.9% w/v) 1ml/kg. A crossover design was used for the administration of drugs (Figure 2-5). Animals administered the saline control or PTZ were not anaesthetised prior to injection.

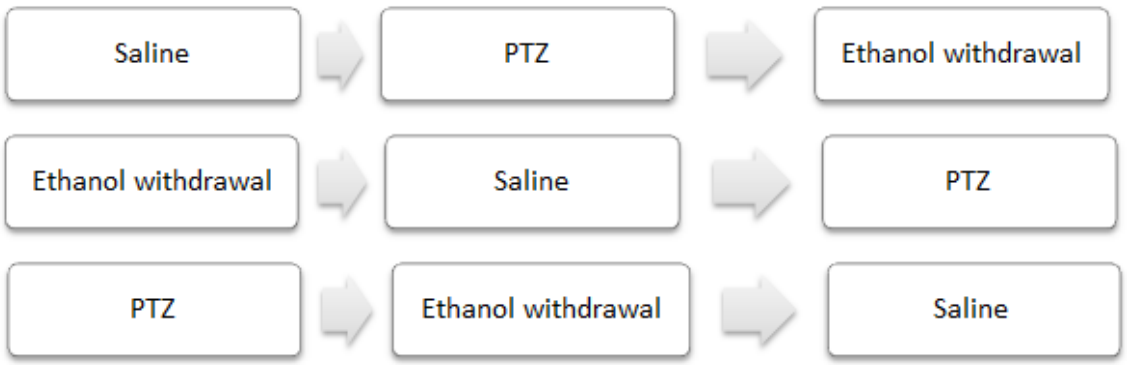


Figure 2-5 ***Schematic of the within-subjects design for drug administration****. Rats received ethanol withdrawal, PTZ or saline with a 2 week washout period and 3 days discrimination retraining between treatments.*

2.II.ii.8 Statistical analysis

Analyses were performed on the proportion of reference-cue and ambiguous-cue trials responded to positively, negatively, or with an omission. Mean values for these variables were calculated for each rat over the three test sessions within each treatment schedule. Data were analysed with Generalised Estimating Equations (GEE's are used to analyse linear data in preference to Generalised Linear Models when repeated measures need to be accounted for)[2]. Rat ID was included as a repeated subject variable and tone frequency as a within-subjects variable. Tones and treatment were included as factors. For all analyses pairwise comparisons were adjusted for multiple comparisons by least significant difference (lsd). An alpha value of 0.05 was used for significance tests. All analyses were performed using IBM SPSS Statistics 21.

2.II.iii Results

2.II.iii.1 Was response extinction observed?

As outlined in Table 2-1, there was a pronounced drop in the number of rats sufficiently meeting performance criteria during the discrimination phase. Further to this, animals that met this criterion showed worsening of performance in the discrimination retesting sessions between test periods. Figure 2-6 shows the drop of response accuracy below criterion over the multiple testing sessions by these 9 animals that initially met the criterion. Animals are typically excluded from analyses if they fail to meet responding criterion during training sessions (e.g. Enkel et al. (2009), Murphy et al. (2013), Chaby et al. (2013)). In this study, 6 of the 9 rats failed to respond with at least 60% accuracy on both trial types during retraining and were excluded from subsequent analyses.

[2] I was unable to use a repeated measures ANOVA for this analysis (as is used in chapter 3 to analyse similar data) as multivariate test statistics could not be produced due to insufficient residual degrees of freedom due to the small sample sizes. A linear mixed model analysis was also unsuitable as the validity of the model fit was uncertain. GEE's are also less sensitive to variance as they are semiparametric tests.

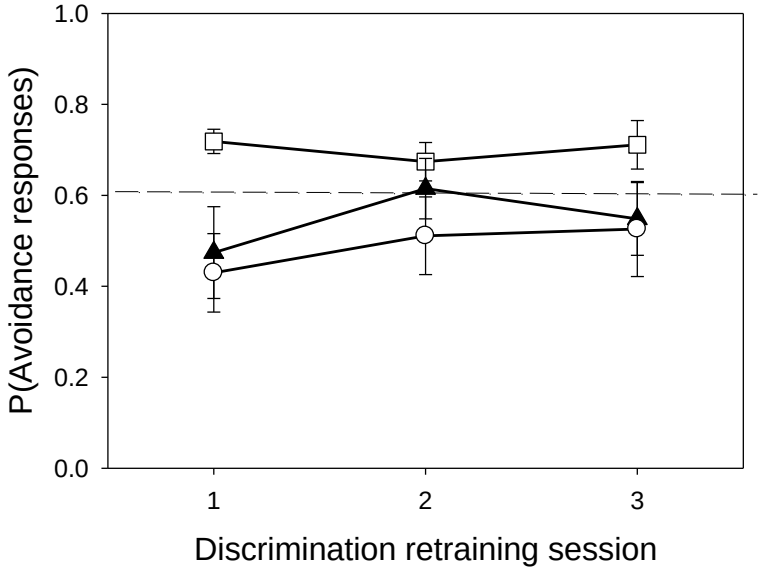


Figure 2-6 ***Proportion of avoidance responses made during retraining sessions.*** *The graph shows the performance of rats during the discrimination retraining periods that preceded ambiguous-cue testing. Each block of discrimination retraining contained three sessions (shown on the x-axis). Squares represent the first block of discrimination training; triangles represent the second block; and circles show rats performance during the third. Symbols show means (±S.E.M. (N = 9). The dashed line represents the 60% response criterion.*

Prior to the first training session, 9 rats met the criterion, and the first results section contains a between-subjects analysis of their responding during this first session. These rats were allocated treatments in a pseudo-randomised but ordered manner, which meant that in this session equal numbers of rats received each treatment. The second results section contains a within-subjects analysis which includes only the 3 rats that maintained the discrimination and met the performance criterion throughout the study. These two analyses were performed to allow us to determine whether the treatment effects were consistent. The order of treatment of these 3 rats was unfortunately not balanced (see Table 2-2), and so a treatment order effect was also investigated.

	Session 1	**Session 2**	**Session 3**
Rat 1	Ethanol	Saline	PTZ
Rat 2	Ethanol	PTZ	Saline
Rat 3	Saline	Ethanol	PTZ

Table 2-2 ***Treatment orders of the rats in the judgement bias study.*** *3 rats were included that displayed above criterion performance during discrimination retraining sessions.*

2.II.iii.2 Did ethanol or PTZ affect rats' responses to ambiguous stimuli? Between-subjects analysis.

A between-subjects analysis of rats' positive responses on the first testing session showed a main effect of the tone played (GEE: $\chi^2 = 5055.27$, df = 6, $p < 0.001$) which indicated that the rats generalised positive responding in accordance with how closely the audio stimulus matched the positive tone. Contrary to that which was hypothesised, rats treated with PTZ performed significantly more positive responses than those administered saline (main effect of treatment: GEE: $\chi^2 = 7.05$, df = 2, $p = 0.029$). An interaction between the tone and treatment (GEE: $\chi^2 = 7328.00$, df = 8, $p < 0.001$) was also observed, and significant probe x treatment differences are highlighted in Figure 2-7 a & b.

Rats also showed generalisation of the tones as indicated by the proportion of negative responses performed, which increased as the tone frequency neared that of the negative tone (tone main effect: GEE: $\chi^2 = 84592.77$, df = 6, $p < 0.001$). Here, however, there was no treatment effect (GEE: $\chi^2 = 2.94$, df = 2, $p = 0.230$), but a treatment x probe interaction was observed (GEE: $\chi^2 = 24.56$, df = 6, $p < 0.001$). Again, significant interactions are indicated in Figure 2-7 c & d.

A main effect of treatment was found in the proportion of trials where no response was made (GEE: $\chi^2 = 14.10$, df = 2, $p = 0.001$). Interestingly, this was also a function of the tone frequency (GEE: $\chi^2 = 133.97$, df = 6, $p < 0.001$). Significant treatment x probe interactions were also observed (GEE: $\chi^2 = 43.74$, df = 6, $p < 0.001$) and are shown in Figure 2-7 e & f.

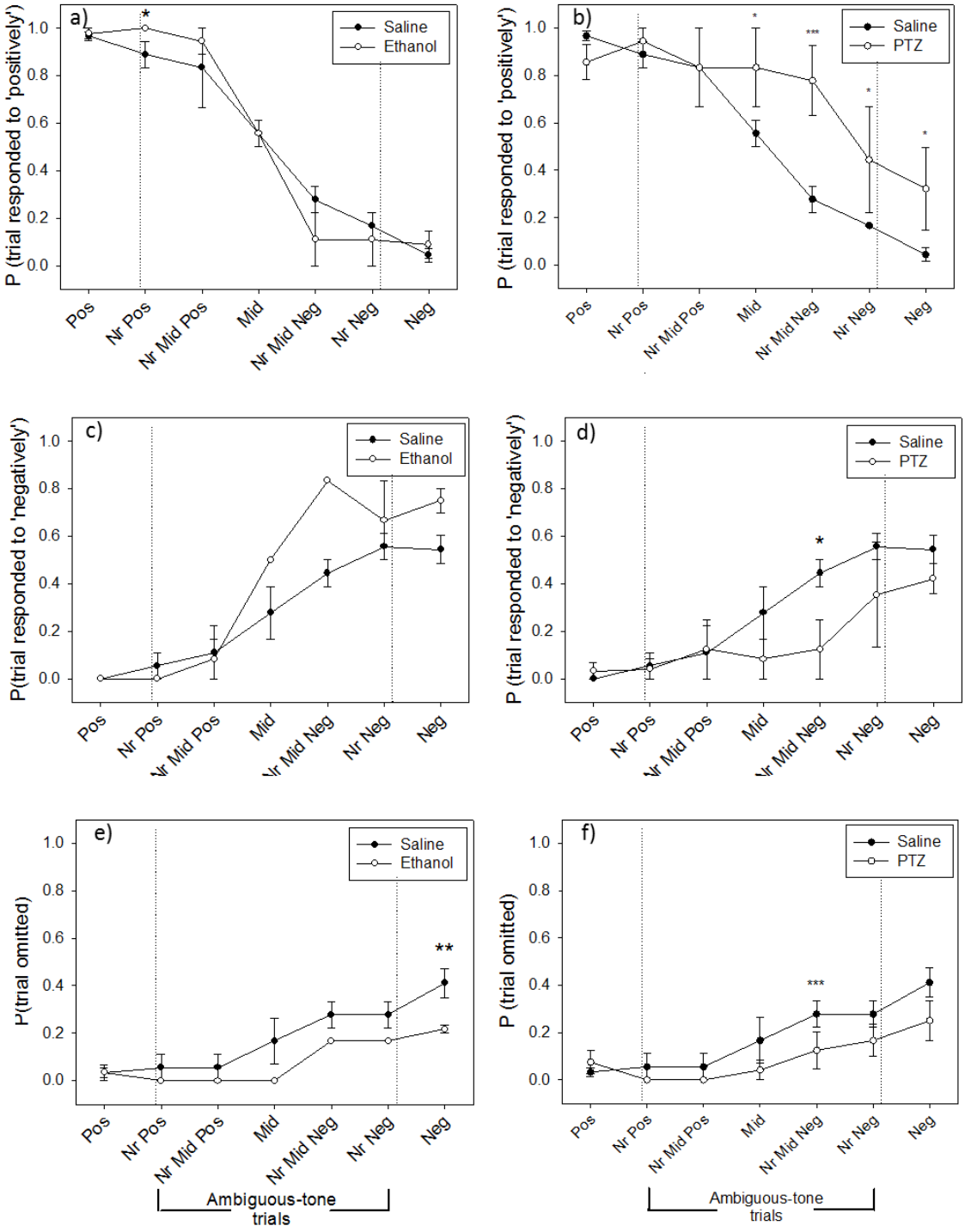


Figure 2-7 ***The pattern of responding by rats during the ambiguous-probe test session – between subjects.*** *Graphs show responding during the reference trials (reference cues: Pos and Neg) and five intermediate ambiguous trials (ambiguous cues: Nr Pos,Nr Mid Pos, Mid, Nr Mid Neg and NrNeg when treated with ethanol hangover (left panel) or pentylenetetrazol (right panel) compared to a saline control. a) & b) show the proportion of 'positive' responses (responses made on the lever predicting a food reward); c) & d) show the proportion of negative' responses (where a response was made on the lever predicting avoidance of mild foot-shock); and e) & f) show the proportion of response omissions (where no response was made within the 10 s tone presentation. Symbols show means +S.E.M. *:p < 0.05; **: p<0.01; ***: p<0.001. (Saline: n = 3, Ethanol hangover: n= 3, PTZ: n = 3).*

2.II.iii.3 Did ethanol or PTZ affect rats' responses to ambiguous stimuli? Within-subjects analysis.

Similarly to the between-subjects analysis, when only subjects that consistently met the criterion for task performance were included in a between-subjects analysis, positive responding was decreased the further the tone deviated from the positive tone (GEE: $\chi^2 = 509.64$, df = 2, $p < 0.001$). A main effect of treatment was also observed on positive responding (GEE: $\chi^2 = 9.42$, df = 2, $p = 0.009$), where rats responded significantly less when administered PTZ than when administered saline (pairwise comparisons: df = 1, $p = 0.005$). The interaction of the tones and the treatments was also significant (GEE: $\chi^2 = 9.71$, df = 2, $p = 0.08$) and these interactions are highlighted in Figure 2-8a & b.

Tones were similarly generalised by rats when responding negatively during the testing sessions (GEE: $\chi^2 = 87.70$, df = 2, $p < 0.001$). The treatment administered also had a strong effect on the negative responses rats made during these sessions (GEE: $\chi^2 = 16.86$, df = 2, $p < 0.001$), where PTZ administration caused rats to respond less than ethanol hangover (pairwise comparisons: df = 1, $p < 0.001$). No treatment x probe interaction was observed (GEE: $\chi^2 = 2.00$, df = 2, $p = 0.368$; Figure 2-8 c & d).

The treatment administered also had a pronounced effect on the proportion of trials where a response was omitted (GEE: $\chi^2 = 191.82$, df = 2, $p < 0.001$), where administration of PTZ caused more response omissions than saline (pairwise comparisons: df = 1, $p < 0.001$) or ethanol treatment (pairwise comparisons: df = 1, $p < 0.001$). Response omissions were also influenced by the tone frequency (GEE: $\chi^2 = 79.21$, df = 2, $p < 0.001$), and presented with a significant interaction with the treatment given (GEE: $\chi^2 = 54.50$, df = 2, $p < 0.001$; Figure 2-8 e & f).

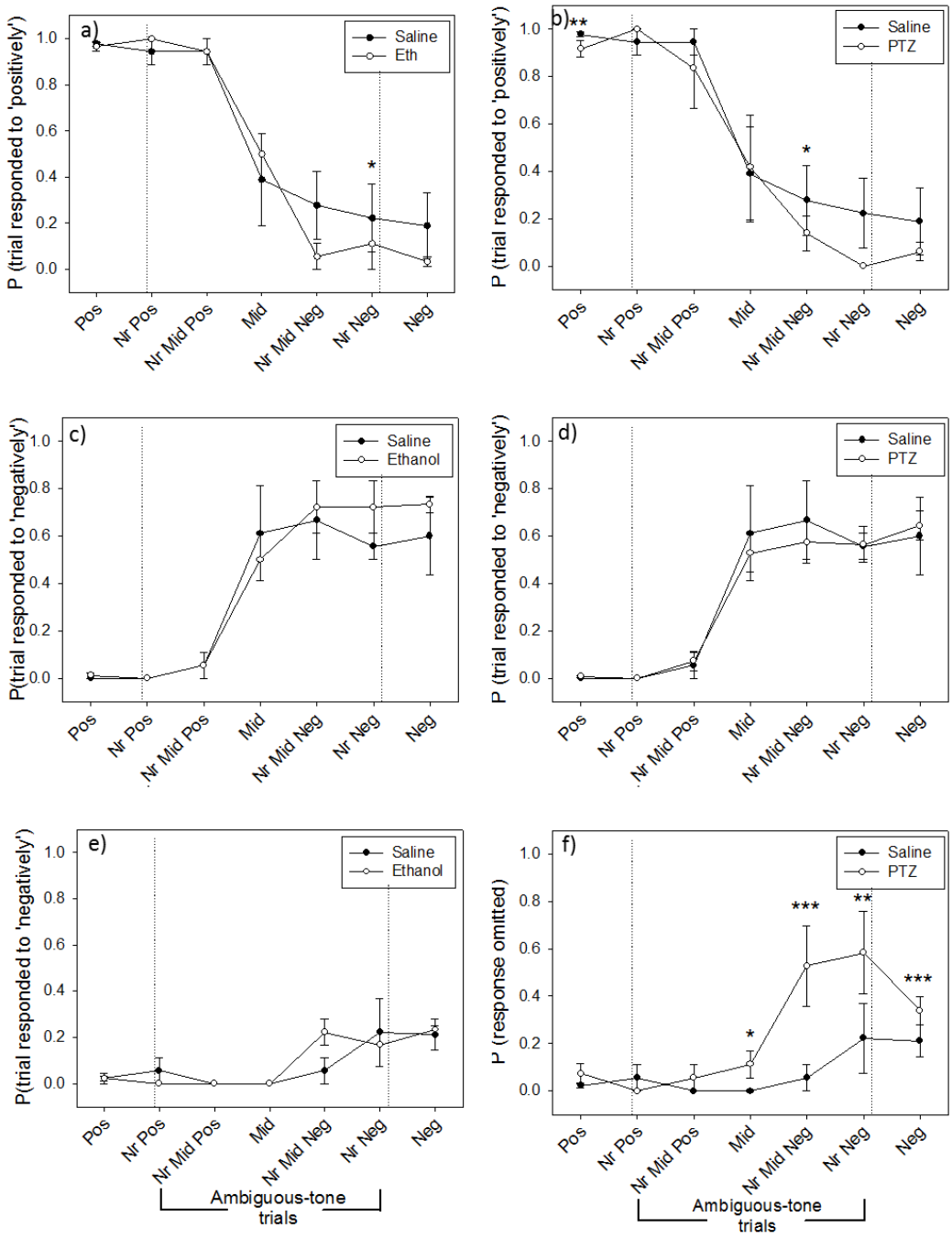


Figure 2-8 ***The pattern of responding by rats during the ambiguous-probe test session – within subjects.*** *Graphs show responding shows the pattern of responding by rats during the ambiguous-probe test sessions during the reference trials (reference cues: Pos and Neg) and five intermediate ambiguous-trials (ambiguous cues: Nr Pos,Nr Mid Pos, Mid, Nr Mid Neg and NrNeg. when treated with ethanol hangover (left panel) or pentylenetetrazol (right panel) compared to a saline control. a) & b) show the proportion of 'positive' responses (responses made on the lever predicting a food reward); c) & d) show the proportion of negative' responses (where a response was made on the lever predicting avoidance of mild foot-shock); and e) & f) show the proportion of response omissions (where no response was made within the 10 s tone presentation. Symbols show means +S.E.M. *:$p < 0.05$; **: $p<0.01$; ***: $p<0.001$. (n = 3).*

As the order of treatment administration was not balanced in this within-subjects analysis, an effect of order was explored separately (Figure 2-9). These data are presented in the form of bars to allow for enhanced visual comparison of the three sessions. The proportion of positive responding was altered depending on the test session (GEE: $\chi^2 = 226.68$, df = 2, $p < 0.001$), but pairwise comparisons did not identify a significant difference between any of the test sessions themselves. There was, however, a trend for rats to perform fewer positive responses in the second session than the first (pairwise comparisons: df = 1, $p = 0.089$). As the model also showed session x probe interactions (GEE: $\chi^2 = 12.92$, df = 2, $p = 0.002$), these have been highlighted in Figure 2-9a.

A much more marked effect of session was apparent when analysing the negative responses that rats made during the three testing sessions (GEE: $\chi^2 = 12.92$, df = 2, $p = 0.002$), where responding dropped significantly in the third session as compared to the first (pairwise comparisons: df = 1, $p < 0.001$). From the presentation of the data showing the tone x session interactions (GEE: $\chi^2 = 2719.12$, df = 3, $p < 0.001$; Figure 2-9b), it could be suggested that responding on the negative and negative-like trials decreased the most substantially.

This decrease in negative responding was accompanied by an increase in response omissions (GEE: $\chi^2 = 21.86$, df = 2, $p < 0.001$). However, pairwise comparisons do not show significant differences between specific sessions. Figure 2-9c and the session by tone interactions (GEE: $\chi^2 = 858.11$, df = 3, $p < 0.001$) indicate that omissions were increased in the final session, similarly to when negative responding was increased.

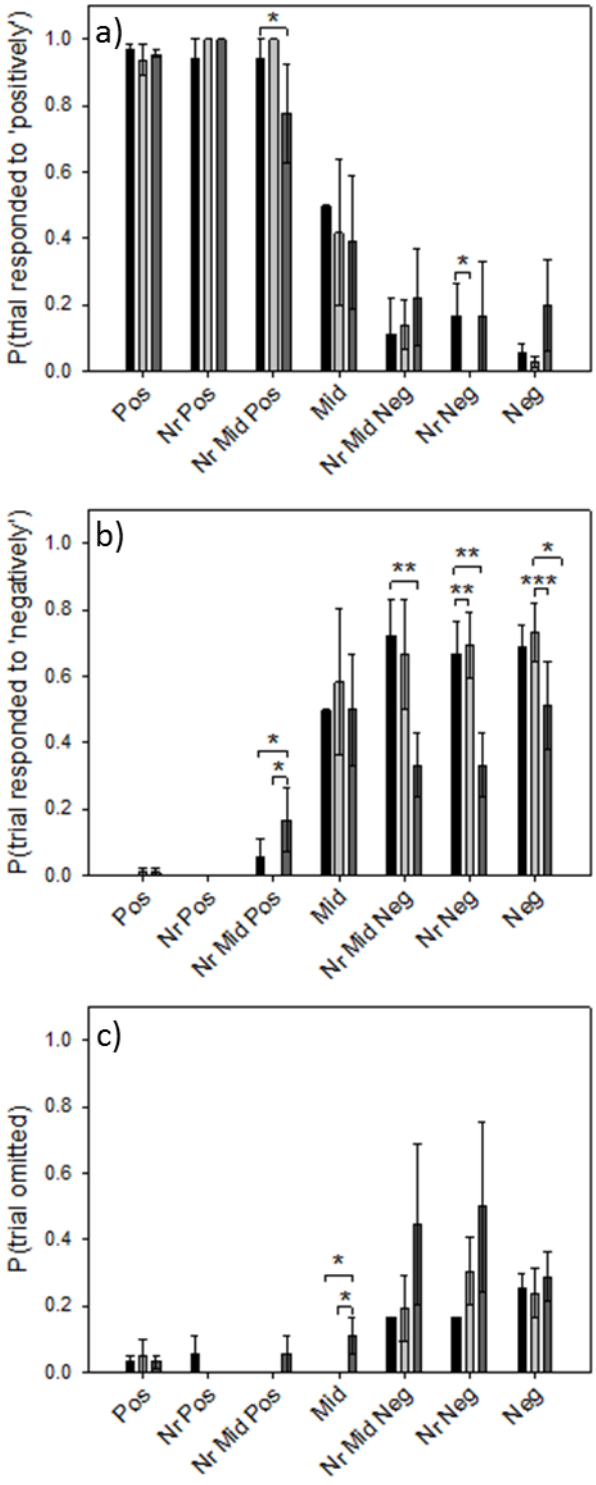


Figure 2-9 ***The pattern of responding by rats during the ambiguous-probe test session – session-by-session analysis.*** *Graphs show responding shows the pattern of responding by rats during the ambiguous probe test sessions during reference trials (reference cues: Pos and Neg) and five intermediate ambiguous-trials (ambiguous cues: Nr Pos, Nr Mid Pos, Mid, Nr Mid Neg and NrNeg) on the first (black), second (light grey) and third (dark grey) testing sessions. a) shows the proportion of 'positive' responses (responses made on the lever predicting a food reward); b) shows the proportion of negative' responses (where a response was made on the lever predicting avoidance of mild foot-shock); and c) shows the proportion of response omissions (where no response was made within the 10 s tone presentation. Symbols show means +S.E.M. *:$p < 0.05$; **: $p<0.01$; ***: $p<0.001$. (n = 3).*

2.II.iii.4 Were intermediate cues sufficiently ambiguous?

Interestingly, we found that many of the five intermediate 'ambiguous' tones were not responded to in a different manner than the learned reference tones. Specifically, these data indicate that there was no difference in the proportion of responses made to the learned cues and to the intermediate cues with similar frequencies (e.g. Pos vs. Near Pos; Neg vs. Near Neg). Table 2-3 and Table 2-4 shows the comparisons between the animals' responses to intermediate cues and the learned positive and negative cues respectively.

Ambiguous cue frequency	**Pairwise Comparisons Compared to positive cue**	
	Between subjects (*P* values)	**Within subjects (*P* values)**
Near Pos	0.920	0.233
Near Mid Pos	0.365	0.492
Mid	***<0.001***	***<0.001***
Near Mid Neg	***<0.001***	***<0.001***
Near Neg	***<0.001***	***<0.001***

Table 2-3 ***shows the responding of animals presented with intermediate cues compared to responding to the learned positive cue.*** *Instances where the proportion of responses was significantly different from that of the positive cue, the P values are highlighted (significance value: P < 0.05). Pairwise comparisons obtained from the output of the GEE analysis of the between subjects (n = 9) and within subjects (n=3) datasets.*

Ambiguous cue frequency	Pairwise Comparisons Compared to negative cue	
	Between subjects (*P* values)	**Within subjects (*P* values)**
Near Neg	0.531	0.303
Near Mid Neg	0.159	0.213
Mid	***<0.001***	***<0.001***
Near Mid Pos	***<0.001***	***<0.001***
Near Pos	***<0.001***	***<0.001***

Table 2-4 ***shows the responding of animals presented with intermediate cues compared to responding to the learned negative cue.*** *Instances where the proportion of responses was significantly different from that of the negative cue, the P values are highlighted (significance value: P < 0.05). Pairwise comparisons obtained from the output of the the GEE analysis of the between subjects (n = 9) and within subjects (n=3) datasets.*

2.II.iv Discussion

The interpretation of rats' biasing of ambiguous-cues differed according to the data that were analysed. However, none of these biases aligned with anxiogenesis which was predicted. When we compare the behaviour of all 9 rats during the first testing session, those treated with ethanol showed a positive bias to positive-like cues, indicating that they were more expectant of reward. There was no biasing of ambiguous-cues when rats responded to avoid expected foot-shock which would be increased if animals were in an anxiety-like state. Interestingly, the number of omissions made by rats treated with ethanol was decreased when compared to those administered saline. It is possible that these particular rats that were treated with ethanol in this first study were simply more efficient at performing the task, so it might reflect a superior task performance rather than an effect of ethanol hangover. A within-subjects analysis circumvents this issue as individual performances do not need to be taken into account. In this case, the responding of the 3 rats that consistently met performance criteria on the judgement bias task showed a less pessimistic biasing of ambiguous-cues with ethanol hangover treatment, but this was not related to the expectation of reward. When rats were administered ethanol, responding was reduced during near negative trials, indicating a reduced expectation of punishing events during the hangover period correlative with a reduction in anxiety. These data therefore suggest that ethanol hangover either has antidepressant or anxiolytic effects, which is in opposition with reported anxiety in other studies (Zhang et al., 2007, Doremus et al., 2003, Lal et al., 1988).

When comparing treatments between subjects, PTZ- treated rats showed a biasing in responding correlative to anxiolysis. Negative responding during negative-like ambiguous-trials was reduced, showing that the rats were less expectant of a punishing outcome. This was concomitant with an increase in positive responding and a decrease in response omissions during these trials. A positive bias in responding to the middle and near negative cues was also observed, indicating that the rats were generally more expectant of reward, as well as reduced punishment. However, responding to the learned cue associated with foot-shock was reduced, which might indicate a general inaccuracy in performance of the discrimination during exposure to

PTZ. We are less able to interpret altered responding towards ambiguous-stimuli when responding to the learned cues is also altered, as this might indicate a change in the motivation to respond rather than biasing of judgement (see Chapter 1). When we instead analysed the responding of the 3 rats that received all treatments in a within-subjects design, we observed the opposite effect. These rats demonstrated a decreased expectation of reward when administered PTZ, which is a hallmark of depression. We also observed a large increase in response omissions during ambiguous-trials which suggests that the consistency in task performance dropped. This increase in response omissions might be an artefact of treatment order where rats learned about the absence of reinforcement of ambiguous-cue trials or a general reduction in negative responding seen over time, rather than an effect of the drug. Two of the 3 rats were administered PTZ during the final testing session, where responding on the negative trials was not maintained (Figure 2-9b). This might explain the increase in response omissions during the negative trials that we observed with the administration of PTZ which was given only on the latter two testing sessions. With such scant data, however, it is not possible to separate an effect of time or treatment.

Additionally, we may not have observed the predicted anxiogenic effect of ethanol hangover due to our modifications of the testing schedule from that outlined in the literature (Zhang et al., 2007, Gauvin et al., 1997). It could be estimated that we surpassed the optimal time to measure anxiety-like behaviour by several hours. According to Morse et al. (2000), BEL reach zero at 10 h with a 3g/kg bolus i.p. injection, and 13 h with a 4g/kg dose. Markers of anxiety peak 3 h after this, and then begin to decline. If we estimate the clearance of BEL to be complete somewhere between the two time periods where clearance of 3g/kg and 4g/kg ethanol take place, peak anxiety would have occurred at approximately 14 h post-injection. With an 18 h period between drug administration and testing, 4 h passed since peak anxiety, which may have been reduced to undetectable levels.

Our protocol also differed from similar studies where five intermediate stimuli were included in test sessions, rather than the 'standard' inclusion of three (Enkel et al., 2009, Rygula et al., 2012). We found that these cues did not produce sufficient ambiguity, where responding was indistinguishable between the learned and

intermediate cues (see Table 2-3 and Table 2-4). Specifically, we found that there was no change in the proportion of responses made to the two cues with frequencies closest to the learned cues, and we only observed ambiguity from the middle stimulus onwards. We cannot obtain information regarding animals' judgements of ambiguity if the cues themselves are not ambiguous, and so these cues will be removed from future studies.

It is also worth considering that the training paradigm itself may have influenced the animals' affective states. Repeated exposure to daily foot-shock is a protocol used to produce learned-helplessness in rats, which is a model of depressive-symptoms (Maier and Seligman, 1976), and according to their performance during training, individual rats received a variable number of foot-shocks during this stage. As this effect was not controlled for, it may have produced variations in underlying affective states of the rats tested.

In this instance, we cannot make any firm conclusions of the effects of ethanol hangover and PTZ on judgement biases. The sizes of the groups were smaller than anticipated and no consistent conclusions were reached from the two analyses performed. The deficits in group sizes were a direct result of the inability for rats to learn and maintain an active avoidance responding schedule to cues predicting foot-shock, and the aspects of this training protocol are dissected and discussed in detail in the next chapter (section 3.V), with an alternative protocol presented.

2.III Proxy measures of affect

2.III.i Rationale

We failed to find an anxiogenic effect of ethanol hangover in the judgement bias task, but this may have been a function of sample size, or it may have been due to the limitation in the dosage that we were able to administer. To determine whether we could reliably measure anxiety-like behaviour in rats following ethanol hangover, we aimed to more closely replicate the protocol used by Zhang et al. (2007). They found that repeated dosing of 2g/kg ethanol increased anxiogenic effects over a period of 3

days 9 h after administration. We were able to condense the gap between ethanol administration and behavioural testing in order to repeat this element of their study. The animals were also tested in an open field test (OFT) which is another commonly used tool in animal models of anxiety to corroborate any effects observed. Additionally we aimed to determine whether there were prominently displayed behaviours that could be related to a hangover at the time-points where ethanol injection was shown to produce anxiety-like effects. In both tasks a within-subjects control design was employed so that the performance of each rat could be compared in ethanol hangover and control conditions.

2.III.ii The open field test

The OFT is one of the most popular procedures used in animal psychology to detect changes in anxiety-like behaviour. The test consists of an unfamiliar, walled arena, in which an animal's behaviour is monitored. This paradigm exploits the conflict between curiosity (exploration) and fear (Belzung, 1999, Russell, 1973). Open spaces are innately stressful to rodents that tend to live in social groups in small habitats, and they subsequently prefer the periphery of open arena and will walk close to the walls, a behaviour called thigmotaxis (Prut and Belzung, 2003). Increased time spent away from these walls and in the centre of the arena is indicative of anxiolysis, where the stress-induced inhibition of exploratory behaviour is decreased (Gould et al., 2009). Less anxious subjects spend more time in the centre of the arena and also enter it more frequently. Similarly, a variety of anxiogenic drugs reduce the time spent away from the walls of the arena (Prut and Belzung, 2003).

Changes in locomotion such as the distance travelled and the amount of time spent mobile can reflect stimulant or sedative effects of drugs (Gould et al., 2009). Ethanol has been shown to produce both sedative effects during intoxication and anxiety-like behaviour during hangover (depending on the dosage and time period after administration (Zhang et al., 2007)), so the OFT is an ideal test to use to identify which effect (if either) is present in this study.

2.III.ii.1 Methods

2.III.ii.1.1 Animals and husbandry

8 experimentally naïve male Lister Hooded rats aged 29 weeks weighing 625g ± 30g at start of testing were used. The husbandry of the animals was as in 2.II.ii.3.

2.III.ii.1.2 Apparatus and procedure

A black 80cm x 80cm open field arena was positioned on the floor. A tape camera was mounted 90cm directly above the centre of the arena. The room lights were off and the arena was lit by a light box to optimise contrast for filming and analysis. At the start of the test the rat was placed in the centre of the arena. The experimenter remained in the room behind a screen for the 5 min filming period. At the end of the test the animals were returned to their homeroom and the arena was cleaned with 50% ethanol. Tapes were converted to mpeg files using Mediacruise 2.2 (Canopus Co., Ltd.) and these were analysed using Ethovision XT (Noldus Information Technology). The rats were tracked from their centre-point by this software.

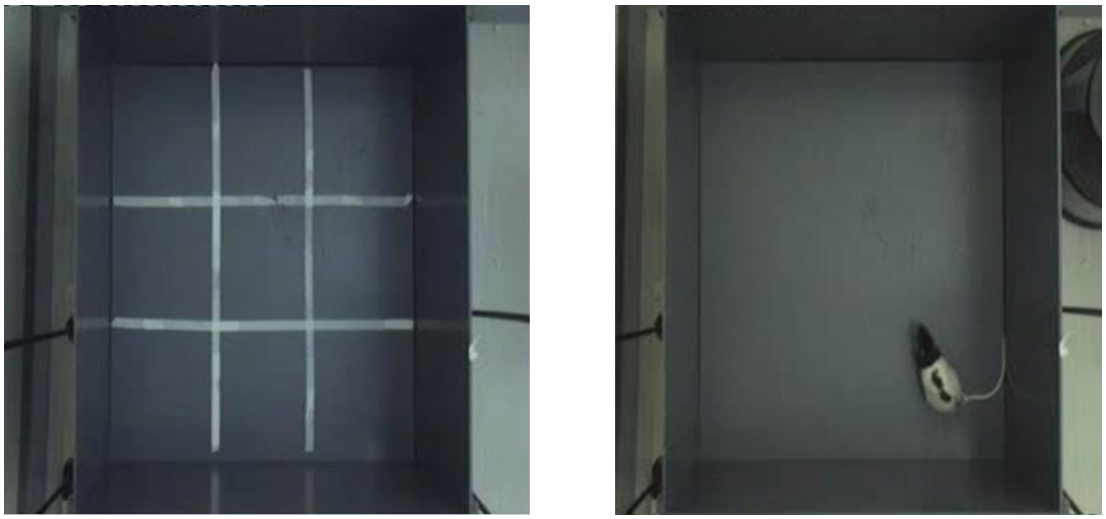


Figure 2-10 ***Photographs of the open field test arena.*** *The arena was calibrated in Ethovision XT to delineate the arena into 9 equally sized zones consisting of 4 corner zones, 4 edge zones and 1 centre zone (left panel). The markers of these zones were removed before animals were tested in the arena (right panel).*

2.III.ii.1.3 Behavioural parameters

The parameters investigated related to exploration and general activity. The total distance moved and the percentage of the observation spent mobile reflected the activity of the animals, whereas the percentage of the time spent in the centre and the number of entries made into the centre of the arena reflected their exploratory behaviour and are indicative of the affective state of the animals (Gould et al., 2009).

2.III.ii.1.4 Ethanol administration

Ethanol was prepared and rats were anaesthetised for injection as in 2.II.ii.7. Rats were given either 15% EtoH at a dose of 2g/kg or saline (0.9% w/v), both at a volume of 16ml/kg. Injections took place 8-10am for 3 consecutive days. On the third day, a rat was injected every 10 min to allow an 8 h gap between injection and the OFT. Five days after the last injection the treatments groups were swapped.

2.III.ii.1.5 Statistical analysis

The videos were analysed to track the location of the animal and secondly to measure their movement. To identify within-subjects effects over the two testing periods, paired t-tests were used to compare each behavioural parameter. Where analyses were performed on individual test sessions, one-way ANOVAs were performed. Levene's test was used to assess the equality of variance of the data. Assumptions of normality were not violated in any of the analyses.

2.III.ii.2 Results

2.III.ii.2.1 Was performance consistent over the two testing sessions?

The time spent in the corners, centre, and the edges of the arena was unaffected $F_{1,3}$ = 0.58, p = 0.500). However, paired t-tests showed that animals in the second test spent less time mobile (t = 2.74, df = 7, p = 0.029) and travelled significantly shorter

differences (t = 2.82, df = 7, p = 0.026). Due to these differences in locomotion between the first and second test, data from the first test only were used for subsequent analyses.

2.III.ii.2.2 Did ethanol treatment affect exploration of rats on the OFT?

Ethanol treatment had no effect on the locomotion of the animals in this study as indicated by the total distance moved ($F_{1,6}$ = 1.58, p = 0.255) and the percentage of the observation that they spent mobile ($F_{1,6}$ = 0.55, p = 0.818; Figure 2-11).

Measures of anxiety, as reflected by animals' exploration of the arena, were similarly unchanged. Rats administered ethanol did not differ in the time that they spent at the centre of the arena ($F_{1,6}$ = 0.01, p = 0.939), or by the number of entries that rats made into this area ($F_{1,6}$ = 2.35, p = 0.176; Figure 2-11).

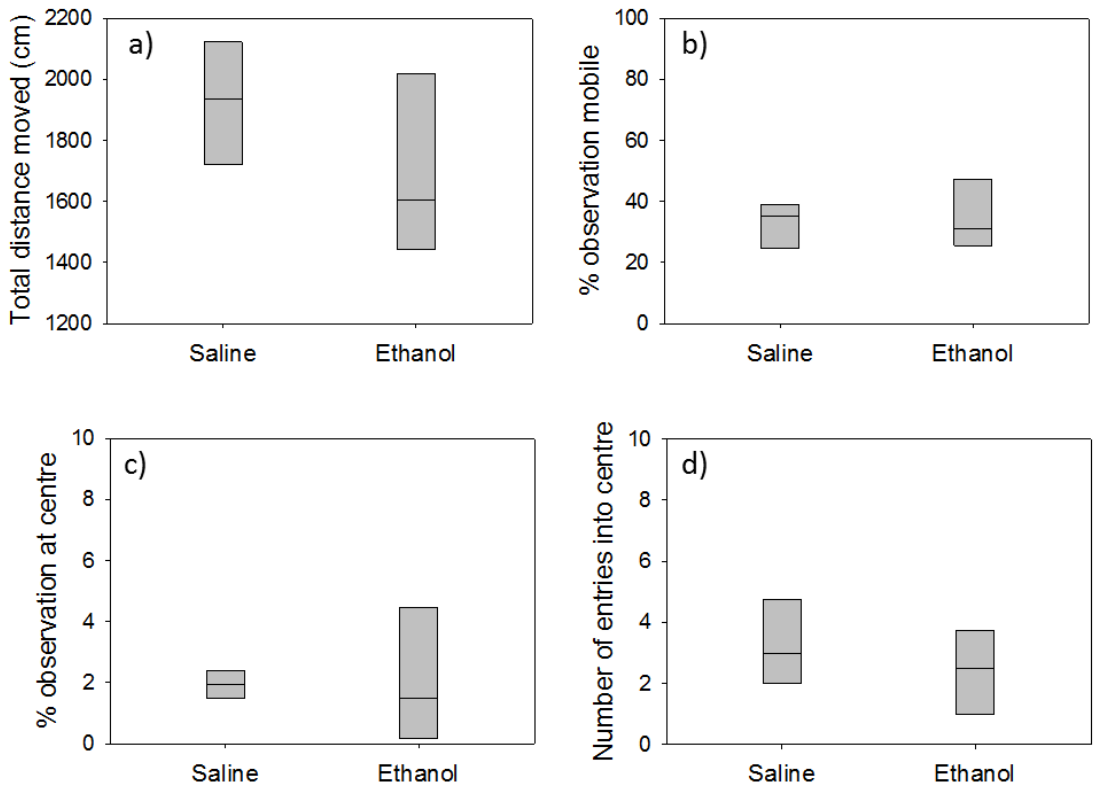


Figure 2-11 ***Exploratory behaviour of rats on the Open Field Test*** *after administration of saline or induction of an ethanol hangover . a) represents the total distance moved (cm) by rats in the 5 min observation; b) represents the percentage of the observation period that rats spent mobile; c) shows the percentage of the observation period that rats spent in the centre of the arena; and d) shows the number of entries that rats made into the centre of the arena. Boxplots show the median and first and third quartiles of the data. (Saline: n = 4; Ethanol: n=4).*

2.III.ii.3 Discussion

The attempt to perform a crossover design was unsuccessful due to test decay, where animals' activity on re-exposure was reduced. Short periods of exposure (2 – 10 min) in the OFT are often considered to measure animals' responses to novelty (Gould et al., 2009) and correlate with the length of exposure used in this experiment (5 min). This meant that the sample size was greatly reduced, with just four rats in each treatment group as compared to the more substantial group size of eight which was proposed. Small sample sizes increase the chance of incurring type II errors, where we fail to identify an effect when one exists. This is perhaps why we did not observe any anxiogenic effects of ethanol hangover in this experiment.

Alternatively, it could be surmised that the OFT was not a suitable task to use to identify anxiety from hangover. There is still discussion as to whether this paradigm effectively models features of anxiety reliably. For example, in a recent review of the effect of drugs on anxiety-like behaviours in the OFT, it was reported that administration of benzodiazepine receptor agonists, an anxiolytic class of drugs, produced anxiolytic effects in only 56% of studies, and counter-indicatively produced anxiogenic effects in 13% (Prut and Belzung, 2003). However, mice given ethanol-induced hangovers via a similar acute bolus injection protocol as used here displayed anxiety-like behaviours in the OFT (Karadayian et al., 2013). They used ten mice per treatment group, more than double the number of rats that were available in this study, further highlighting the insufficient group size.

2.III.iii The elevated plus maze as a measure of affect

The elevated plus-maze (EPM) is used to identify anxiety-like behaviours in rodents, exploiting the conflict between an innate tendency to avoid open spaces associated with increased risk of predation, and exploratory behaviour (Pellow et al., 1985). The activity of the rat in the exposed and sheltered areas (the open and closed arms) is thought to give an indication of whether the animals are in a high or low state of arousal. The time spent in the open arm as a percentage of the total time spent in the open and closed arms is among the most reliable indices of anxiogenic-like behaviour

in rodents, where reduced activity reflects a heightened state of fear (Pellow et al., 1985). The percentage of the entries into the open arms from a total number of entries into open and closed arms is also a measure that loads highly onto anxiety-like dimensions in factor analyses (Fernandes and File, 1996). Additionally, risk-assessing behaviours such as stretch-attends and another behaviour conveniently termed risk assessment can be observed during the test, adding to the cohort of measures of affect.

A general measure of locomotive behaviour can be obtained by comparing the number of entries made onto the closed arms during the test and the percentage of the test spent walking to identify whether reduced activity could influence the other measures. Rearing, or 'vertical exploratory behaviour', is also associated with general locomotor activity.

Data of an animal's spontaneous behaviour can also be extracted from EPM tests that can give us additional information about their affective state or of general activity. For example, excessive grooming is proposed to reflect an anxiety-reducing behaviour following stressful events in animals (Spruijt et al., 1992), and is a behaviour that can be measured on this task.

2.III.iii.1 Methods

2.III.iii.1.1 Animals and husbandry

The animals used in 2.III.ii underwent testing on the EPM 20 days after the OFT, and at the start of testing the animals were aged 35 weeks and weighed 640g ± 30g. Husbandry was as in 2.II.ii.3.

2.III.iii.1.2 Apparatus and procedure

The plus maze consisted of two opposing open arms (length: 50cm, width: 10cm) and two opposing closed arms of equal length and width but with 40cm high walls surrounding the edges. The centre of the maze was 10cm x 10cm from which the four

arms were connected at an angle of 90^{o} relative to the adjacent arms. The apparatus was elevated 77cm from the floor.

Filming in the EPM took place 8 h post-injection. To begin a trial the rat was placed in the centre of the maze facing a closed arm and filmed for a 5 min observation period. Filming took place by a camera in an aerial position for Ethovision analysis later. The experimenter remained in the room where a screen blocked the view of the observer from the rat. The arena was cleaned with 50% ethanol after each animal.

2.III.iii.1.3 Ethanol administration

Ethanol and saline treatments and administration were as in 2.III.ii.1.4.

2.III.iii.2 Definitions and descriptions of behaviours – Elevated plus maze

The exploratory parameters investigated were the percentage of time spent in the open arms, the percentage of entries into the open arms and the number of entries into the closed arm. Video-tracking software Ethovision XT (Noldus Information Technology) was used to track the animal's position in the arena. Behavioural parameters measured were the percentage of time spent in the following behaviours: Walking, Scanning, Risk assessment, Grooming, Rearing, Stopped (Table 2-5). These behaviours were scored manually by the observer from videos using Observer XT (Noldus Information Technology).

2.III.iii.2.1 Statistical analysis

To identify within-subjects effects over the two testing periods, repeated measures general linear models were used (RM-GLM) and significant main effects and interactions were compared with paired t-tests. Where analyses were performed on individual test sessions, one-way ANOVAs were performed. Levene's test was used to

assess the equality of variance of the data. Assumptions of normality were not violated in any of the analyses.

Exploration	**Description**
Proportion of time spent in open arms	(Time in open arms / total time exploring open and closed arms).
Proportion of entries in open arms	(Number of open arm entries/total number of arm entries).
Number of closed arm entries	The number of entries made into closed arms. This is a general measure of locomotion.

Behaviour	**Description**
Walking	Forwards or backwards movement.
Rearing	Rat standing on hind legs with the front paws not on the floor, may be touching walls.
Still	No movement.
Grooming	Rubbing face or body with paws.
Risk Assessment	Exiting enclosed arm with forepaws and head, investigating surroundings.
Stretch-attend	Body elongation with all paws on the floor.

Table 2-5 ***The behaviours observed and scored from video recordings of rats on the elevated plus maze.***

2.III.iii.3 Results

2.III.iii.3.1 Was performance consistent over the two testing sessions?

An RM-GLM analysis showed significant differences in the performance of rats on the first and second exposures to the EPM ($F_{1,7}$ = 7.95, p = 0.026). On the second exposure, the exploratory behaviour of the rats was significantly reduced, where rats spent less time exploring the open arms (t(7) = 5.65, p < 0.001), and also less time scanning (t(7) = 4.33, p = 0.003). General locomotive behaviour, as indicated by the time spent walking during the observation period, was also reduced (t(7) = 7.12, p < 0.001) and was accompanied by a correlative increase in the time spent stopped (t(7) = -2.68, p =0.032). There was also a trend for grooming behaviour, an indicator of stress (Spruijt et al., 1992), to be increased (t(7) = -2.02, p = 0.083). In light of these differences, only the data from the first EPM exposure were used in the subsequent analyses to avoid confounding results.

2.III.iii.3.2 Did ethanol treatment affect exploration and behaviour of rats on the EPM?

A one-way ANOVA identified no significant differences in the exploration of rats following ethanol or saline administration in terms of the time spent on the open arms: ($F_{1,6}$ = 0.62, p = 0.462; Figure 2-12b); their scanning ($F_{1,6}$ = 0.53, p = 0.495), rearing ($F_{1,6}$ = 0.50, p = 0.506); and risk assessing behaviour ($F_{1,6}$ = 0.11, p = 0.753; Figure 2-13 c- e). However, there was a trend for rats that had been administered ethanol to make more entries onto open arms than rats administered saline ($F_{1,6}$ = 4.60, p = 0.07, 6; Figure 2-12a).

Locomotion was not altered, where rats spent a similar percentage of the observation walking ($F_{1,6}$ = 0.55, p = 0.486) or stopped ($F_{1,6}$ = 0.89, p = 0.383; Figure 2-13 a- b), and did not differ in the number of entries they made onto closed arms of the maze ($F_{1,6}$ = 3.93, p = 0.095; Figure 2-12c). Grooming behaviour was similarly unaffected ($F_{1,6}$ = 0.29, p = 0.611; Figure 2-13f).

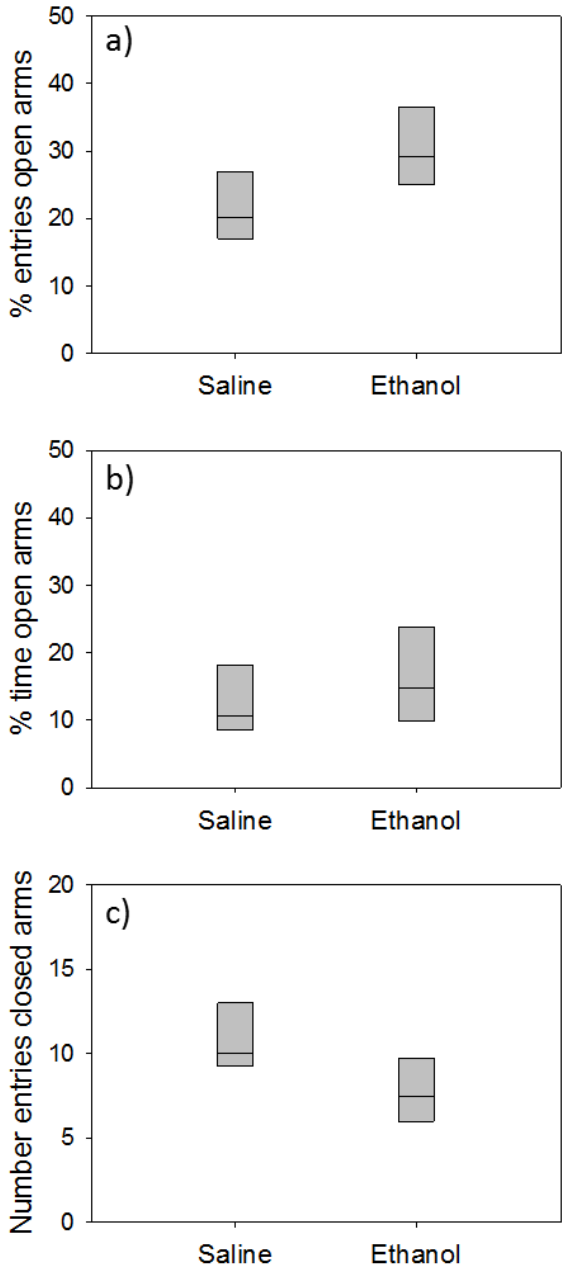


Figure 2-12 ***Exploratory behaviour of rats on the Elevated Plus Maze*** *after administration of saline or induction of an ethanol hangover . a) represents the entries that rats made onto open arms as the percentage of all entries made on to open and closed arms; b) represents the percentage of the 5 min observation period that rats spent on open arms; c) shows the number of entries that rats made onto closed arms. Boxplots show the median and first and third quartiles of the data. (Saline: n = 4; Ethanol: n=4).*

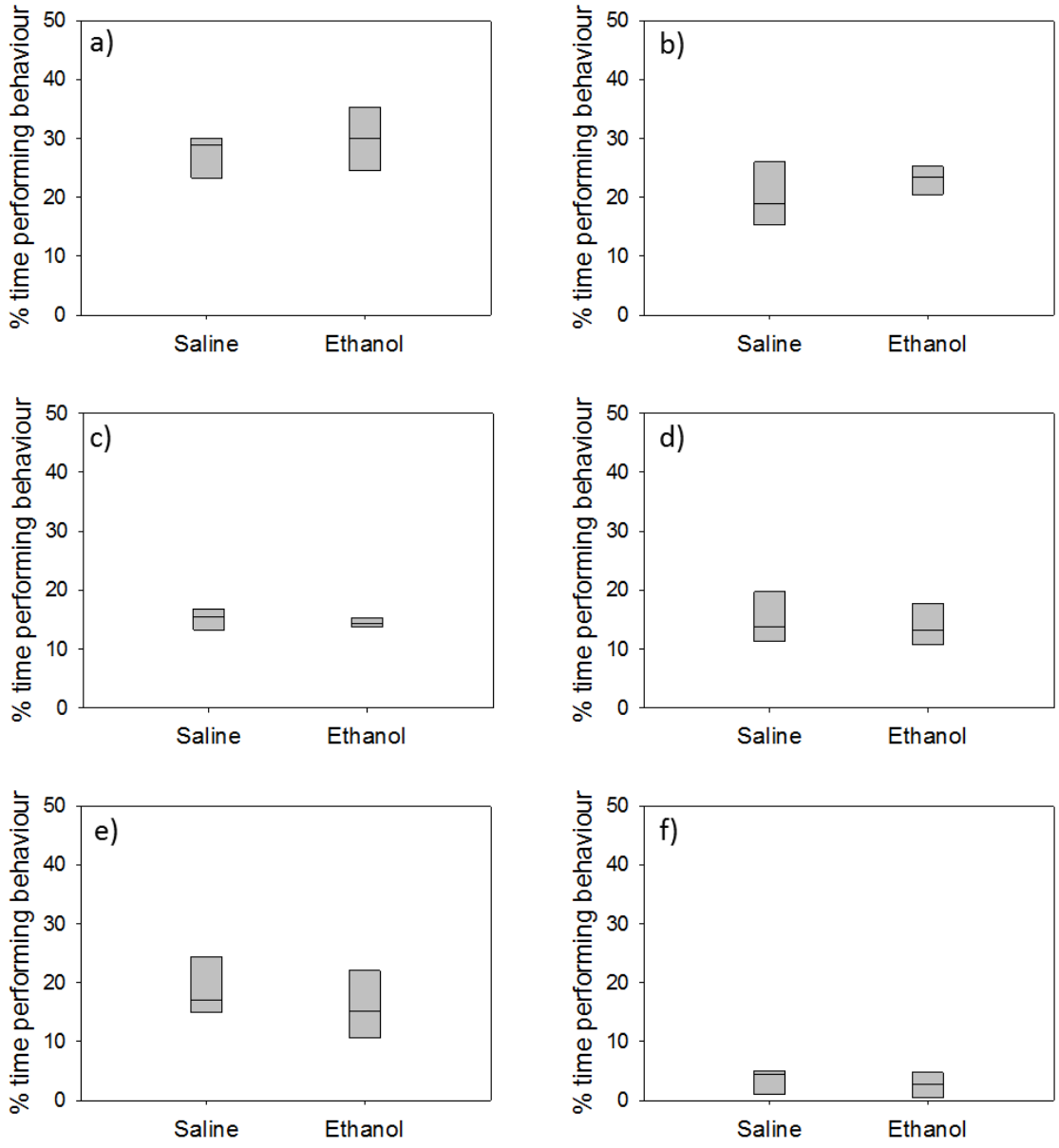


Figure 2-13 ***Behavioural observations of rats on the Elevated Plus Maze*** *after administration of saline or induction of an ethanol hangover. The percentage of a 5 min observation period that rats spent performing the following behaviours after administration of saline or induction of an ethanol hangover: a) Walking; b) Stopped; c) Scanning; d) Risk assessment; e) Rearing; f) Grooming. Boxplots show the median and first and third quartiles of the data. (Saline: n = 4; Ethanol: n=4).*

2.III.iii.4 Discussion

Like with the OFT, test decay of the EPM led to the halving of intended sample sizes. We found no difference in the locomotor or exploratory activity, or in the spontaneous behaviours displayed by rats on this test, which might be an artefact of this small sample size. Zhang et al. (2007), who found pronounced changes in activity with ethanol hangover, used 8 - 11 animals for each treatment group. However, if we refer to Figure 2-12, it appears that our data show an increase in the number of entries and the time spent on open arms which is an indicator of anxiolysis; this is contrary to our predictions that hangover would produce anxiety and therefore reduce open arm activity. It is then perhaps unlikely that increasing the sample size would result in acceptance of our hypothesis. On the other hand, the data from the open field test (Figure 2-11) suggest that rats administered ethanol show changes in activity that are in line with an anxiogenic effect; the number of entries into the centre of the arena and the time spent there are both slightly reduced. However, the distance travelled following ethanol administration seems to be shorter, which might indicate a sedative effect. Again, increasing the sample size in this study might not be conducive to proving our hypothesis.

Also, Zhang used male Wistars to look at hangover anxiety in the EPM whereas we used Lister Hooded rats. However, McDermott and Kelly (2008) found that the Lister Hooded rats show significantly greater levels of activity than Sprague Dawleys or Wistars, which would suggest that it may in fact have been more likely for us to identify changes in active behaviour in this strain when the rats are experiencing hangover.

2.IV General discussion

Our tests did not show a consistent effect of ethanol hangover on judgement bias or other behavioural measures. This is interesting as the tests used (OFT:Karadayian et al. (2013); EPM: Zhang et al. (2007)), and the strain (File et al., 1992) have previously been shown to be compatible with the measurement of anxiety-like effects following

hangover. Further to this, adult rats were used as adolescent rats have been shown to clear ethanol from their blood faster than adult rats (Doremus-Fitzwater and Spear, 2007) and thus the anxiogenic effects are likely to present for longer periods in adult rats.

One way in which the protocol used in this study differed from those listed above was the use of anaesthesia in the injection of ethanol. In no other study are the animals anaesthetised prior to injection nor were they placed in incubators after ethanol administration. Although it is unlikely that this brief anaesthesia negated the hangover effects of alcohol, it is important to note the divergence in the methods used.

The doses and administration of ethanol in these experiments were consistent with other studies, where Zhang et al (2007) observed anxiety-like behaviour on the EPM in rats with three daily doses of 2g/kg i.p., and Karadayian et al. (2013) found that mice injected with 3.8g/kg ethanol displayed anxiety-like behaviours on the OFT. The dose used should therefore have been sufficient to precipitate alcohol hangover, so it is likely that our lack of observed effect was the result of small sample sizes or inadequacies with our experimental set-up.

The cohort of experiments in this chapter suffered from inadequate sample sizes throughout, a consequence of which is a reduced sensitivity to identify treatment effects. It is quite possible that the data obtained from the proxy measures study were subject to type II error, and as such we failed to find an effect of ethanol hangover, and it is also possible that we incurred type I errors in the analysis of the judgement bias data, where unusual and contradictory effects were found.

By the end of the judgement bias testing and retraining, only 3 of an original cohort of 24 rats were displaying responding that met the criterion set out for responding. This demonstrates that the protocol, as is, is not suitable for the training of judgement bias paradigms. The protocols employed in this task and others in publication were dissected and reviewed to identify possible areas for optimisation. These are discussed in the following chapter (section 3.V), as are the resulting alterations made to the training and testing methods. Similarly, the methods used in the proxy measures of effect also caused a reduction in the overall sample sizes. The test decay observed with

the EPM is in line with other reports of habituation where rats spent less time exploring the open arms on a second exposure, as occurred in this study ((File and Gonzalez, 1996, Hogg and File, 1994); but see Hogg (1996) for a comprehensive review of behavioural changes with repeated testing). Similarly, where the OFT is used to measure behaviour in a novel situation, habituation can also occur on subsequent exposures (Gould et al., 2009). We therefore conclude that a within-subjects design was not suitable for these tests and a between subjects design would be more appropriate for future experiments.

Chapter 3 - The affective component of sickness

3.I Introduction

Summary: Animals are typically used in drug development to assess effectiveness and to identify adverse effects of novel compounds. Assessment of these adverse effects in rats, the third most commonly used vertebrate species in animal research, is notoriously difficult due to their inability to vomit. It is, however, postulated that a reduction in affect could be an indicator of sickness. It may therefore be possible to identify this concurrent symptom of low mood in sickness in order to identify unsuitable compounds.

The emetic drug lithium chloride (LiCl) is widely used to induce states of sickness in animal models, particularly to act as a punisher in conditioned avoidance paradigms. Although the behavioural and appetitive outcomes of LiCl administration have been well documented, the affective component that manifests with avoidance has yet to be identified. It is unclear to what extent negative affect is experienced by animals during periods of sickness after administration of this drug. In this chapter I explore the behavioural and cognitive effects of LiCl-induced nausea in the rat. I used a judgement bias task to establish the affective states associated with LiCl administration, and I also explored whether a bias in judgement caused by LiCl can be reversed using an anti-emetic drug, ondansetron.

3.I.i Anxiety- and depression-like behaviours observed during sickness

Sickness in humans often involves a subjective experience induced by nausea (the 'feeling' of being ill), which encompasses a strong emotional component. This emotional component is often characterised by a depressed mood and involves a loss of interest in normally pleasurable activities (anhedonia; Ngampramuan et al., 2013). Furthermore, the psychological symptoms of sickness can be compared to many of the symptoms of major depressive disorder (MDD). Fatigue, reduced cognitive function, and the aforementioned anhedonia are often present in both circumstances (Schiepers

et al., 2005). In addition to similarities in psychological measures of mood, there are also a number of physiological correlates that are shared by depression and sickness. Specifically, inflammation is a common component of many disease states, and it is also implicated in some neuropsychiatric disorders. Many of the key immunomodulators released by activation of the immune system have been shown to be involved in precipitation of the anhedonia observed during sickness (Schiepers et al., 2005). Many authors have reviewed this phenomenon, leading them to propose the link between inflammation and depression as 'the inflammatory response system model of major depression' (Maes et al., 1995), 'the macrophage theory of depression' (Smith, 1991) and 'the cytokine hypothesis of depression' (Yirmiya et al., 1999). These all consider the role of inflammatory molecules in the development and maintenance of inflammatory components of disease and depressed moods. Depressed patients have been found to exhibit many of the biomarkers of inflammatory processes, including increased levels of cytokines and inflammatory mediators (Pace et al., 2006), and indeed patients with diseases which have a major inflammatory component display symptoms of depression (Capuron et al., 2000). It has also been shown that administration of cytokines produces depression-like episodes in rats (Schiepers et al., 2005), and similar effects are seen in humans treated with pro-inflammatory cytokines in immunotherapy, who display side effects common with depression (Table 3-1). Likewise, antidepressants can be used to directly treat or to improve the efficacy of other treatments of disorders with inflammatory components (e.g. O'Malley et al. (2000), Rahimi et al. (2012)).

Behavioural parameters of sickness are induced in animal models using the pro-emetic agent LiCl, where administration produces a range of physiological and behavioural outcomes including reduced locomotion, reward devaluation and anorexia. LiCl is used as an acute aversive stimulus in animal behaviour studies, where its aversive properties are considered to be analogous to nausea in humans[3]. The modulatory effect of LiCl administration on inflammatory cascades may mediate its aversive properties, but it is unclear whether these are akin to the changes seen in sickness. LiCl

[3] The term nausea is also used to describe the experience of sickness in animals (e.g. Parker et al., 2008), but its use does not assume the experience of a similar subjective component as exists in humans.

has been shown to potentiate production of TNF-α, IFNγ and IL-8 *in vitro* (Schiepers et al., 2005), all of which are components of the inflammatory cascade during sickness (Yirmiya et al., 1999).

Immunotherapy	Neuropsychiatric side effects	Clinical condition treated
IFN-α	Fatigue	Cancer
	Psychomotor slowing	Multiple sclerosis
	Depressed mood	Chronic hepatitis C, other viral infections
	Anxiety	
	Social withdrawal	
	Irritability	
	Anorexia	
	Cognitive disturbances (mental slowing, lack of concentration, memory impairment)	
IFN-β	Fatigue	Multiple sclerosis
	Depressed mood	
	Cognitive impairment	(Metastatic) cancer
IL-1	Cognitive impairment	(Metastatic) cancer
IL-2	Fatigue	
	Anhedonia	
	Dysphoria	
	Cognitive impairment (mental slowing)	
TNF-α	Fatigue	Cancer
	Anorexia	

Abbreviations: IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.

Table 3-1 ***Neuropsychiatric side effects of immunotherapies based on the administration of proinflammatory cytokines.*** *Reproduced from Schiepers et al. (2005).*

3.I.ii Hypothesis

As sickness in humans consists of a negative affective state with similarities to depression, I hypothesised that depression-like symptoms may be evident in the cognition and behaviour of rats treated with LiCl. I predicted that a sickness induced by

LiCl injection would produce symptoms similar to those seen in negative affective states, such as a pessimistic biasing of ambiguous information.

The ultimate objective of this chapter was to measure this bias as indicated by rats' responses in a judgement bias task. This was preceded by preliminary studies where a dose suitable for use in this task was identified. It was necessary to select a single dose due to the extensive training requirements necessary for the judgement bias task and the possibility that response extinction might not allow for multiple test sessions using different doses (see Chapter 2). The suitable dose induced sickness in the rats as indicated by the presence of sickness behaviours, but importantly did not affect their motivation to obtain reward. The outcomes of the judgement bias task are sensitive to changes in motivation, where reward anticipation is an integral measurement. Motivation to obtain reward was investigated with a range of LiCl doses using a progressive ratio operant task in conjunction with a spontaneous behaviour study. These studies were undertaken concurrently but will be discussed separately in sections 3.II and 3.III.

3.II Spontaneous behaviour as an indicator of LiCl-sickness[4]

3.II.i Rationale

Humans and other animals display characteristic behaviours that are indicators of illness, for example, sleeping more and moving less. Benjamin Hart proposed in his seminal 1988 paper that, in animals, a reduction in grooming and general movement, and a curled posture are hallmarks of sickness and have the function of conserving heat and energy in order to mobilise immune responses (Hart, 1988). LiCl-induced sickness has infrequently been characterised in terms of spontaneous behaviour, however there tends to be agreement that locomotion, grooming and exploratory behaviours such as rearing are suppressed in the few studies that exist (Ishii et al., 2004, Parker et al., 1984). Conversely, Cappeliez and White (1981b) recorded increased

[4] A note on unit notation: LiCl is used in a diverse range of fields and the dosage is expressed in a variety of ways. For ease of readership of this chapter I have converted all of the dose rates to a common notation of mg/kg (1mEq = 1mM = 1mmol = 42.4mg).

locomotion when rats were administered 6.4 – 31.8mg/kg, and increased walking and rearing behaviour at the lowest dose. They did, however, observe the rats over substantially shorter periods, recording the behaviour of the animals every 3 s in a 5 min period, whereas Ishii et al. (2004) scored behaviour continuously over a 60 min period. An additional behaviour has been identified that is characteristic of LiCl injection where rats lie with a flattened belly pressed to the floor, which has been termed 'lying-on-belly' (LOB; Meachum and Bernstein (1990), Meachum and Bernstein (1992), Parker et al. (1984), Tuerke et al. (2012)).

The amount of time spent performing behaviours often associated with sickness (grooming and general locomotion) were recorded over a fixed observation period, as were the additional behaviours (rearing and LOB) as highlighted in the aforementioned studies. Coprophagy (ingestion of faeces) was also recorded as abnormal levels of this behaviour may be analogous to pica – a proxy measure of nausea in non-vomiting species (Takeda et al., 1993).

3.II.ii Methods

3.II.ii.1 Animals

Animals were 16 male Lister Hooded rats (Charles River, Margate, Kent, UK) aged 16 weeks and weighed 320g ± 19g. Rats were housed in groups of four in standard RC2 cages with sawdust as bedding ('Aspen', BS and S Ltd, Edinburgh, UK) in a temperature controlled (21+/- 2 ^{o}C) room with a 12 h light cycle with lights on at 0700h. Standard rat diet (RM3, Special Diet Services, Essex, UK) was fed at a restricted rate to maintain rats at no less than 85% of their free-feeding body weight. Water was provided *ad libitum.* Tests were run in the light period between 12-6pm. The animals were free from any common pathogens according to the FELASA Health Monitoring Recommendations. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and approved by the local ethical review process (project licence number: 60/3793). On completion of all experiments, rats were humanely euthanized with slow rising concentration of CO2.

3.II.ii.2 Apparatus and software

Rats were filmed in transparent filming boxes in a room separate to the holding room, where the observer was not present, with a Panasonic SDR-S26 SD video camera. The rats had previously been habituated to the filming room and filming boxes for 1 h the day prior to the first filming. The room was separated with dividers to ensure that the rats were unable to see each other during filming. Observations of 10 min lengths were recorded 5 min after injection with LiCl or vehicle. One video recording was made of each rat on treatment days, with a total of four recordings.

All videos were scored by a primary observer. A pseudo-random selection of these videos was also scored by an additional observer, blinded to the treatments to measure the reliability of the observations. The second observer scored three videos of each of the four treatment groups, each of the twelve videos containing a different rat.

3.II.ii.3 LiCl administration

LiCl (Sigma - Aldrich, Dorset, UK) was diluted in saline (0.9% sodium chloride) and was administered by injection i.p. at a volume of 10ml/kg. Animals were administered 12.7mg/kg, 31.8mg/kg or 63.5mg/kg LiCl or a saline (0mg/kg) control in a pseudorandomised order. Treatments were administered in the holding room and the rats were replaced in their homecage. 5 min after injection rats were transferred to a filming room and placed in a clear plastic box and filmed for 10 min. A battery of tests occurred every three days, and Figure 3-1 outlines the schedule of drug administration and behavioural testing on test days.

3.II.ii.4 Definitions and descriptions of behaviours – spontaneous behaviour

Behaviour	Description
Walking	Mobile, moving one paw in front of the other.
Body dragging	Abdomen on floor, mobile. The body is elongated and the belly dragged along the floor by the front paws.
Rearing	Forepaws off the floor simultaneously, not grooming, may be touching walls. Includes climbing.
Still	At least three limbs touching the floor. Head moving.
No Movement	At least three limbs touching the floor. No head movement.
Lying on Belly	Flattened torso, limp limbs, Paws forward, abdomen on floor.
Grooming	Rubbing face, body with paws.
Coprophagy	Eating faeces.

Table 3-2 ***Descriptions of spontaneous behaviours scored in a 10 min observation period following LiCl or saline treatment.***

3.II.ii.5 Experimental procedure

A progressive ratio (PR) task immediately followed the spontaneous behaviour study, and baseline PR sessions were run on the days intermediate to the test days (see 3.III). Additionally, as part of the PR study, a novel flavour (~0.1ml diluted peanut butter) was offered to the rats via a 1ml pipette 15 min prior to administration of LiCl or saline. Whether or not they would voluntarily eat the peanut butter was recorded. Rats were not force fed the peanut butter if they did not take it.

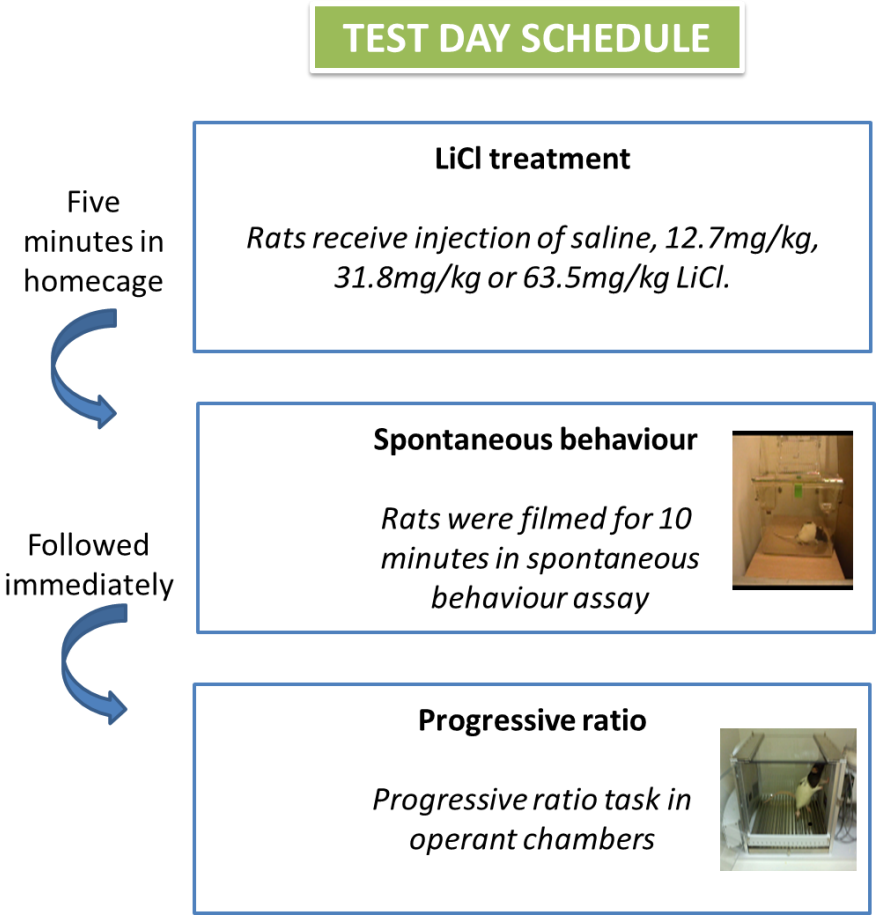


Figure 3-1 ***Schedule of experimental procedures.*** *Every third day rats were treated with a dose of LiCl (0 – 63.3mg/kg). Spontaneous behaviours were filmed for 10 min and then rats underwent a progressive ratio session. In the intermittent days progressive ratio sessions occurred with no filming or treatments in order to re-establish baseline responding. Rats were run in the same operant boxes and filmed in the same filming boxes throughout the study.*

3.II.ii.6 Statistical analysis

The time spent performing each mutually exclusive behaviour in the spontaneous behaviour study was recorded as a percentage of the total observation period. A one-way ANOVA was performed on these percentage data with LSD post-hoc tests to further assess significant differences between doses. Pearson's correlations were calculated to assess the inter-observer reliability of scoring. Levene's test was used to assess the equality of variance of the data. Assumptions of normality were not violated in any of the analyses.

3.II.iii Results

3.II.iii.1 Was spontaneous behaviour altered by LiCl injection?

Inter- and intra-observer reliability The behaviours in the sample of videos were correlated highly between the two observers in the following categories: walking, rearing, grooming, lying on belly, coprophagy and abdomen dragging (Pearson's R's > 0.9). The correlations of still behaviour and no movement, which coded for two behaviours where rats were not mobile and differed only with movement of the head, were insufficient (Pearson's R = 0.757 and R = 0.765 respectively). When the two behaviours were combined into a single category, the correlation was very high (Pearson's R = 0.984). Subsequently, there will be no reference to these separate behaviours, and they will instead be referred to and analysed in combination in a new behavioural category, 'immobile'.

Spontaneous behaviour An ANOVA showed significant effects of dose on the display of spontaneous behaviour. Dose-dependent increases in immobile and coprophagy behaviours were identified (ANOVA: Immobile: $F_{3,59} = 4.17$, $p = 0.010$; coprophagic behaviour: $F_{3,59} = 4.48$, $p = 0.007$), whereas rearing and grooming were reduced (ANOVA: Rearing: $F_{3,59} = 6.44$, $p = 0.001$; Grooming: $F_{3,59} = 11.15$, $p < 0.001$). There was no change in the amount of time rats spent walking in a normal manner or walking whilst dragging their abdomen (ANOVA: Walking: $F_{3,59} = 0.93$, $p = 0.430$; abdomen dragging: $F_{3,59} = 0.71$, $p = 0.553$), but there was a trend for rats to spend more time lying on their bellies when administered LiCl (ANOVA: LOB: $F_{3,59} = 2.37$, $p = 0.080$). Results of the post-hoc tests are displayed in Figure 3-2.

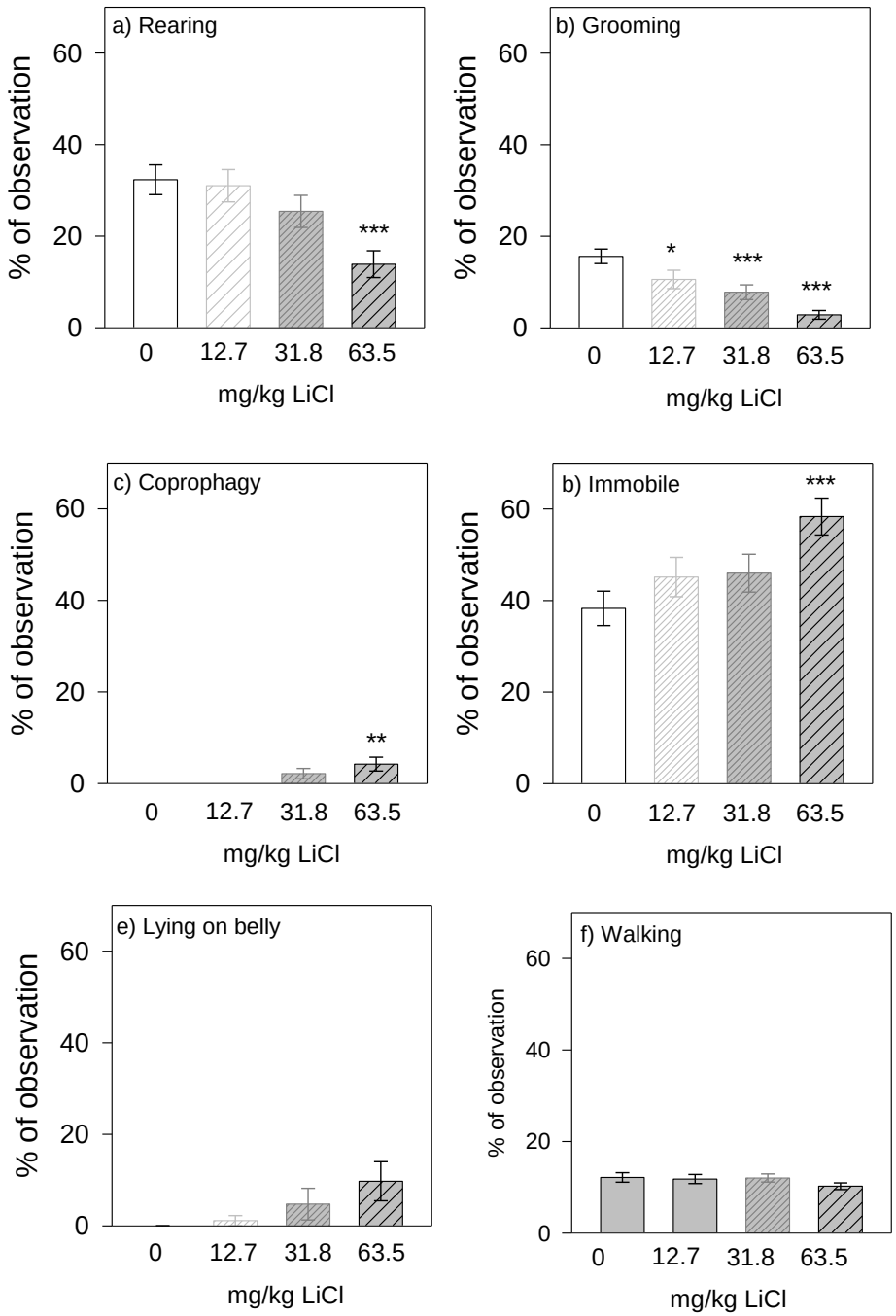


Figure 3-2 ***Behavioural observations of rats during a 10 min period*** *The graphs show the proportion of the observation period that rats spent engaged in each behaviour following administration of LiCl or saline vehicle. (P values denoted as: * p < 0.05, **p < 0.01, ***p < 0.001). Bars are means + S.E.M. (n = 16).*

3.II.v Discussion

The spontaneous behaviour assay identified several of the behavioural indices of sickness as reported by Hart (1988) following injection with LiCl, such as a reduction in grooming and general locomotion. The rats also displayed lying on belly behaviour, another accepted postural index of sickness. The prevalence of these behaviours increased in a dose-dependent manner, and were most obvious following the highest dose of LiCl (63.5mg/kg). The rats also showed substantially shorter grooming behaviours following the medium dose of 31.8mg/kg, and performed coprophagy and lying on the belly which were not recorded following saline injection. These two highest doses were therefore considered to induce sickness.

3.III Appetitive responding following LiCl administration

3.III.i Rationale

A pilot study was conducted to determine whether appetitive responding could be maintained whilst rats were experiencing LiCl-induced sickness as it has been reported to reduce operant responding for reward at doses as low as 5mg/kg (Hernandez et al., 2011). A decrease in the motivation to respond for reward could confound and confuse the outcomes of the judgement bias paradigm. A PR task was chosen to investigate a rat's motivation to obtain a reward and to participate in an operant task (Hodos, 1961). In a PR session, the number of responses (nose poke, lever press etc.) required to obtain a reward increases incrementally on successive trials (PR*e;* Table 3-3). The session ends after a fixed period of time when one trial has ended if another has not yet been completed, indicating that the rat had discontinued responding. The ratio or trial number at which the rat ceases to respond is referred to as the 'breakpoint'.

Trial number	1	2	3	4	5	6	…	17	18	etc.
Responses needed to complete trial	1	2	4	9	12	15	…	178	219	…
Cumulative responses	1	3	7	16	28	43	…	885	1104	…

Table 3-3 ***Progressive ratio schedule .*** *The table shows the number of responses required to complete a trial in the progressive ratio task. The progressive-ratio schedule was based on the following exponential progression: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, …, derived from the formula [(5×e0.2n)−5], rounded to the nearest integer, where n is the position in the sequence of ratios (Roberts and Richardson, 1993).*

A number of measures can be extracted from a progressive ratio task which can indicate reinforcing efficacy of drugs and other substances, and also a rat's motivation to consume food (Hodos, 1961, Richardson and Roberts, 1996). These include the number of rewards received, the speed at which an animal will respond for a reward, and the number of responses a rat will make before it reaches its breakpoint (e.g. LeSage et al. (2004)). In the absence of a change in the number of trials completed, the total number of responses during a session is a sensitive measure of an animal's motivation to respond for reward, perhaps even more so[5].

3.III.i.1 Prevention of LiCl-conditioned taste aversions

In conditioned aversion paradigms lithium injection is paired with something that is normally hedonic (e.g. peanut butter), and on reintroduction it can cause avoidance, aversion or rejection, indicating an aversive experience (Parker, 2003, Garcia and Koelling, 1967). LiCl has aversion-inducing properties for novel and known flavours (Benoit et al., 2003, Fenwick et al., 1975), and produces reductions in reward valuation (Hernandez et al., 2011). In the context of this study, where I aimed to explore the

[5] For example, if two rats completed sessions with a mean of 16 trials per session, their responding could differ by up to 178 responses (see Table 3-3), and hence the discrimination between the motivation states of the two rats would be diluted.

features of sickness, it was important to prevent development of an aversion to the food reward delivered in the judgement bias task.

There is a wealth of literature regarding the prevention of taste aversions, particularly in the realm of cancer therapies where aversions to foods eaten in temporal proximity to radiation or chemotherapy are frequently encountered (Symonds and Hall, 2002). Measures are often taken to preserve appetitive preferences for these foods (referred to as 'target tastes'). Taste aversions are also well established in rodent models, and have therefore been exploited in order to devise methods for their prevention. There are two methods that are adopted to attempt to alleviate aversion: overshadowing and latent inhibition.

Overshadowing taste aversions involves exposing the subject to a more salient cue in addition to the target taste prior to the sickness event, where increased salience is achieved via novelty. Attenuated aversions have been demonstrated via conjunctive administration of a range of novel flavours (Symonds and Hall, 1997), or interference with contextual stimuli (Kwok and Boakes, 2012).

Latent inhibition is a process of pre-exposing an animal to the target taste prior to induction of sickness. A taste encountered more frequently before sickness is protected from becoming aversive for a greater number of sickness events, whereas if sickness is paired with the initial exposure aversions can be produced immediately (Nachman and Ashe, 1973). Within the judgement bias and progressive ratio tasks discussed in this chapter, rats were frequently exposed to the food reward during the training phase, effectively pre-exposing them to the target taste.

A number of researchers have attempted to assess the effectiveness of combining these two methods in preventing aversions (Nagaishi and Nakajima, 2008, Nakajima and Nagaishi, 2005, Blaisdell et al., 1998). This method of preserving appetite for previously encountered foods via pairing with a non-target flavour with the sickness event has been coined the 'scapegoat technique'(Broberg and Bernstein, 1987). Nagaishi and Nakajima (2008) effectively demonstrated that rats both pre-exposed to the target taste and given a distinct novel taste in conjunction with a LiCl injection, consumed more of the target taste than when one method was adopted

independently. This theory has been contested by Blaisdell et al. (1998) who argued that summation of latent inhibition and overshadowing facilitates formation of avoidances, a phenomenon referred to as the 'comparator hypothesis'. Blaisdell et al reasoned that the cue competition effects of combining the methods counteract each other, rather than summate. This hypothesis is controversial as it is inconsistent with classical Pavlovian theory (e.g. Frey and Sears (1978), Mackintosh (1975), Wagner (1981)) and has been subsequently challenged by Nakajima's group (Nagaishi and Nakajima, 2008, Nakajima and Nagaishi, 2005). In the present study the 'scapegoat technique' was adopted, and was given in the form of peanut butter administered orally prior to a LiCl injection.

3.III.ii Methods

3.III.ii.1 Animals

Animals were as 3.II.ii.1. The PR study took place concurrently with the spontaneous behaviour study (Figure 3-1). Training of the rats on the PR task began when the rats were aged 12 weeks.

3.III.ii.2 Apparatus and software

Eight identical operant boxes were used. Each chamber measured 300mm x 245cm x 200cm and had a metal rod flooring. An automatic feeder at the top of the unit fed down into a magazine where the food was dispensed (45mg dustless precision pellets, Bio-Serv, LBS, Surrey, UK). A tone generator, speaker and attenuator produced and controlled the volumes of the tones. The speaker and house light were located on the back wall of the chamber. Responses were measured via entries into nose-poke holes. Two nose-poke holes (left and right) were accessible to the rats. A PC running MED-PC IV software (Med Associates Inc., Sandown Scientific, Middlesex, UK) was used to control the operation of the tone generators, speakers, attenuators and feeders. The programming was written with MedState-notation.

3.III.ii.3 Progressive ratio training

Rats were initially habituated to the operant chambers and sucrose rewards for 40 min with 15 sucrose pellets in the magazine over two consecutive days. On the third day rats were run on a 30 min variable interval schedule (VI20) where they received one reward in the magazine every ~20 s. Following habituation and magazine training, the rats were run on a 30 min FR1 (fixed ratio-1) schedule with a 5 s time-out between trials signalled by the houselight switching off. Responding on only one of the holes was reinforced and this was counterbalanced between subjects. A response was made by poking the nose into the hole and resulted in the delivery of a sucrose pellet. Nose poking when the trial was in a time-out period had no outcome. Rats that were not reliably responding were given further training by directly reinforcing entries into the nose-poke holes (a pellet was placed inside the hole). Once rats met the criterion of ≥80 responses on three consecutive sessions they continued to the next ratio of reinforcement, FR3, where the rat made three responses to receive a reward. This was followed by a FR5 schedule once they had completed 80 trials on three consecutive sessions on the previous schedule. Once all of the rats had completed at least 80 trials per session on the FR5 schedule they were trained on a progressive ratio session (Pr*e,* Table 3-3), with a break point of 20 min. Rats underwent 20 sessions before testing, which ensured a substantial pre-exposure to sucrose. Rats performed on a Pr*e* schedule for five sessions to establish baseline responding, and then for a further ten sessions with treatments administered on the first, fourth, seventh and tenth day.

Parameters measured were the number of rewards received, the number of responses made during active and time-out periods, the duration of the session, the latency between trials, and the accuracy of responding (i.e. the proportion of responses into the correct hole within a session).

3.III.ii.4 LiCl administration

Drug administration was as in 3.II.ii.3.

3.III.ii.5 Statistical analysis

Repeated measures ANOVA was used to analyse break-points, responding during sessions, accuracy and session length, with LiCl dose as a within-subjects factor. Where assumptions of sphericity were violated a Greenhouse-Geisser correction was adopted. Where a significant difference was observed ($p < 0.05$) paired t-tests were used. The lengths of each progressive ratio schedule were compared using a GLM with dose and trial number as factors, with a Greenhouse-Geisser correction used where assumptions of sphericity were violated (where $\varepsilon < 0.75$). An ANOVA was performed to highlight the trials where durations were significantly different. In order to gauge whether the consumption of peanut butter or ingestion of faeces in the spontaneous behaviour study affected appetitive responding in the PR task, a linear mixed model was run with dose, coprophagy and peanut butter as fixed effects and rat as a random effect. Estimations were restricted maximum likelihood estimations and a Bonferroni confidence interval adjustment was applied to *post-hoc* analyses.

3.III.iii Results

3.III.iii.1 Were the aversive properties of LiCl established by taste aversion?

Twelve of the sixteen rats displayed an aversion to the peanut butter in the session immediately following either 32.2 or 63.5mg/kg dose (whichever came first), indicating that the sickness experience had been paired with the taste. Two rats displayed aversions after the 12.7mg/kg when this was the first LiCl dose administered. Two rats did not develop an aversion throughout the study. There was no reversal of the aversion (i.e. once a rat had demonstrated an initial rejection of the peanut butter, they would not eat it on later occasions when it was offered).

3.III.iii.2 Did LiCl affect rats' responding on a progressive ratio task?

There was no effect of dose on the break-points within a progressive ratio session (RM-ANOVA: $F_{3,45} = 0.24$, $p = 0.869$; Figure 3-3), nor was there any difference in the number of responses that a rat made within a session (RM-ANOVA: $F_{3,45} = 0.11$, $p = 0.955$; Figure 3-4). The lengths of the sessions, however, were significantly lengthened following LiCl injection (RM-ANOVA: $F_{3,45} = 8.06$, $p < 0.001$), specifically following injection with the highest dose use (63.5mg/kg: $t(15) = 3.79$, $p = 0.002$; Figure 3-5).

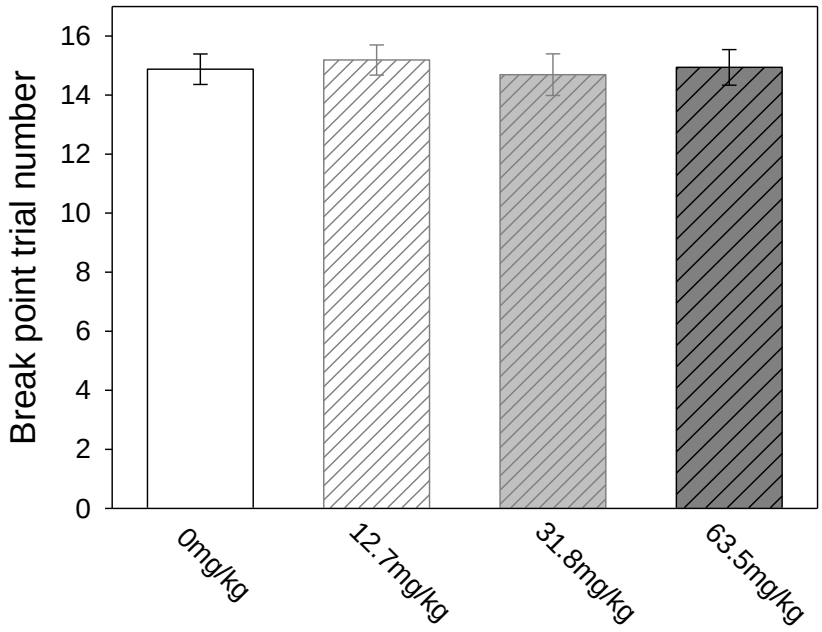


Figure 3-3 ***Mean number of trials completed before reaching breakpoint on a progressive ratio schedule*** *following LiCl or saline injection. Bars show means +S.E.M. (n = 16).*

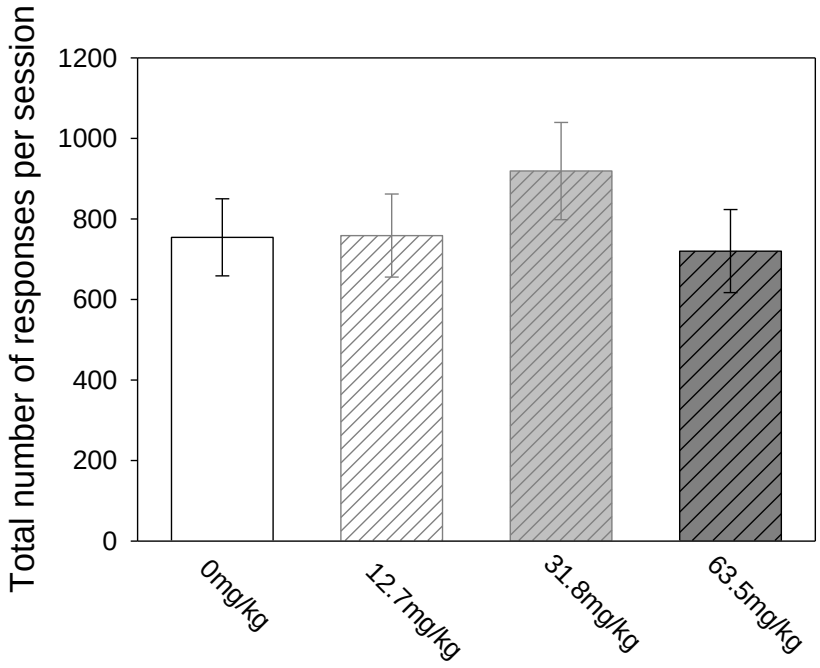


*Figure 3-4 M**ean number of responses performed during a progressive ratio test session.** Rats did not differ in their responding during a session when administered LiCl as compared to the vehicle saline. Bars show means +S.E.M. (n = 16).*

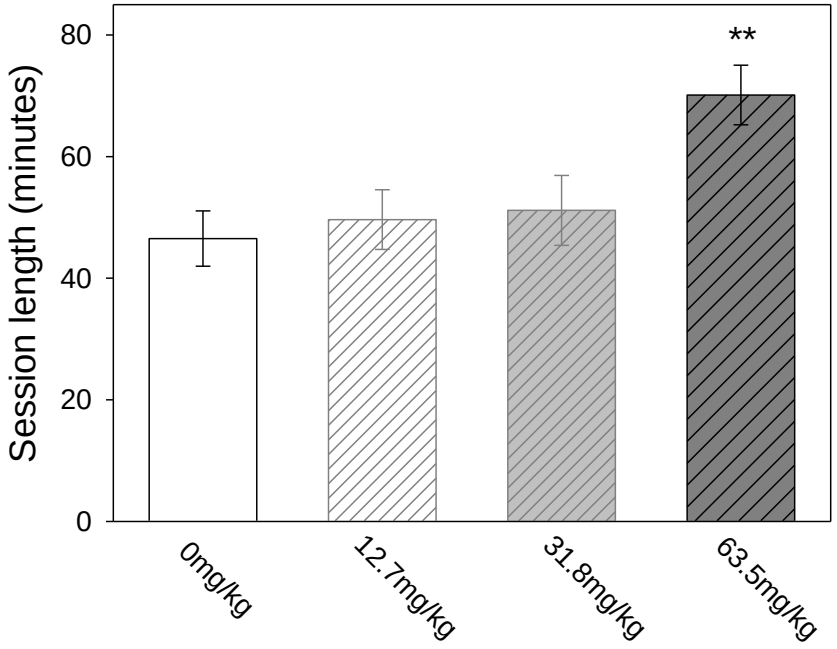


*Figure 3-5 **Mean length of progressive ratio sessions** following LiCl or saline injection. The session ended 20 min after the last reward was received. Rats treated with the highest dose of LiCl (63.5mg/kg) had significantly longer session lengths than rats injected with the lower doses of LiCl, or the vehicle saline. (P values denoted as: **p < 0.01). Bars show means +S.E.M. (n = 16).*

The analysis of the length of time that rats took to complete trials was truncated at the largest PR*e* ratio that was completed by all of the rats (trial 10). Rats took longer to complete a trial as the number of responses required to obtain reward increased (GLM: $F_{2.1,31.8} = 30.48$, $p < 0.001$, Greenhouse-Geisser correction applied), and also took significantly longer to complete a trial depending on the dosage of LiCl received (GLM: $F_{1.3,20.0} = 9.82$, $p = 0.003$, Greenhouse-Geisser correction applied). As a significant interaction was highlighted between dose and trial number (GLM: $F_{2.9,43.7} = 4.39$, $p = 0.009$, Greenhouse-Geisser correction applied) the within subjects contrast was referred to. Between the sixth and tenth trial, the time taken to complete a trial was significantly longer when rats were administered 63.5mg/kg LiCl, whereas rats administered 12.7mg/kg and 31.8mg/kg LiCl did not differ from the control rats. Significant differences between trial completion latencies are shown in Figure 3-6.

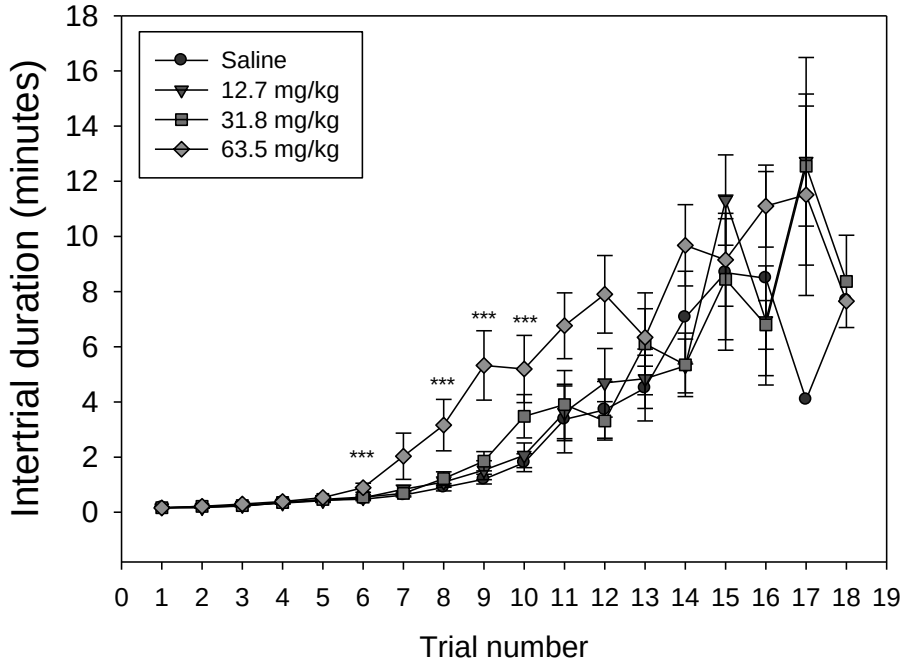


Figure 3-6 ***Mean length of trials within a progressive ratio session*** *The graph shows the mean amount of time for a rat to complete a trial. On subsequent trials the number of responses required to obtain a reward increased exponentially (see Table 3-3). During the 6th to 10th trial within a session, rats that received the highest dose of LiCl (63.5mg/kg) took longer to complete trials than rats treated with the vehicle saline. (P values denoted as: ***p < 0.001). Symbols show mean + S.E.M. p values show differences compared to saline-injected animals. (n = 16).*

3.III.iii.3 Did the occurrence of coprophagy or ingestion of peanut butter before a session effect responding?

Coprophagy was not observed in rats when administered saline, or the lower dose of LiCl (12.7mg/kg), however all of the rats readily ate the peanut butter on at least one occasion. To determine whether ingestion of either of these substances affected the levels of responding, and whether this was correlated to the dose of LiCl, a mixed model analysis was performed. Like the repeated measures analysis in the previous section, a mixed model analysis identified no effect of dose on the number of responses made (Linear Mixed Models: $F_{3,47} = 0.29$, $p = 0.835$). Ingestion of peanut butter was similarly found not to effect responding (Linear Mixed Models: $F_{1,49} = 1.67$, $p = 0.202$), but a marginal, near-significant effect of coprophagy was seen (Linear Mixed Models: $F_{1,57} = 3.74$, $p = 0.058$; Figure 3-7) where it appears that this behaviour was correlated with an increase in responding at the highest dose.

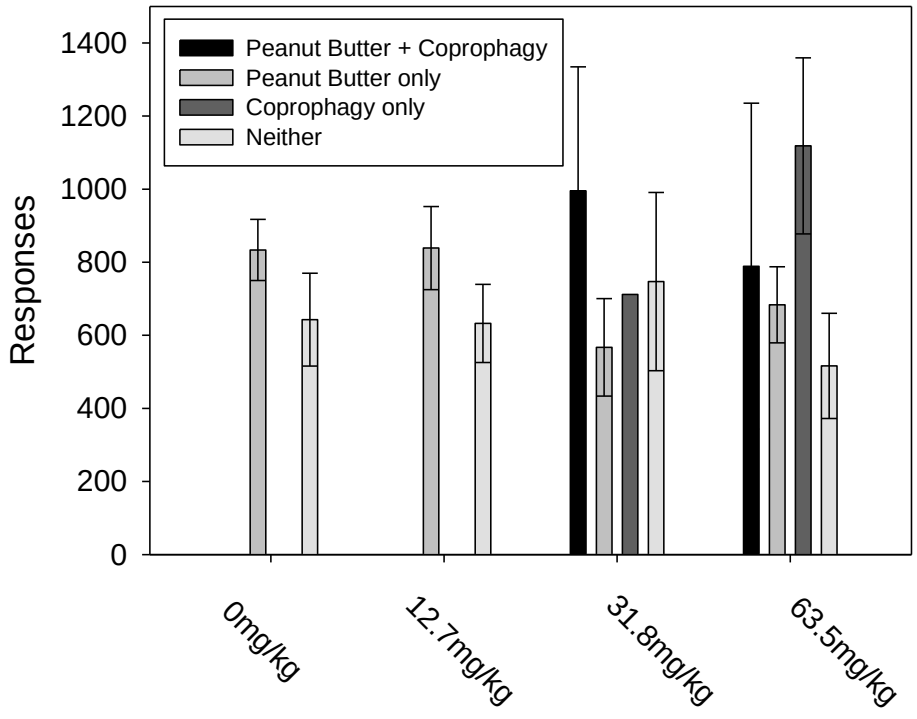


Figure 3-7 ***Responses following coprophagy or ingestion of peanut butter.*** *The graph shows the number of responses made during a progressive ratio session where peanut butter was eaten or coprophagy occurred before testing sessions after saline or LiCl injection. Bars show means +S.E.M.(n = 16).*

3.III.ivDiscussion

Our behavioural data indicated that the two highest doses of LiCl produced sickness behaviours. 63.5 mg/kg LiCl injection resulted in a reduction in grooming and rearing and a concomitant increase in coprophagy and still behaviour in the spontaneous behaviour assay, all strongly indicating the induction of sickness. The sickness behaviours were not as pronounced with a 31.8 mg/kg dose, but included distinct reductions in grooming behaviour. Coprophagy was also recorded (a nausea-related behaviour) which was not observed in the rats following a 12.7 mg/kg LiCl or saline injection. Aversion to peanut butter also occurred following administration of these higher doses. When comparing the effect of these two doses in performance on the PR, rats spent significantly longer completing trials in the progressive ratio task when administered 63.3 mg/kg LiCl, but not after a 31.8 mg/kg dose. Increased latency to respond would inherently effect lever-pressing on the judgement bias task where responding is time-dependent so the higher dose was not selected for the judgement bias study. Responding on the progressive ratio task was conserved when rats were administered 31.8mg/kg LiCl, so this was selected as a suitable dosage for use in the judgement bias task.

The total number of responses on the PR task was unchanged after LiCl challenge (Figure 3-4), indicating that aversion to the sucrose reward was prevented. However, this responding could be viewed as an artefact of the extensive training received beforehand, where responding can be maintained for devalued rewards due to habitual responding, rather than a motivation to obtain the reward itself (Hitchcott et al., 2007). Habitual responding to devalued rewards can be identified by pharmacological intervention. Dopaminergic neurons in the ventromedial prefrontal cortex (vmPFC) are indicated in outcome valuation and goal-directed behaviour, and an infusion of dopamine into the vmPFC of animals promotes a more accurate outcome valuation. This treatment adjusts response behaviour, where responding for devalued rewards is decreased by dopamine infusion, and conversely increased for a ‘valued’ reward. Hitchcott et al. (2007) suggested that dopamine infusions in this area ‘over-rides’ habitual responding and instead elicits the ‘correct’ response behaviour.

A highly hedonic sucrose solution was devalued by pairing a 127.2mg/kg LiCl injection with 30 min of free access to the solution. The control group received an unpaired LiCl injection 24 h after sucrose exposure. Responding for this reward was measured before and after dopamine infusion into the vmPFC. As predicted, the authors found that after dopamine infusion responding for this reward was decreased in the rats that had been given the paired injection, suggesting responding was of a habitual rather than a motivational nature (Hitchcott et al., 2007). This gives rise to the possibility that rats continued to respond for sucrose reward in our study out of habit despite its association with sickness. I am, however, confident that devaluation of the sucrose reward did not occur due to an overshadowing of sickness pairing with a novel flavour (peanut butter). Although aversions can be formed to known flavours (Benoit et al., 2003, Fenwick et al., 1975), they tend to be paired with more salient cues where salience is often achieved by novelty (Kalat, 1974). The rats in our study had been exposed to sucrose over 25 sessions before LiCl injection, whereas the peanut butter flavour was entirely novel. By receiving a substantial number of pre-exposures to the reward during training, a devaluation of the sucrose reward was avoided. In addition, aversion to sucrose was overshadowed. A taste of peanut butter preceded the LiCl injection by 15 min, whereas the sucrose reward was encountered after the injection. In classical conditioning the conditioned stimulus (CS) precedes the unconditioned stimulus in a CS-US pairing, so in this case the rats were conditioned to associate the sickness event (US) with the novel peanut butter flavour (CS). The rejection of this flavour on subsequent test days indicates that the adverse effects of LiCl were attributed to the peanut butter and that the rats were protected from developing aversion to the sucrose reward.

I was concerned that ingestion of peanut butter or faeces prior to the PR task may have altered the rats' appetitive motivation and therefore their behaviour on the task. Foodstuffs high in calorific value, such as peanut butter, are capable of stimulating appetite and thereby could increase responding for food. The ingestion of faeces, on the other hand, might serve to reduce sickness symptoms. In the absence of a vomiting response, nausea is typically measured in rats by levels of the behaviour pica. Pica is defined as the ingestion of non-nutritive substances and this behaviour is increased

with exposure to nausea-inducing agents. Although coprophagy cannot technically be defined as pica as faeces contain a nutritive content, it could be argued that excessively high levels of coprophagy in the absence of any other non-nutritive substance are equivalent. A reduction of nausea could therefore be responsible for the lack of change in response levels when rats were administered high doses of LiCl. An analysis of responding including coprophagy and ingestion of peanut butter as factors did not indicate that either influenced the number of responses made or trials completed, although there was a marginally significant trend for rats to respond more when they engaged in coprophagy after a 63.5 mg/kg dose of LiCl. This dose was not used in the judgement bias study, but nevertheless, precautions were taken to ensure that this behaviour did not occur. Access to faeces was reduced in the operant chambers via a metal bar flooring elevated from the bottom of the chamber which the faeces pass through.

To summarise, the objectives of this preliminary study were achieved where a dose was identified that induced sickness behaviours without significant changes in responding in the PR task. The selected dose of 31.8mg/kg was then used to identify sickness-related changes in affective state in the elevated plus maze and judgement bias tasks.

3.IV Proxy measures of affect - the elevated plus maze

3.IV.i Rationale

In addition to the judgement bias task, a proxy measure of affective state was also performed using the elevated plus-maze (EPM) with the view that more confident conclusions could be made by comparing the cognitive and behavioural effects of LiCl. Whilst it might appear more appropriate to use a paradigm that measures more depressive-like behaviours in animals, many of these require repeated exposures to aversive experiences (e.g. Forced Swim test). In addition, the coexistence of symptoms of anxiety in depressed patients are extremely common (Mineka et al., 1998), so it reasonable to predict that they are also displayed in sick animals. Anxiety-like symptoms can be identified in animals after a single and short exposure to the EPM,

and it was expected that they would be displayed in rats administered LiCl compared to control animals.

3.IV.i.1 Elevated plus maze as a measure of affect

As was described in detail in section 2.III.iii, the elevated plus-maze is used to identify anxiety-like behaviours in rodents, exploiting the conflict between an innate tendency to avoid open spaces associated with increased risk of predation, and exploratory behaviour (Pellow et al., 1985). Additionally, spontaneous behaviours can be measured to identify nausea-related behaviours such as grooming and time spent still (see Table 3-4 for descriptions of behaviours).

The EPM is subject to test decay with repeated exposure (as was seen in Chapter 2) so pre-exposure to or repetitions of the test were not performed within this study.

3.IV.ii Methods

3.IV.ii.1 Animals

The animals used in this study were reused from the PR and spontaneous behaviour study in 3.II.ii.1. Housing conditions were identical to section 3.II.ii.1. The rats were 22 weeks and 442g ± 27g at the time of testing on the EPM. There was a two week period between the PR task and the EPM where no testing took place. The rats were assigned to two groups and activity was filmed on the EPM following either injection with saline vehicle or 31.8mg/kg LiCl. Rats were randomly allocated to two groups, SAL and LiCl, with half receiving a saline injection (SAL, n=8) and the other half receiving a 31.8mg/kg injection of LiCl (LiCl, n=8).

3.IV.ii.2 Apparatus and equipment

Apparatus was as described in Chapter 2 (section 2.III.iii.1.2). The maze was cleaned with 50% ethanol after each rat.

3.IV.ii.3 Operational procedure

The rats were injected in the home room and replaced to their homecage. After 15 min they were transferred to the filming room. The camera was positioned overhead of the EPM and operated by remote control. The observer was not in the room during filming session. Rats were identified by a number shown to the camera before the start of the test, so the observer was semi-blind to the treatments. The observer was not present in the room during the test period. The videos were scored manually using Observer software (Version 5, Noldus Information Technology). To begin a test session, an animal was placed in the centre of the maze facing a closed arm. The exploratory and behavioural parameters measured are described in Table 3-4.

3.IV.ii.4 Statistical analysis

Each video was scored twice for analyses of behaviour and exploration. The percentages of the observation spent engaged in each behaviour or in each location of the maze were calculated. These percentage data were analysed using a one-way ANOVA with lsd post-hoc tests to further assess significant differences between doses. Levene's test was used to assess the equality of variance of the data. Assumptions of normality were not violated in any of the analyses.

Behaviour	*Description*
Proportion of time spent in open arms	(Time in open arms / total time exploring open and closed arms).
Proportion of entries in open arms	(Number of open arm entries/total number of arm entries).
Number of closed arm entries	The number of entries made into closed arms. This is a general measure of locomotion.
Walking	Forwards or backwards movement.
Rearing	Rat standing on hind legs with the front paws not on the floor, may be touching walls.
Still	No movement.
Grooming	Rubbing face or body with paws.
Head-dips	Head protruding over open arm.
Risk Assessment	Exiting enclosed arm with forepaws and head, investigating surroundings.
Stretch-attend	Body elongation with all paws on the floor.

Table 3-4 ***The behaviours observed and scored from video recordings of rats on the elevated plus maze.***

3.IV.iiiResults

3.IV.iii.1 Did LiCl influence rat behaviour on the elevated plus maze?

The number of rats included in the analysis was reduced by two in the saline group as they fell from the maze during testing (final group numbers: SAL = 6, LiCl = 8).

The general levels of locomotion were not affected by a LiCl injection, as indicated by the number of entries made onto the closed arms of the maze (ANOVA: $F_{1,12} = 2.94$, $p = 0.112$; Figure 3-8c) and also the percentage of the observation period that the rats spent walking (ANOVA: $F_{1,12} = 0.4$, $p = 0.382$; Figure 3-9a).

Anxiety-like activity was no different in rats experiencing LiCl-sickness, where the time spent on the open arms and the percentage of entries onto them were similar to that of the control rats (ANOVA: Percent entries: $F_{1,12} = 0.13$, $p = 0.724$; Figure 3-8b; Percent time: $F_{1,12} = 0.69$, $p = 0.424$; Figure 3-8a). Measures of risk assessment were similarly unaltered by LiCl (ANOVA: head dipping:, $F_{1,12} = 0.15$, $p = 0.712$; Figure 3-9b; risk assessment: $F_{1,12} = 1.32$, $p = 0.280$; Figure 3-9c) as were exploratory measures (ANOVA: rearing: $F_{1,12} = 1.71$, $p = 0.224$; Figure 3-9d). Sickness-related behaviours such as grooming and time spent still were also not altered by LiCl administration (ANOVA: grooming: $F_{1,12} = 0.57$, $p = 0.470$; Figure 3-9e; still: $F_{1,12} = 1.14$, $p = 0.314$; Figure 3-9f).

Scanning and the stretch attend posture were displayed negligibly by rats given either injection, and were similarly unchanged (ANOVA: scanning: $F_{1,12} = 0.25$, $p = 0.630$; Figure 3-9g; stretch attend: $F_{1,12} = 0.82$, $p = 0.389$; Figure 3-9h).

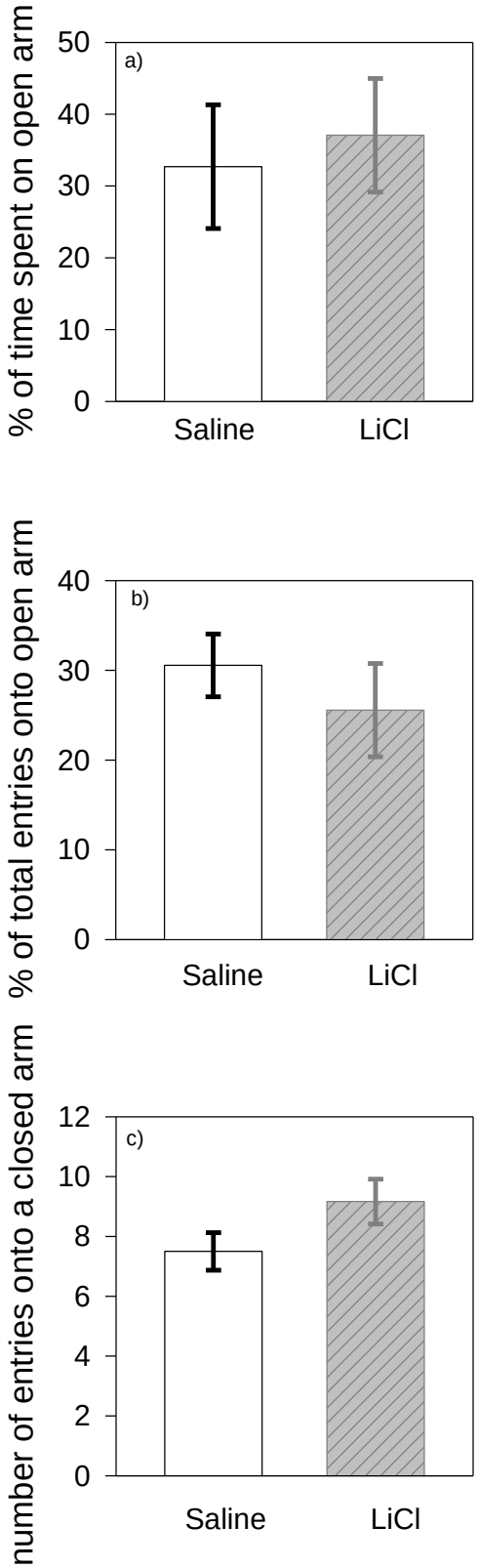


*Figure 3-8 E**xploratory behaviour of rats on the Elevated Plus Maze** Panels show a) the percentage of time that rats spent exploring the open arms of the EPM; b) the percentage of entries made onto the open arm; and c) the total number of entries made onto the closed arm following injection with 31.8mg/kg LiCl or saline.Bars show mean +S.E.M. (n = 16).*

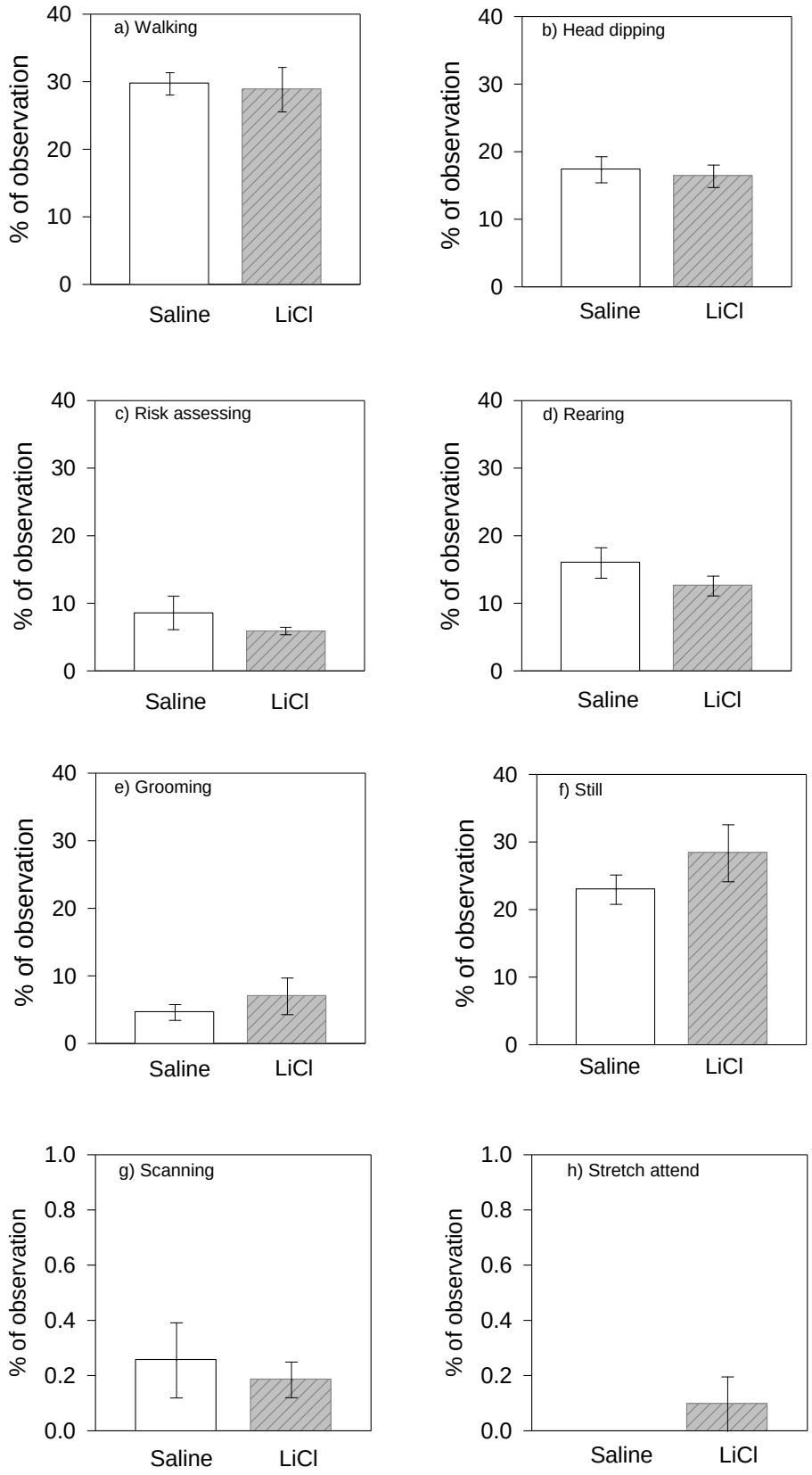


Figure 3-9 ***Behavioural observations of rats on the Elevated Plus Maze.*** *Panels show the proportion of the EPM task that rats spent engaged in each behaviour following administration of 31.8mg/kg LiCl or saline vehicle (note the change in scale in g) and h)). Bars show means +S.E.M. (n = 16).*

3.IV.iv Discussion

The outcomes of the EPM in this study suggest that LiCl has no effect on anxiogenic behaviour. Although the number of subjects was reduced due to technical difficulties, there is no indication that the negative findings are due to a lack of power. In fact, the choice of task may not have been the most suitable measure of LiCl induced changes in affect. The EPM is most-suited to identifying anxiety-like behaviours, whereas we predicted that LiCl might produce behavioural symptoms on the depressive scale. Alternative proxy measures of affect that classify depression-like activity may have been more appropriate such as the forced swim test, but were considered too costly to the animals' welfare.

Studies reviewing the effects of LiCl on animals on the EPM are scarce, and tend to observe its effects in combination with other agents. A Medline search combining the terms 'lithium chloride' and 'elevated plus maze' resulted in just 4 outcomes, none of which studied the effects of LiCl alone on animals on the maze. The effects of other key sickness-inducing agents (lipopolysaccharide (LPS) and interleukin-1β (IL-1β)) have been investigated using the EPM at doses sufficient to induce sickness behaviours. In mice, these agents reduced the percentage of entries onto and activity on the open arms in a dose-dependent manner, but had varying effects on general activity as identified by the number of entries onto the closed arms (Lacosta et al., 1999, Swiergiel and Dunn, 2007). Therefore the data allude to, but do not unequivocally prove, the contribution of cytokine-sickness to anxiety.

3.V Methodological development of the rat judgement bias task

3.V.i A review of current methods

In 2009, a judgement bias protocol was published that assessed both expectation of reward and punishment in the rat (Enkel et al., 2009). More specifically, the study assessed the motivation to gain a food reward vs. a motivation to avoid aversive foot-shock. This paradigm holds appeal as it allows for the estimation of affective valence related to both anxiety and depression. In 2012 and 2013, three further studies were

published employing near-identical training methods (Rygula et al., 2012, Rygula et al., 2013, Papciak et al., 2013), along with another that employed considerably different training and testing procedures (Anderson et al., 2013). A detailed summary of the methods in these five published studies can be found in Table 3-5. Although these studies appear superficially comparable by adopting a similar tone range as audio cues to stimulate lever pressing for reward or avoidance of foot-shock, each author used a different trial structure (i.e. length of CS, inter-trial interval, type of food reward, intensity and duration of shock) and varied in the number of trials in testing and training sessions. The design of my first study largely reflected the methods outlined in Enkel et al. (2009). In my study, rats were quick to perform an operant response to gain food reward, however relatively few rats sufficiently learned to actively avoid foot-shock by lever pressing, and this was amplified when rats were given the choice of responding on both levers (Table 2-1). The same criterion of response accuracy whereby rats were required to respond to at least 60% of the negative trials training was adopted (as in Enkel et al. (2009) and Anderson et al. (2013)). However, the number of trained rats I successfully trained was markedly reduced compared to those in publication (43% as compared to 69 – 95%), and in addition the number of training sessions far exceeded those Enkel et al. (2009). In the first version of this task only 9 of a total of 24 rats achieved a 60% response rate on the negative trials, following ~22 training sessions (Chapter 2). Due to the insufficient levels of uptake of the task, I modified the task in the first experiment in this chapter to include aspects of the methods from Anderson et al. (2013), specifically the incremental titration of foot-shock intensity over sessions upwards from an initially low voltage. The purpose of this was to identify a voltage that would cause each rat to reliably respond, while avoiding the use of more intense shock that might cause excessive pain or the rat to perform a freezing response. This process was said to cause rats to respond maximally during the negative trials (Anderson et al., 2013). However, when I used this procedure in the training of the second task (Figure 3-14), no improvement in responding was observed. As before, the rats were unable to satisfactorily perform active avoidance responses to the shock-predicting cues, with a plateau of correct responding on negative trials at just 20% (Figure 3-14), which continued for 25 sessions. This led to further review of the training paradigm.

From the summary of these judgement bias studies (Table 3-5), it is important to note the variability in terms of the learning and retention of the task which suggests a need for an improved, standardised protocol. In the studies by Enkel et al. (2009) and Anderson et al. (2013) there was additional exclusion of rats from their analyses to avoid confounding results if their performance accuracy dropped below criterion during the ambiguous-cue testing sessions. As Anderson et al. (2013) progressed through their studies, the number of excluded rats increased, indicating that performance became less stable over time. In the study reported by Rygula et al. (2012) there was no indication that the responding of the rats fell below criterion during the testing sessions. I attempted to address both the inconsistencies in training the rats to perform avoidance responses, and the extinction of responding in testing sessions. In the next two sections of this chapter I will explore the potential modifications to the training and testing parameters to provide a more optimal training paradigm. First I discuss training of the avoidance response, and later the reinforcement outcomes of responses to ambiguous tone trials in the testing sessions.

	Enkel et al., 2009	**Rygula et al., [1] 2012, [2] 2013 [3] Papciak et al., 2013**	**Anderson et al., 2013**
Affect manipulation	[1a] Genetic model of depression [1b] Pharmacologically-induced stress	[1]Tickling [2] Chronic psychosocial stress [3]Chronic restraint stress	[1a]Acute diazepam, [1b] Acute reboxetine, [1c] Acute fluoxetine [1d] Chronic fluoxetine
Rat strain, sex and age/weight at start of training	[1a] 16 eight-week old, [1b] 16 (age not specified) male Sprague Dawleys	[1]26, [2] 40, [3] 32 male Sprague Dawleys(175-200g)	20 male Lister Hooded rats, 12 weeks
Food restrictions in homecage	*Ad libitum* access to food	85% free-feeding weight	*Ad libitum* access to food
Tone frequencies (amplitude)	2– 9kHz (64-77dB)	2 - 9kHz (75dB)	2 – 8kHz (66-77dB)
Maximum tone duration	30 s	50 s	5 s

	Enkel et al., 2009	Rygula et al., [1] 2012, [2] 2013 [3] Papciak et al., 2013	Anderson et al., 2013
Number of ambiguous-cues	Three:3kHz, 5kHz,7kHz (6 of 25 trials)	One: 5kHz, (10 of 50 trials)	[1a] One:5kHz (20 of 100 trials), [1b] three: 4Kz,5kH,6kHz (30 of 100 trials)
Number of trials per session	Positive training: n/a (time-limited) Negative training:20 Discrimination training: 20 Testing: 24	Positive training: n/a (time-limited) Negative training:40 Discrimination training:40 Testing:40	All Training:100 Testing:100
Session frequency	Daily	Daily	Weekdays
Positive reinforcer	80µl 33% sweetened condensed milk	[1,3] 100µl 20%, [2] 100µl 5% sucrose solution	One food pellet
Foot-shock intensity and maximum duration	0.7mA 60 s	0.5mA 10 s	0.23-035mA (incremental titration individual to each rat) 1 s
Escape response	Lever press during foot-shock	Lever press during foot-shock	None

	Enkel et al., 2009	Rygula et al., [1] 2012, [2] 2013 [3] Papciak et al., 2013	Anderson et al., 2013
Ambiguous-cue reinforcement	None	None	50% –food pellet, 50% - foot-shock
Positive training criteria	None	[1] "stable performance", [2,3] ≥200 responses maintained over 3 consecutive sessions	None
Negative training criteria	≥60% correct responses	[1] ≥60% correct responses , [2,3] ≥60% correct over 3 consecutive sessions	≥60% correct responses
Discrimination training criteria	≥70% correct responses on both levers	≥70% correct responses on both levers	≥60% correct responses on both levers over 2 consecutive sessions

Table 3-5 ***A summary of the methods in published judgement bias studies using food and foot-shock as response outcomes.***

3.V.ii Challenges of active avoidance of noxious stimuli in operant responding

The drive to eat and forage for food is a natural, innate response with immediate effects on an animal's survival. The training of operant responding for food reward is thus relatively fast and simple; and can be achieved in few sessions. Adaptation of behaviour to escape painful stimuli is also described as an innate response (Miller, 1948), and rats will readily avoid pain in a passive manner (i.e. run to a safe place to avoid shock e.g. Dieter (1976)). However, rats are not documented to instinctively respond to noxious stimuli by actively responding to avoid them (D'Amato and Schiff, 1964, Meyer et al., 1960, Modaresi, 1990, Feldman and Bremner, 1963). This response deficit is not limited to rodents, with failures to learn avoidance also observed in dogs (Brush, 1957) and humans (Turner and Solomon, 1962). Rats will instead wait until the onset of painful stimuli before making the appropriate response to escape it (Meyer et al., 1960, D'Amato and Schiff, 1964). This can continue over thousands of trials, with excessive training further detrimental to performance (Coons et al., 1960, Baum, 1968, Feldman and Bremner, 1963). Consequently, training rats to press a lever in order to avoid a mild foot-shock requires a more complex paradigm. It is apparent that we are still slightly off the mark in developing this paradigm when relating to the training rates seen in the most recent judgement bias studies cited in Table 3-5. The difficulty in training rats to press a lever to avoid shock was labelled "The Avoidance Barpress Problem" by Modaresi (1990), where this paradox was reviewed. He discussed a cohort of studies completed in the 1960's designed to identify potential causes and solutions to this problem which I expand on in this section.

Many aspects of the training paradigm of avoidance responding can be manipulated, ranging from: the presentation and discriminability of the CS; the duration, persistence and amplitude of shock; the possibility of an escape response; the number and length of the trials and inter-trial intervals; and the inclusion of 'warning signals'. The strain, sex and age of rats have also been assessed for any features that might improve responding. I describe each potential modification in detail and conclude by discussing those that were included in my final study design.

3.V.ii.1 Conditioned stimulus

Berger and Brush (1975) broadly studied the temporal effects of a warning stimulus (analogous to the tone CS in the judgement bias study) on avoidance performance in a lever-pressing task in terms of duration and consistency (whether the CS duration was fixed or variable). They found that greater avoidance responding occurred with longer CS presentations, and also when this duration was fixed. In particular, increasing the duration of the tone from 10 to 20 s significantly increased the number of avoidance responses, but they found that tone durations from 20 up to 60 s to have asymptotic effects on the animals' responding. After ~ 40-50 trials, all of the animals in the 20, 30, 40, 50 and 60 s groups were performing avoidance lever presses in ~90% of the trials. This would suggest that providing rats with a longer CS improved responding, and CSs with durations shorter than 20 s should be avoided.

3.V.ii.2 Foot-shock presentation

There are considerations of both welfare and task performance that arise with using shock as a reinforcer. Excessively high levels of shock may not only be painful to the rat, but may also result in a freezing response which would serve to reduce active avoidance behaviours. As a result there is a responsibility to ascertain suitable shock levels to train rats efficiently, to effectively use a minimal number of animals in our studies (Russell and Burch, 1959).

Increasing the amplitude of shocks was a core component of the methods in Anderson et al. (2013). Increases in shock produce greater responding in unsignalled shock avoidance paradigms (Myers, 1977, Powell, 1970) but in the area of signalled shock avoidance (as with a CS in the judgement bias task) there is consensus that conditioning of active avoidance is actually inverse to the intensity of shock (Bolles and Warren Jr, 1965, D'Amato and Fazzaro, 1966), where high-intensity shocks serve to hinder responding even further. In fact, D'Amato et al. (1968) advised that as low a voltage as possible should be used in order to successfully produce avoidance responding. Minimally effective intensities were reported in Bolles and Warren Jr

(1965) and D'Amato and Fazzaro (1966) as 0.2mA. Enkel et al. (2009) used a shock amplitude 3.5 times higher than this recommendation.

There is also consensus in the literature with reference to the presentation of the shock itself. Intermittent shocks potentiate responding as compared to continuous shock, and more so when there are frequent pulsation (e.g. 0.2 - 0.5 s every 5 - 20 s; D'Amato et al. (1968), Servatius et al. (2008)). This is more apparent when milder shocks are applied (D'Amato et al., 1968, D'Amato and Fazzaro, 1966). In contrast, when shocks are of a greater intensity, this improvement in responding disappears. For example, Berger and Brush (1975) compared responding in tasks where shock was continuous vs. intermittent, and found that responding was not improved when shocks were pulsed. They did, however, use comparatively high voltages of shock (2mA) and longer pulses (0.5 s every 60 s). The presentation of shock in pulses rather than continuous is supposed to improve avoidance responding as the shock-shock intervals provides the rat time to 'consider' an alternative behavioural response, rather than to just 'sit-out' the shock (Berger and Brush, 1975). Pulsing of shock is used in current research involving active avoidance paradigms, overcoming the excessive training requirements seen in earlier studies. For example in Servatius et al. (2008), Sprague Dawley rats were effectively lever pressing to avoid shock after just four training sessions of 20 trials when a 1.0mA shock was pulsed for 0.5 s every 3 s.

Also, in Anderson et al. (2013) the foot-shocks were short but inescapable. Inescapable shock is inherently stress-inducing (Van der Kolk et al., 1985) and is used to produce models of depression (Maier, 1984), so rendering shocks inescapable in a training paradigm might inadvertently produce negative affect in control animals. This may have been inferred by a measure of affect that they used (midpoint bias score) where control animals showed a negative affect during baseline measurements.

3.V.ii.3 Number of trials and intertrial interval

Another successful manipulation of the paradigm includes shortening the intertrial interval (ITI). This was first reported by (Pearl, 1963) who compared ITIs of 40, 80 and

180 s, and found that avoidance responding was greater with shorter ITIs. This effect was replicated in a later study by Pearl and Fitzgerald (1966). Morris (1974) further concluded that responding could be increased four-fold by reducing the ITI from 90 s to 10 s. Consequently, other authors have adopted short ITIs (20-80 s) in active avoidance studies (e.g. Van Oyen et al. (1980), Bolles et al. (1966), Rakover (1980)). This is interesting as it does not agree with traditional learning theory where longer ITIs are associated with improved learning. It is hypothesised that shorter ITIs facilitate avoidance responding particularly after animals received shock on the previous trial, as the increase in shock-induced activity counteracts the immobility aroused by fear. Furthermore, this is transferred from escape responses to avoidance responses as training continues (Rakover, 1980).

3.V.ii.4 Effect of strain and sex

Differences in avoidance behaviour have been highlighted between a number of strains of rats. Both Long-Evans and Wistar Kyoto rats have been reported to show better acquisition and more prolonged active avoidance responding than Sprague Dawley rats (Nakamura and Anderson, 1962, Servatius et al., 2008, Beck et al., 2010). Wistar Kyoto rats are perhaps more capable at learning this task as they display more anxiety-like behaviours compared to Sprague Dawleys (Carr and Lucki, 2010) and subsequently pay greater attention to threat-like cues. This is highlighted in a quicker acquisition of lever-pressing behaviour in an active shock avoidance lever-press task and a greater resistant to extinguishing of this behaviour (Jiao et al., 2011). In the present day, this strain is more frequently used in research of active avoidance, and might be a more suitable choice for the judgement bias task in terms of reducing the training requirements.

Sex differences have also been identified in uptake of active avoidance tasks, where females tend to attend more than male rats to cues predicting threat. This is due to a greater susceptibility of females to demonstrate anxiety-like tendencies (Gray and Lalljee, 1974, Servatius et al., 2008), and additionally a propensity for males to reduce their activity when exposed to stressors (Steenbergen et al., 1990). These features

have been identified in female Sprague Dawley, albino and Wistar strains (Van Oyen et al., 1981, Beck et al., 2010, Heinsbroek et al., 1983). Differences between sexes in active avoidance behaviour in the Wistar Kyoto strain are surprisingly not exhibited, which is attributed to overshadowing of the female trait influences by the overall strain-induced trait influences (Beck et al., 2010).

3.V.iii Partial reinforcement of learned cues

A decline in performance, referred to as response extinction, was a major setback in the judgement bias study in Chapter 2. Here, two thirds of the rats successfully trained to discriminate two cues showed a drop in performance below criterion during subsequent test sessions. The non-reinforcement of ambiguous trials often causes extinguishing of responses, as the animals learn that these trials have no outcome. This phenomenon is unlikely to be a problem where the animals are used only once for testing (e.g. Papciak et al. (2013)), but more prevalent when animals are tested repeatedly (e.g. Enkel et al. (2009)).

The non-reinforcement of probe trials often causes extinguishing of responding, as the trials are learned to have no outcome. This, however, is less likely to be a problem where the animals are used only once or very few times for testing. In my own study and in that of Anderson et al. (2013) the rats were used in subsequent experiments. Anderson et al. (2013) achieved a high inclusion rate which declined only marginally over subsequent studies (from 19 to 17 from an original group of 20 rats over three studies) which meant that many treatment combinations could be tested in the same cohort of rats. This was achieved by randomly reinforcing ambiguous-cue trials, where the response outcomes were shock or food presented in a 50:50 ratio. It is, however, not possible to rule out that some learning of the outcomes had occurred. Their task might have measure risk taking (i.e. responding when animals know there is a 50% distribution of an aversive outcome) rather than measuring the interpretation of ambiguity. Animals are said to respond differently towards uncertainty and risk, and this is acknowledged by Anderson et al who refer to their task as an affective tone discrimination task rather than as a classic judgement bias task. Although it appealed

to replicate the inclusion rates obtained in Anderson's study by randomly reinforcing probe trials, it may have prevented from answering the questions laid out in this thesis.

In other studies, no schedule of reinforcement was used (Rygula et al., 2013, Papciak et al., 2013, Rygula et al., 2012, Enkel et al., 2009). In the experiments by Rygula and Papciak, animals were tested only during one session and no rats fell below responding criterion. However, in Enkel et al. (2009), the rats were tested repeatedly over a period of 6 weeks, and subsequently 10 of the original group of 32 subjects did not continue to meet the performance criterion over this time.

Another option to consider in the reinforcement of trials is partial reinforcement. Here animals are trained on a schedule where a proportion of the trials during training sessions are not reinforced. It is thought that the animals become less expectant of reinforcement during training and are less sensitive when exposed to non-reinforced trials in testing sessions as a result. These animals can be tested for a greater number of sessions before response extinction occurs. Therefore in the subsequent judgement bias studies in this chapter, partial reinforcement was used in order to prevent rats from extinguishing responding over multiple testing sessions.

3.V.iii.1 My paradigm

The amendments to the training paradigm included pulsing shock on and off for 0.2 s every 2 s for a maximum of 80 s, moderate shock levels of 0.35mA, and a longer CS of 30 s (see 3.VI.ii.3). Trials were partially reinforced in later discrimination training sessions. The strain of rat used would be a major consideration if this study were to be replicated, but was not possible during this experiment.

3.VI Judgement bias experiment 1 - Pharmacological induction of nausea

3.VI.i Rationale

By measuring the judgement of ambiguity in animals, we can make an estimation of the valence of their affective state (i.e. whether they perceive their environment in a positive or negative manner). Rats that had learned to associate two cues with a 'positive' and a 'negative' outcome were exposed to cues of an ambiguous nature in a judgement bias task. Interpretation of these cues was measured in terms of whether they were responded to as the positive cue or the negative cue, and their responses compared following injection with LiCl or saline. The rats were expected to respond in a more negative manner to the ambiguous-cues following injection with LiCl as this is reported to produce an aversive state.

3.VI.ii Methods

3.VI.ii.1 Animals

Experiment 1 24 male Lister Hooded rats (Charles River, Margate, Kent, UK) aged 8 weeks 210g ± 10g at the start of training were used in this study. Animals were weighed and handled daily. Housing conditions were as 3.II.ii.1.

3.VI.ii.2 Apparatus and software

The apparatus and software used was as in section 2.II.ii.4.

3.VI.ii.3 Training and testing protocol

Habituation and magazine training In the first session rats were introduced to the operant chambers and allowed 30 min to explore and eat as many as 50 pellets from the food magazine. They were then trained to associate reward delivery to the magazine on presentation of a tone in one 34 min sessions of 50 trials. Trials consisted of a 10 s tone followed by pellet delivery and a 20 s intertrial interval (ITI). Here rats

were randomly assigned a 'positive tone' that signalled food delivery (2 kHz or 8 kHz – the tones were altered to match those used in published judgement bias studies; see Table 3-5), and this tone continued to signal food delivery throughout the study.

Lever training Rats were trained to press levers using a continuous reinforcement schedule where both levers were present throughout the session. Each lever press resulted in delivery of one food pellet, either until 50 pellets had been received or 30 min had elapsed, whichever occurred soonest. Once rats had obtained all 50 pellets in two consecutive sessions, lever preferences were analysed to identify any lever bias. Rats were ranked by the number of responses made on the left lever, then alternately assigned either the right or left lever to represent the positive lever in order to counterbalance any bias. The positive lever refers to the lever that resulted in food delivery when depressed during a positive trial. The other lever was assigned to be the negative lever which was pressed to avoid foot-shock during the negative trials.

Positive lever training In the positive trials a 2 kHz or 8 kHz tone predicting a food reward (referred to as the positive tone) was played for up to 10 s. A lever press within this time terminated the tone and a food pellet was immediately delivered to the magazine. If a lever press was not made during this time the tone was terminated and no pellet was delivered. Only the positive lever was extended during these sessions. Trials were separated by a 20 s ITI. Responding during this interval added a 5 s time-out to the ITI to discourage inactive responding. A response made during the time-out period had no outcome. The session ended either when 50 rewards had been received or 50 min had elapsed, whichever was soonest.

Negative lever training Like in the positive lever training sessions, a tone was presented for up to 10 s in which time the rat had to make a response on the negative lever. The tone played (2 kHz or 8 kHz) and the lever (left or right) was the opposite of those in the positive lever training sessions. Only the negative lever was extended during these sessions. If the rat pressed the lever during this time the tone ceased, ending the trial, and an avoidance response was recorded. If the rat did not press the lever during this time, the tone ceased after 10 s and a mild foot-shock was delivered through the grid floors immediately after. This was recorded as a response omission.

The shock could be terminated by pressing the negative lever (making an escape response) or would terminate after 80 s if a response was not made.

Initially, shocks were titrated upwards daily in intervals of 0.01mA from 0.15mA to a maximum of 0.35mA. There was a maximum of 10 trials per session with a 150 s ITI to minimise the number of shocks rats received while learning. Sessions ended when the maximum number of trials had been reached, or when 50 min had elapsed, whichever was soonest. Following five sessions of unsuccessful training on this schedule, the number of trials in a session was increased to 20, and the ITI reduced from 150 s to 20 s. After an additional 25 negative trial training sessions following this schedule, responding had not reached the criterion of ≥ 60% avoidance responses and the shocks had reached 0.35mA intensity.

At this stage the methods were revised, and the intertrial interval was increased to 60 s, the tone presentation was increased to 30 s and the shocks were altered so that they pulsed on and off for 0.2 s every 2 s, for a maximum of 80 s. Once avoidance responding was ≥ 60% on three consecutive sessions, the rats were moved to the discrimination sessions. This took a further 12 training sessions (Table 3-6).

Discrimination sessions (forced and choice, partial reinforcement) Discrimination sessions consisted of 40 trials (20 positive and 20 negative) presented in a pseudo-randomised order, with no more than two of the same trial presented in succession. The first discrimination sessions consisted of forced trials (i.e. at the beginning of the trial, the incorrect lever was retracted, leaving only the correct lever). Once rats had responded with ≥ 60% accuracy on both positive and negative trials within a session, the rats were presented with choice trials as opposed to forced trials. If the incorrect lever was pressed during a trial (i.e. the negative lever pressed during a positive trial or the positive lever pressed during the positive trial), the outcome would mirror that of an omission trial in the previous sessions (cessation of tone and no food pellet if an incorrect response was made on a positive trial, tone cessation and foot-shock escapable with a lever press if the incorrect response was made on a negative trial).

In order to ensure that the ambiguous trials within the testing sessions were not learned to be unreinforced, and therefore different to the training sessions, I adopted

a schedule of partial reinforcement during the discrimination training schedule. Once rats were reliably responding to the trials (≥ 60% accuracy on both trial types) the reinforcement schedule was reduced to 90% for one session, 80% the next and then to 75%. During the unreinforced trials, a correct response, an incorrect response or a response omission had no outcome (Figure 3-10).

Training schedule	Mean number of sessions to reach criterion (± s.d.)
Positive lever	6
Negative lever	37.5 ± 3.2
Forced choice	2.5 ± 0.7
Discrimination	3.5 ± 0.8
90% reinforcement	1
80% reinforcement	1
75% reinforcement	3

Table 3-6 ***The number of sessions required to meet response criterion on each training schedule*** *of the judgement bias task. Values are means ± s.d. Where the s.d. is not indicated, a fixed number of session were given for that schedule.*

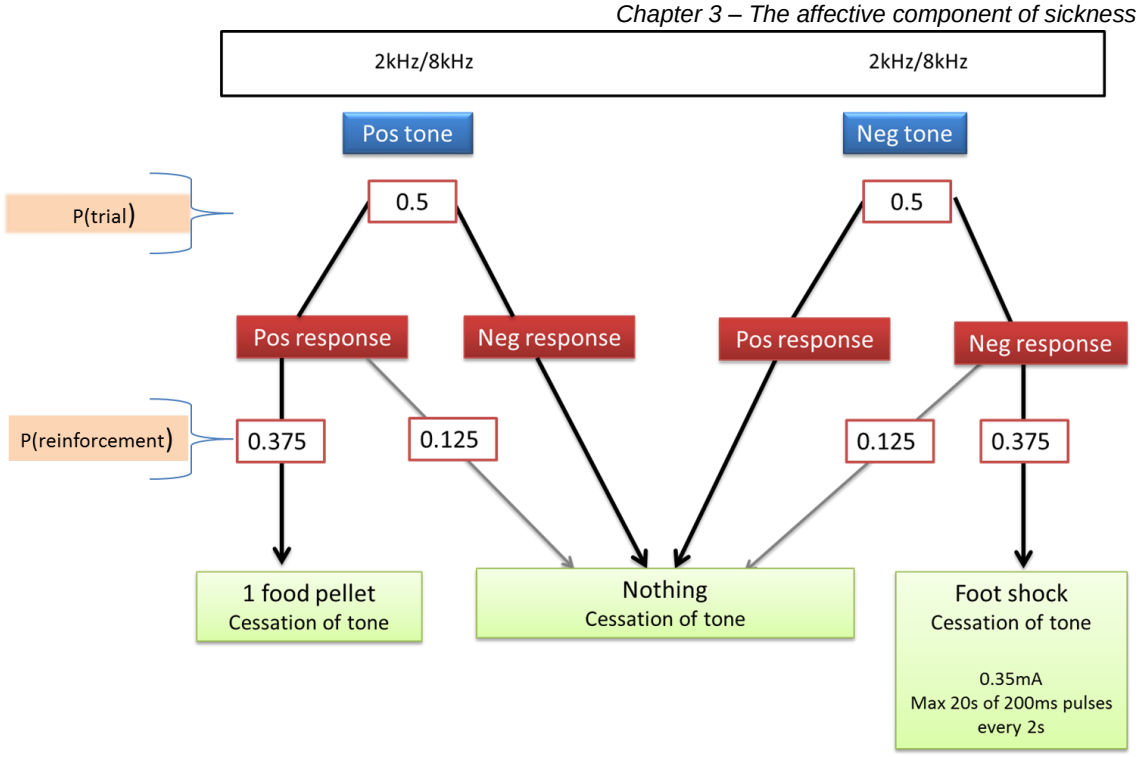


Figure 3-10 ***Schematic of the final discrimination training sessions*** *where 75% of the reference trials were reinforced. 12.5% of each set of positive and negative trials were not reinforced.*

Ambiguous-cue test sessions These were as the discrimination sessions, with 40 trials in an ambiguous-cue testing session. 17 of these were positive trials, 17 were negative, with 15 of each trial type reinforced to match the 75% reinforcement schedule encountered in the training sessions with partial reinforcement. The remaining 6 trials were comprised of 2 of each ambiguous-cue. The ambiguous tones were 4 kHz, 5 kHz and 6 kHz. Responding on these caused the trial to end and the tone to cease but had no further outcome. If no response was made to these trials the tone ceased after 20 s but again there was no further outcome.

The lever chosen on each trial and the latency to choose was recorded as well as the number of response omissions. Additionally the number of escape responses was recorded.

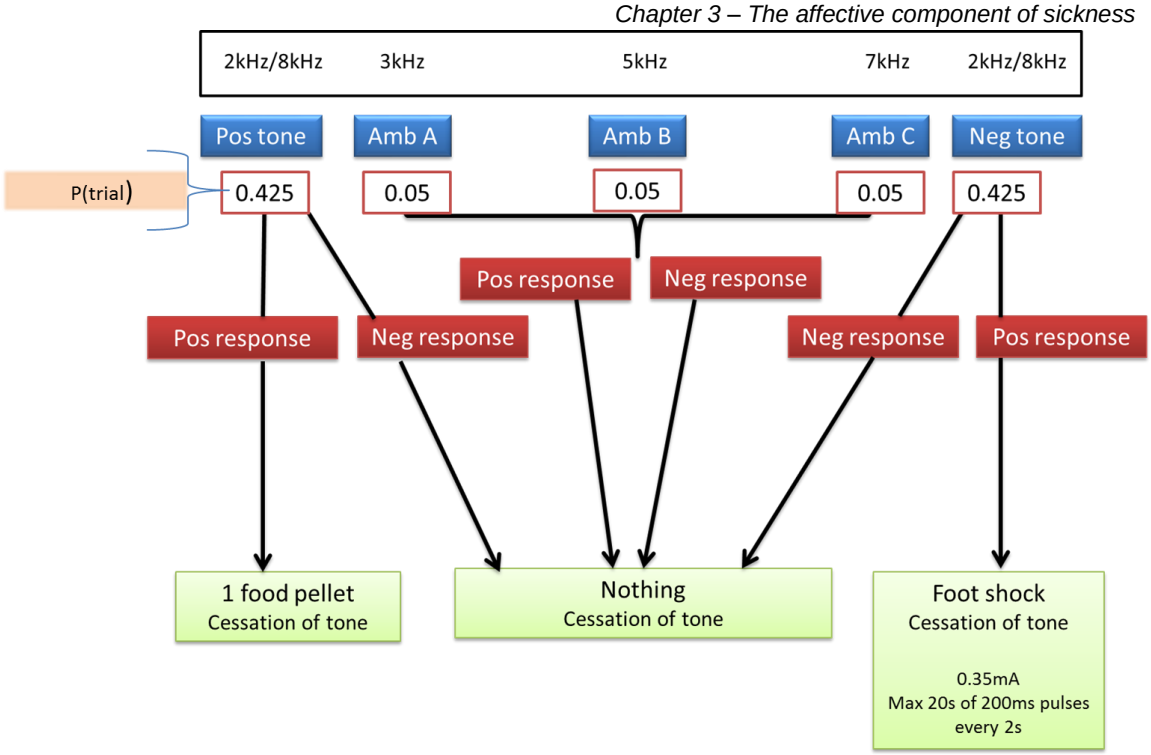


Figure 3-11 ***Schematic of the ambiguous- cue testing session*** *. The figure shows the response outcomes when a rat was presented with a positive, negative or ambiguous-cue trial. Positive and negative trials were always reinforced, whereas ambiguous-cue trials (Amb A, Amb B, Amb C) were never reinforced.*

3.VI.ii.4 LiCl treatment

LiCl and saline treatments were as 3.II.ii.3. Injections of 31.8 mg/kg LiCl or saline were administered at a volume of 10ml/kg in the experimental room and rats were placed into the operant chambers immediately after injection. Test sessions began 15 min after injection.

3.VI.ii.5 Experimental procedure

Rats were administered both saline and LiCl treatments in a crossover design (Figure 3-11). Half of the rats received daily saline injections 15 min before testing in the first three sessions and daily LiCl injections in the final three, and the other half had the reverse order of treatments. The rats were allocated to one of the two groups by matching for accuracy of responding, and this was also balanced within cages. 16 rats met the criterion to be included in the study, so 8 rats were allocated to each group.

The rats underwent three daily sessions of baseline discrimination sessions prior to 3 daily sessions of ambiguous-cue testing where treatments were administered. Following one day of drug 'wash-out', the treatments were reversed and this schedule was repeated.

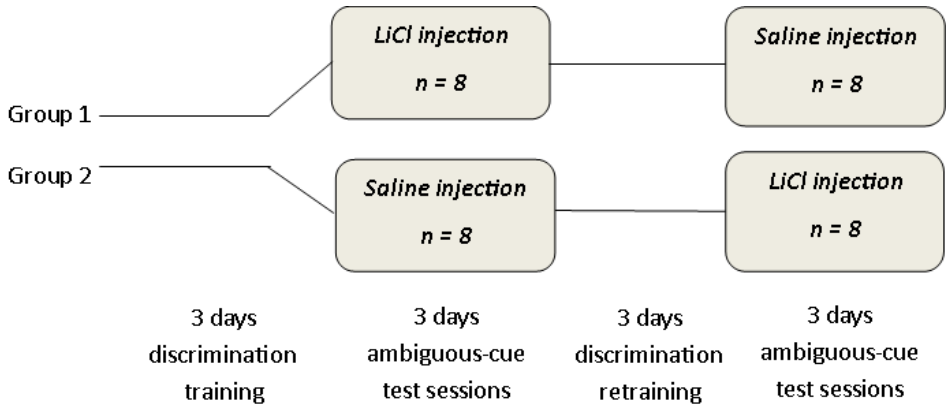


Figure 3-12 ***Timeline of testing and treatments***

3.VI.ii.6 Statistical analysis

Analyses were performed on the mean proportion of reference trials and ambiguous trials responded to positively, negatively, or whether a response was omitted. Mean values for these variables were calculated for each rat over the 3 test sessions within each treatment schedule. A repeated measures ANOVA (RM-ANOVA) was performed for each variable with tone frequency and treatment type included as within-subjects factors (Anderson et al., 2013, Enkel et al., 2009). Greenhouse-Geisser corrections were used where assumptions of sphericity were violated (when $\varepsilon < 0.75$). Where levels of significance were obtained ($p < 0.05$) paired t-tests were performed (two-tailed). The mean latency to make a positive or negative response to each tone was analysed using a Linear Mixed Model as some data sets were incomplete (i.e. no latencies were recorded when there were no responses), where dose and probe values were entered as fixed effects and rat as a random effect. Main effects were assessed with a Bonferroni correction for multiple tests.

3.VI.iiiResults

3.VI.iii.1 Training data

The rats showed fast acquisition of appetitive responding on the positive training sessions, with complete responding on all trials attained by the fourth training session (Figure 3-13). Conversely, rats were slower to learn during the negative training sessions (Figure 3-14). Responding plateaued at 20-25% correct responding for 30 training session before the methods were amended (see 3.VI.iii.1). Figure 3-14 shows the performance during this training of the rats that met criterion and were included in the final judgement bias experiment, and those that were excluded. Following an additional 7.5 ± 3 training sessions on this schedule (mean ± s.d.), 20 rats met the criterion to move onto the next stage of training. During the discrimination training phase, the reinforcement of the trials during a session was gradually reduced to 75% to match the reinforcement that was encountered in the ambiguous-cue testing sessions. Figure 3-15 shows the responding on the positive and negative trials during this training phase.

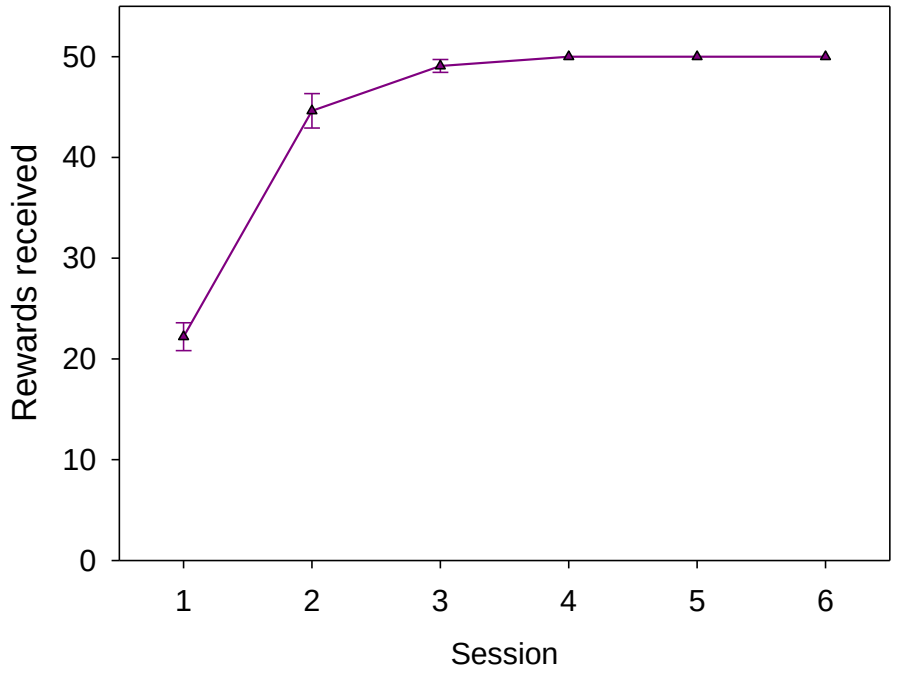


Figure 3-13 ***Rewards received on the positive training sessions*** *A lever press during a 10 s tone presentation resulted in delivery of a food pellet. A maximum of 50 rewards were available during these sessions (n=24).*

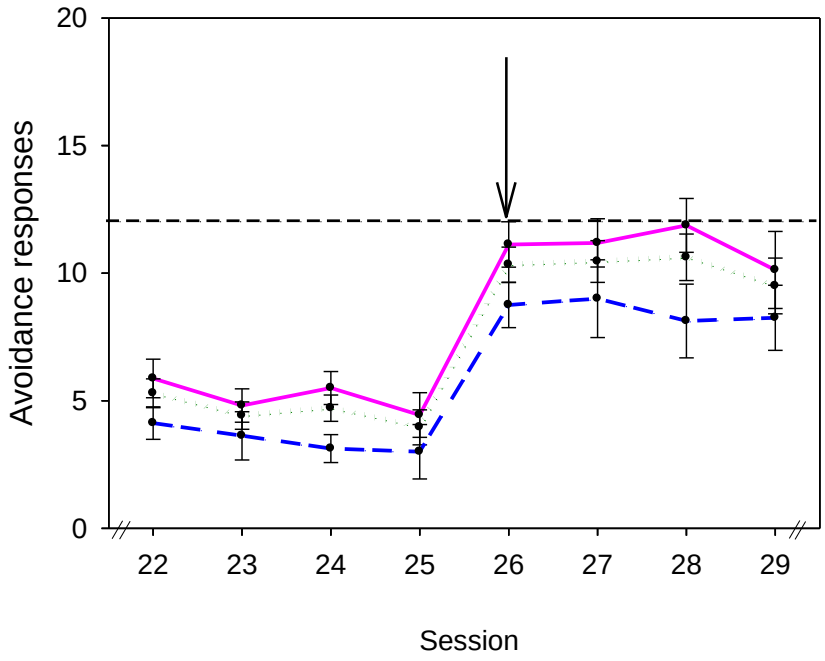


Figure 3-14 ***Active avoidance responses made during negative training sessions*** *in the 4 sessions immediately before and after the presentation of the foot-shock was altered from continuous to pulsing and the tone duration was increased to 30 s (black arrow). The horizontal dashed line represents the threshold for response accuracy of 60% (≥12 avoidance responses within a session). Symbols show means +S.E.M. Dotted line shows all rats trained (n=24), solid line shows rats that met training criterion and were included in the experiment (n=16), and the dashed line shows the rats that did not meet criterion and were excluded from the experiment (n=8).*

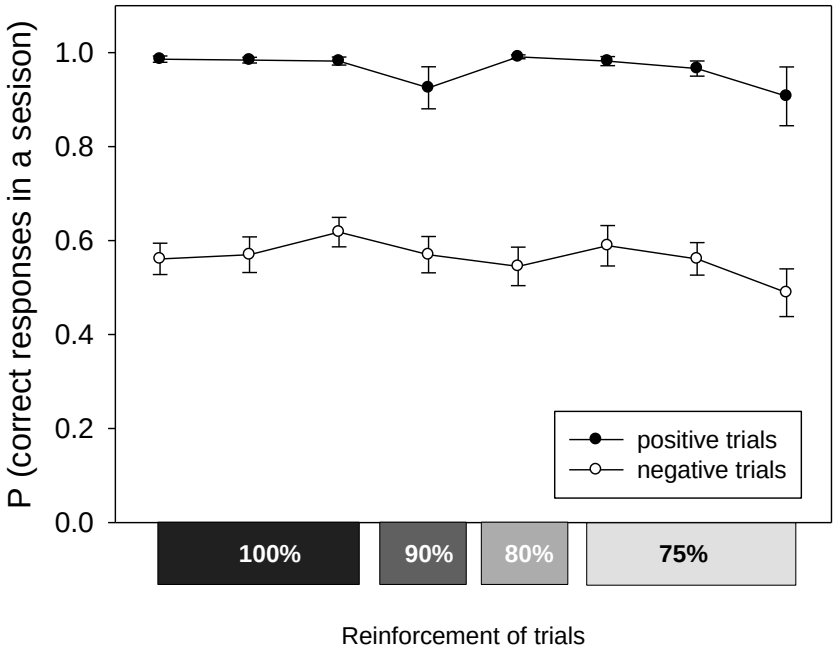


Figure 3-15 ***Proportion of correct responses in discrimination sessions*** *during the instatement of partial reinforcement. Each point shows the mean and standard error of responding during a single session. Symbols show means + S.E.M. (n=20)*

During the baseline discrimination sessions that rats underwent before the ambiguous-cue testing periods there were no differences between the rats' accuracy of responding prior to the first and the second testing periods (Figure 3-16).

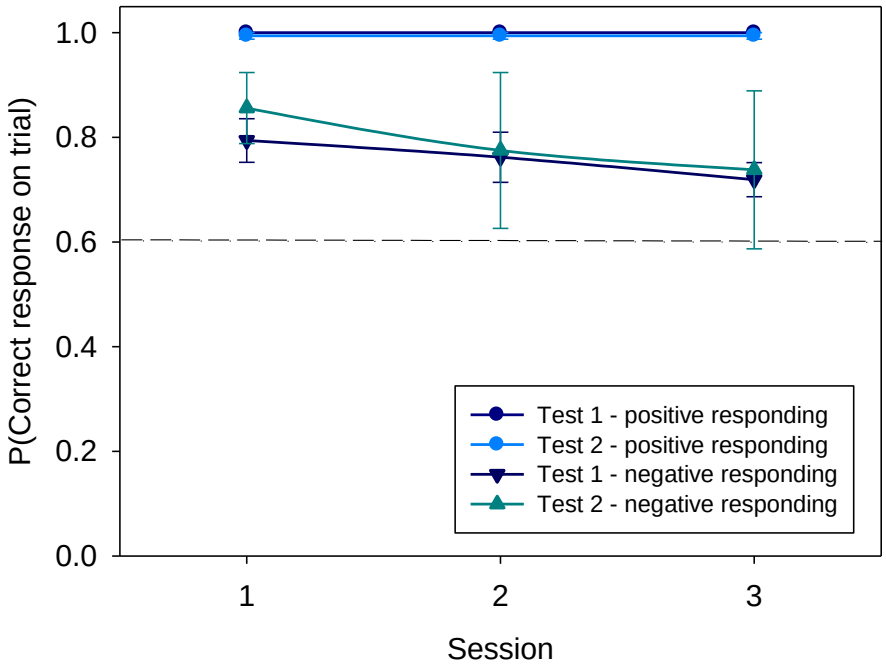


Figure 3-16 ***Performance of rats in the discrimination retraining sessions*** *preceding the ambiguous probe testing sessions. There is no difference between the accuracy of responding on each trial type during the sessions. Symbols show means + S.E.M. (n=16).*

3.VI.iii.2 Did LiCl injection produce a biased judgement of ambiguous-cues?

Anticipation of reward (positive responding) A RM-ANOVA showed a significant effect of tone frequency on the proportion of ambiguous tone trials that were responded to positively (RM-ANOVA: $F_{2,14} = 87.26$, $p < 0.001$; Figure 3-17). Near-negative tones were responded to much less than the midpoint tone ($t(7) = -7.90$, $p < 0.001$) and near-positive tone ($t(7) = -18.0$, $p < 0.001$), and rats also responded less to the midpoint tone than the near-positive tone ($t(7) = -2.83$, $p = 0.025$). Similarly, the frequency of the ambiguous-cue tone also significantly affected the speed at which the rats made a positive response (Linear Mixed Model: $F_{2,60} = 14.7$, $p < 0.001$; Figure 3-22).

Specifically, rats were quicker to respond positively to the near-positive and midpoint tones than the near-negative tone (Nr Pos vs. Nr Neg: t(61) = 3.27, p = 0.002; Mid vs. Nr Neg: t(61) = 3.09, p = 0.003). This indicates the generalisation of the ambiguous tones to the positive learned tone.

Rats injected with LiCl responded a greater number of times in response to ambiguity than rats injected with the saline vehicle (RM-ANOVA: $F_{1,7}$ = 19.64, p = 0.003), but there was no interaction between the treatment and the tones (RM-ANOVA: $F_{2,14}$ = 0.61, p = 0.558). The time taken to respond positively during a trial was not affected by the treatment rats received (Linear Mixed Model: $F_{1,60}$ = 1.96, p = 0.279), nor was there any specific difference in the time taken to respond to the individual tones between the treatments (Linear Mixed Model: $F_{2,59}$= 0.45, p = 0.640).

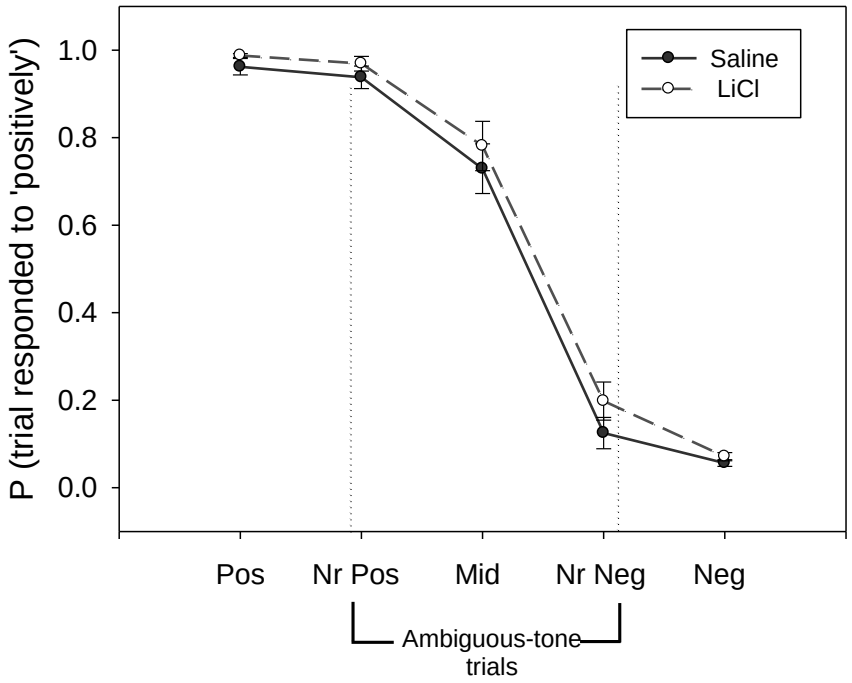


Figure 3-17 ***Proportion of 'positive' responses during the ambiguous probe test sessions*** *(responses made on the lever predicting a food reward) to the trained audio cues (reference cues: Pos and Neg) and three intermediate audio cues (ambiguous-cues: NrPos, Mid, and NrNeg) following administration of saline or 31.8mg/kg LiCl. Symbols show means +S.E.M. (n = 16).*

Anticipation of foot-shock (negative responding) The performance of negative responses was influenced by the frequency of the tone played (RM-ANOVA: $F_{2,14}$ = 50.80, $p < 0.001$). A significantly greater number of 'near-negative' ambiguous tone trials were responded to negatively than the 'near-positive' (t(7) = 8.23, $p < 0.001$) and 'midpoint' (t(7) = 7.49, $p < 0.001$) tone trials (Figure 3-18). No differences were observed in the proportion of ambiguous tone trials responded to when rats were administered LiCl or saline (RM-ANOVA: $F_{1,7}$ = 1.75, p = 0.227). There was also no effect of treatment on responding to the different ambiguous tones (RM-ANOVA: tone x treatment interaction: $F_{1.11,14}$ = 0.28, p = 0.638, Greenhouse-Geisser correction applied). The negative and near-negative tones were not generalised (t(31) = 2.67, p = 0.012). The speed at which a rat responded negatively to an ambiguous-cue tone was not influenced by the frequency of the tone (Linear Mixed Model: $F_{2,31}$ = 0.714, p = 0.498; Figure 3-21) and injection with LiCl did not affect these latencies (Linear Mixed Model: $F_{1,29}$ = 0.48, p = 0.493). There was also a lack of an interaction between the tone and treatment received (Linear Mixed Model: $F_{1,29}$ = 2.28, p = 0.142).

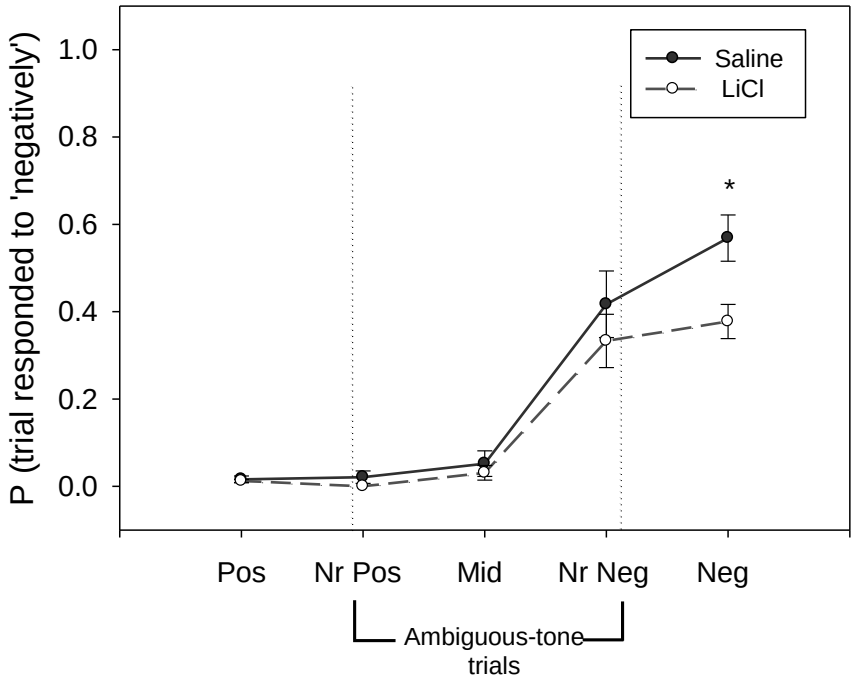


Figure 3-18 ***Proportion of 'negative' responses during the ambiguous probe test sessions*** *As Figure 3-17 but showing negative' responses (where a response was made on the lever predicting avoidance of mild foot-shock). (P values denoted as: * p < 0.05). Symbols show means +S.E.M. (n = 16).*

Response omissions A significant effect of tone frequency on the proportion of trials where a response was not performed (RM-ANOVA: $F_{2,14} = 20.72$, $p < 0.001$) showed that responses were more likely to be omitted during ambiguous trials with tones more closely resembling the negative tone (Nr Pos vs. Mid: t(7) = 3.74, $p = 0.007$; Nr Pos vs. Nr Neg: t(7) = 9.26, $p < 0.001$; Nr Neg vs. Mid: t(7) = 2.20, $p = 0.064$; Figure 3-19). LiCl injection had no effect on the number of ambiguous tone trials where an omission was recorded (RM-ANOVA: $F_{1,7} = 0.54$, $p = 0.485$), and there was also no interaction between the treatment that the rat received and the tone frequency on response omissions (RM-ANOVA: $F_{2,14} = 0.43$, $p = 0.661$).

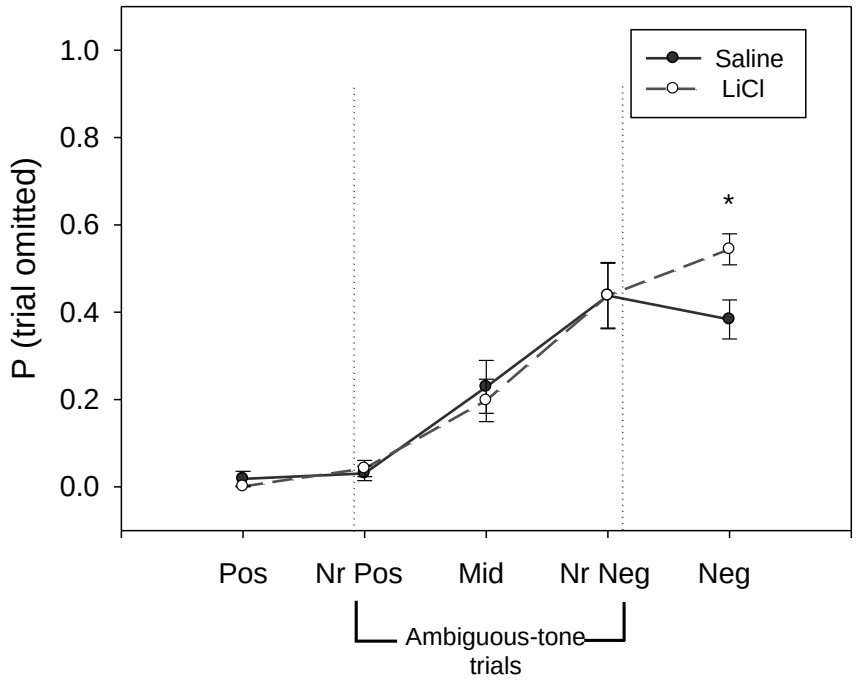


Figure 3-19 ***Proportion of response omissions during the ambiguous probe test sessions*** *As Figure 3-17 but showing response omissions (where no response was made within the 30 s tone presentation). (P values denoted as: * p < 0.05). Symbols show means +S.E.M. (n = 16).*

3.VI.iii.3 Did LiCl effect responding to the learned reference cues?

Figure 3-17 shows that differentiation of positive and negative trials was conserved in test sessions where rats made significantly more positive responses during positive trials than during negative trials (RM-ANOVA: $F_{1,7} = 5345.71$, $p < 0.001$). Rats also responded on the positive lever markedly faster during a positive trial than during a negative trial (RM-ANOVA: $F_{1,45} = 49.9$, $p < 0.001$; Figure 3-20). LiCl injection did not affect the proportion of these trials responded to with a positive lever press (RM-ANOVA: $F_{1,7} = 1.57$, $p = 0.251$) or the response times during reference tone trials (Linear Mixed Model: $F_{1,45} = 2.01$, $p = 0.164$). There was, however, a marginally significant interaction between tone and treatment (Linear Mixed Model: $F_{1,45} = 3.25$, $p = 0.078$), where rats that had received LiCl were slower to make a positive response on the negative trials.

Rats responded to more of the negative trials negatively than the positive reference tone trials during the test sessions (RM-ANOVA: $F_{1,7} = 397.71$, $p < 0.001$). LiCl injection caused an overall reduction in the proportion of these reference trials that were responded to negatively (RM-ANOVA: $F_{1,7} = 6.18$, $p = 0.042$). A significant interaction of trial type and treatment (RM-ANOVA: $F_{1,7} = 7.06$, $p = 0.033$) was investigated and showed a reduction of negative responding on the negative trials following LiCl injection ($t(7) = -2.59$, $p = 0.036$), but there was no difference in negative responding on positive trials ($t(7) = 0.75$, $p = 0.476$). Rats were significantly faster at responding negatively on the positive trials than the negative trials (Linear Mixed Model: $F_{1,31} = 53.2$, $p < 0.001$; Figure 3-21). There was a marginally significant effect of treatment on these latencies (Linear Mixed Model: $F_{1,35} = 3.71$, $p = 0.062$), with a significant probe and treatment interaction (Linear Mixed Model: $F_{1,35} = 4.16$, $p = 0.049$) where rats were quicker to make a negative response to positive-cues when injected with LiCl rather than saline.

The trial type influenced the proportion of reference tone trials where a response omission was made (RM-ANOVA: $F_{1,7} = 98.04$, $p < 0.001$), where more omissions occurred on negative than positive trials. There were also significantly more omissions following LiCl injection (RM-ANOVA: $F_{1,7} = 5.74$, $p = 0.048$), which mirrored the

reduction in negative responding seen during these trials. A RM ANOVA identified a significant treatment and tone interaction (RM-ANOVA: $F_{1,7} = 13.72$, $p = 0.008$), with *post hoc* tests indicating more negative trials omitted but not positive trials following LiCl injection (paired t-tests: negative trials: t(7) = 3.40, $p = 0.011$; positive trials: t(7) = 0.99, $p = 0.353$).

To further investigate the unforeseen reduction in 'negative' responding on the negative reference trials after injection with LiCl, a session effect was assessed (Figure 3-22). A significant effect of session was found following LiCl injection on the proportion of negative trials omitted ($F_{2,14} = 4.15$, $p = 0.038$), with decreased omissions between the first and subsequent sessions (second session: t(15) = 3.61, $p = 0.003$; (t(15) = 3.98, $p = 0.001$). There was no effect of session on positive ($F_{2,14} = 1.27$, $p = 0.312$) or negative ($F_{2,14} = 2.26$, $p = 0.141$) responding on negative trials.

A session effect was also observed after saline injection, where rats were less likely to make a positive response on negative trials during later sessions RM-ANOVA: ($F_{2,14} = 5.70$, $p = 0.015$). More specifically, fewer errors were made in the third session compared to the first (t(7) = -3.42, $p = 0.011$). There was no effect of session on negative (RM-ANOVA: $F_{2,14} = 0.56$, $p = 0.585$) or omitted responding of rats following a saline injection (RM-ANOVA: $F_{2,14} = 0.28$, $p = 0.760$).

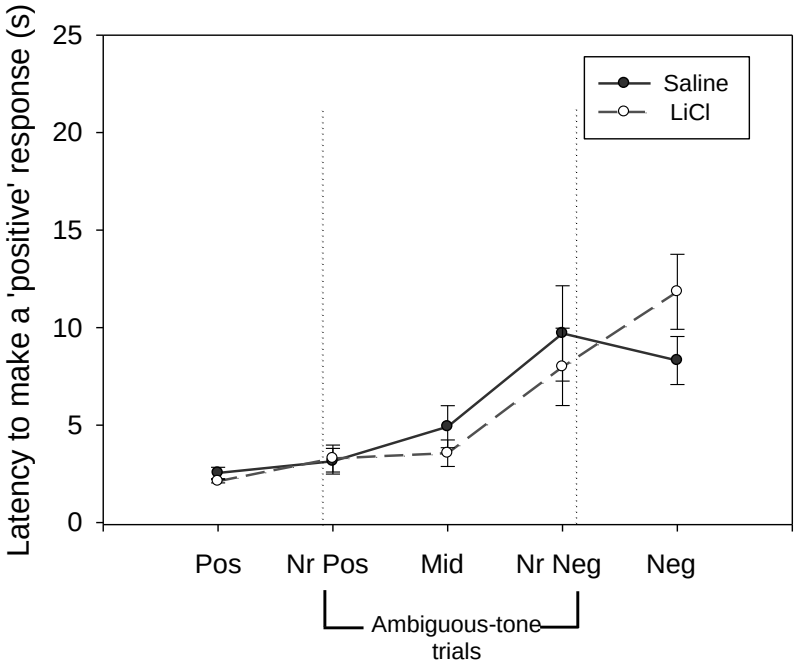


Figure 3-20 ***Mean latencies to respond 'positively' during the ambiguous probe test sessions*** *following administration of saline or 31.8mg/kg LiCl. Symbols show means +S.E.M. (n = 16).*

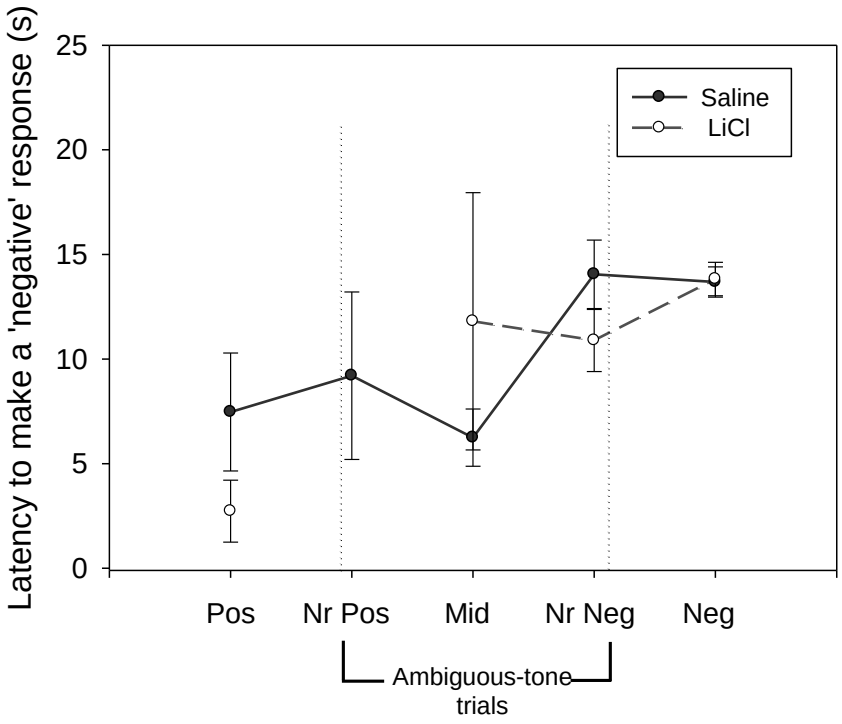


Figure 3-21 ***Mean latencies to respond 'negatively' during the ambiguous probe test sessions*** *following administration of saline or 31.8mg/kg LiCl. Symbols show means +S.E.M. (n = 16).*

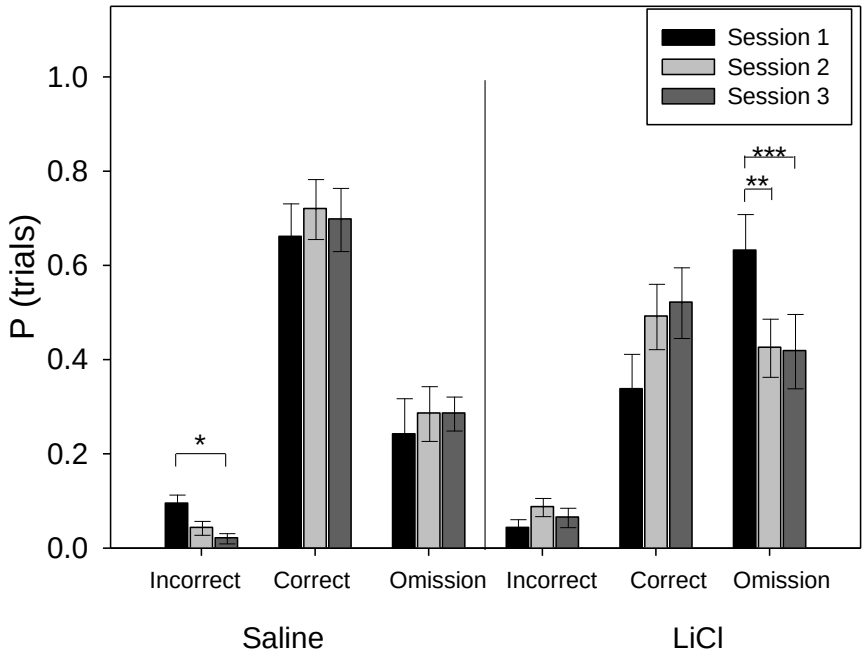


Figure 3-22 ***A session-by-session analysis of responses on negative trials within ambiguous testing sessions.*** *following administration of saline or 31.8mg/kg LiCl. (P values denoted as: * p < 0.05, **p < 0.01, ***p < 0.001). Bars show means +S.E.M.(n = 16).*

3.VI.iv Discussion

3.VI.iv.1 Accuracy and learning

During the training sessions the rats were able to differentially respond to each tone (Figure 3-15). Although the levels of responding were relatively lower on the negative trials, there were very few incorrect responses made to either of the reference tones (i.e. positive responses to the negative tone or negative responding to the positive tone), indicating the discrimination was learned.

3.VI.iv.2 Rats met the response accuracy criterion required by published studies

All of the subjects consistently responded correctly during the positive trials (Figure 3-13). Sixteen of the 24 rats trained on this task also achieved a stable rate of responding of ≥60% on the negative trials in the discrimination sessions, and eight continued to show responding above 70% during the retraining sessions which is congruent with criterion set out in previous studies (Enkel et al., 2009, Rygula et al., 2012).

3.VI.iv.3 Avoidance bar-press problem was improved by amending the methods

The insufficiency in training an association with a predictor of punishment is apparent in Figure 3-15. The rats underwent 30 sessions of negative training with relatively poor levels of responding before the methods were revised. These revisions produced an immediate increase in avoidance responses during these sessions. Most significantly, the shock was amended to be pulsed rather than continuous, and the CS duration was increased. Although this led to an improvement in avoidance responding, the potential increase may have been limited due to the extensive prior training which involved repeated exposure to foot-shock. The avoidance bar-press problem continued to be a factor, where a number of rats that had initially improved avoidance responding, showed decreasing responding over later sessions.

3.VI.iv.4 LiCl caused a positive biasing in interpretation of the ambiguous trials

The increase in positive responding to ambiguous-cue trials (Figure 3-17) indicates that rats injected with LiCl were more expectant of reward, suggesting an improvement of mood. This is in direct conflict with the hypothesis that rats experiencing acute symptoms of sickness experience a concurrent depression of their mood state.

A possible explanation for this result is that the predictions were incorrect, and LiCl instead elicits an acute anti-depressant action. It is, however, difficult to support this conclusion when referring to the literature. In a range of experimental paradigms, LiCl did not produce stable and predictable changes in mood, and opposing outcomes have been observed following different doses and treatment regimens. For example, when investigating the effects of acute LiCl on performance on the forced-swim test (FST) Tomasiewicz et al. (2006) found that 30mg/kg LiCl (a similar dose to that used in the current main study) did not produce depressant- or antidepressant-like effects. They also stated that a single administration of 100mg/kg LiCl decreased mobility time on the FST and subsequently concluded that this dose of LiCl had depressant-like effects. A number of other authors have reported no effects of LiCl on rats in the FST (Hata et al., 1995, Overstreet et al., 1995, Kitamura et al., 2002, Wegener et al., 2003). However, when administered acutely to mice, LiCl administered i.p. at doses of 30mg/kg and 100mg/kg reduced immobility in the FST, which is indicative of antidepressant action (Ghasemi et al., 2008). In contrast, Nixon et al. (1994) who used doses ranging from 21.2mg/kg to 339.2mg/kg in the mouse forced swim test concluded that none had any effect, either anti- or pro- depressant.

When rats are exposed to other paradigms designed to measure emotional response, LiCl produced similarly counter-indicative outcomes. Rats injected with 17.0mg/kg LiCl twice daily showed no antidepressant effect on the learned helplessness paradigm after 4 days or 25 days treatment (Geoffroy et al., 1991), yet a 25mg/kg dose of LiCl reduced fear in an acoustic startle response (ASR) task where a reduction in fear is associated with positive affect (Rana and Parker, 2007). Tomasiewicz et al. (2006) recorded a concurrent increase in reward thresholds in an intracranial self-stimulation (ICSS) task with a 100mg/kg dose in rats, an indicator of a depressed mood, which was

correlated with the previously mentioned increase in immobility in the FST seen in their study. The underlying toxicity of both acute and chronic dosing has made it difficult to interpret therapeutic effects of LiCl in animal models, which may account for the disparity in experimental outcomes.

It is worth considering that the treatment outcomes could be reliant on the original mood state of the animal. The rats in this study may have exhibited an underlying negative mood state as was also seen in Anderson et al. (2013). Repeated shocking is used to condition stress and anxiety in the rat (e.g. Pietersen et al. (2006)), so inevitably the training protocol may have induced an underlying negative affective state. The influence this may have on the directional effect of LiCl on the rats' mood needs to be further examined.

3.VI.iv.4.1 This study used sub-therapeutic doses of LiCl

Clinically, LiCl is used to treat mania in patients with bipolar disorder, where elevated moods are stabilised by lowering them to normal levels. It is not, however, counterintuitive that an increase in the affective states of the rats was observed, as LiCl also has major acute antidepressant effects in humans. Antidepressant effects can be achieved in humans even with short term (3 week) dosing, but antidepressant effects are not seen until after the 1st week (Worrall et al., 1979). It is interesting to note that antidepressant-like effects seen in the study in this chapter occurred at a sub-therapeutic dose as compared to dosing in humans. O'Donnell and Gould (2007) recommended doses of between 63.6 – 127.2mg/kg given i.p. and 1 h before testing to achieve serum levels of lithium that are comparable to therapeutic levels in humans (21.2 – 50.9mg). I administered 31.8mg/kg 15 min before testing, so it is likely that serum levels did not reach this therapeutic level. However, as discussed earlier, use of higher dose rates would have influenced task performance and so were avoided.

3.VI.iv.4.2 Pharmacological agents can produce concurrent CTAs and positive affect

The results appear to indicate that lithium chloride produces both positive and negative shifts in affect. The positive change was identified with the judgement bias task in response to ambiguity and translates as an antidepressant response, and the negative was observed as an induction of a CTA. It is possible that LiCl exerted both sickness-inducing effects that provoked aversion and an increase in positive affect at the same time, as has been found with other drugs which have aversion-inducing properties. Within the same behavioural paradigms, some drugs can produce behavioural changes associated with both reward and aversion simultaneously (e.g. Verendeev and Riley (2011), Wise et al. (1976)). It is also relevant to note that many drugs of abuse with highly rewarding properties are also capable of producing CTAs. These include nicotine, morphine, cocaine, heroin, amphetamine, caffeine, alcohol and THC (see Verendeev and Riley (2012) for review). The authors comprehensively reviewed the paradox of rewarding and aversive properties of drugs of abuse, although they vehemently oppose LiCl being included in this class of drugs, describing it as a "classical toxin" and made a clear distinction that this drug does not possess the same rewarding outcomes. This does not, however, exclude the possibility that LiCl is also capable of concurrently mediating positive affect and aversion, just not via classical reward pathways.

The pathway by which LiCl affects mood (via modulation of inositol and serotonin) is different to that by which LiCl produces a sickness (via the inflammatory pathway), and within this chapter we found evidence suggesting that both were activated. An explanation of the increase in positive rather than negative affect in the judgement bias task is that the mood-enhancing effects of LiCl trumps the negative effects produced by sickness. It was therefore predicted that administering an anti-emetic with the LiCl would attenuate the sickness-effects, strengthening the positive bias.

3.VI.iv.4.3 The judgement bias task does not always support a priori predictions of mood

The increase in positive responding to ambiguous-cue trials (Figure 3-17) indicates that rats injected with LiCl were more expectant of reward, suggesting an improvement of mood. This is in direct conflict with the hypothesis that rats experiencing acute symptoms of sickness would experience a concurrent depression of their mood state, and so interpretations should be made with caution. The results mirror outcomes of other studies where stressors have produced positive biases, conflicting with *a priori* predictions (Doyle et al., 2010a, Briefer and McElligott, 2013, Burman et al., 2011). This phenomenon tends to be encountered following acute stressors, where a 'relief' is experienced following the stressors removal (Spruijt et al., 2001). For example, Doyle et al. (2010a) found that sheep that had undergone a period of stressful restraint, and had elevated levels of serum corticosterone (a common marker for stress) as a result, actually displayed a positive bias in a judgement bias task. They explained that this exposure to a stressful event decreased their risk-taking threshold, which was subsequently mapped onto their performance on the task where more risky decisions were made in response to ambiguity. Risk-taking can be defined in terms of impulsivity, where less risk-averse subjects take more impulsive actions. Acutely administered LiCl reduces impulsive behaviours in the rat (Ohmura et al., 2012), as does chronically administered LiCl in the mouse (Halcomb et al., 2013), which would suggest an increase, rather than a decrease, in their risk-taking threshold, so we cannot draw the same conclusions from the outcomes of this study.

3.VI.iv.5 Rats increase response omissions during negative reference trials.

When rats were cued to respond on negative trials that predicted shock, an active avoidance response was made ~70% of the time. This was reduced to ~45% following an injection with LiCl (Figure 3-18). This was not due to errors in responding, nor a more impulsive responding pattern whereby animals responded in a manner to obtain more food, but instead coincided with an absence of responding. This pattern of responding was not repeated by the rats during the retraining sessions that occurred

before subsequent testing periods, where response levels returned to pre-injection levels (see Figure 3-16). The abstinence in responding was also not generalised to the near-negative ambiguous-cue trials, but limited to responding just on negative trials. This behaviour would appear to be maladaptive and is difficult to interpret. It could be postulated that LiCl is capable of making foot-shocks less aversive, or that LiCl reduced the amount of attention paid to shock-predicting cues. There was no change in responding during the negative trials when rats were administered saline, indicating that this phenomenon was specific to LiCl treated rats.

3.VI.iv.5.1 Pain sensitivity and reactivity

An increase in the pain threshold would render the foot-shocks less aversive, and could be responsible for the reduced responding observed in the judgement bias study. LiCl administration has been reported to alter the perception of pain in this way. For example, chronic LiCl administration prolongs shock-induced hypoalgesia after inescapable shock (Teixeira et al., 1995), thus reducing the experience of pain. However, In Teixera's study, adult female Wistars were fed LiCl in tap water (20mM) for 28 days, equivalent to a serum lithium concentration of 0.5mEq/L. The acute administration of 31.8mg/kg LiCl used in this study is unlikely to reach this concentration in the blood (O'Donnell and Gould, 2007). On the contrary, acute LiCl has been shown to possess hyperalgesic properties. However, this has not been reported at the dose used in this study (31.8mg/kg), but instead at twice this dose(McNally and Westbrook, 1998). If shocks were more painful after LiCl injection, we might expect more freezing behaviour and thus a reduced number of lever presses made by the rats to shock-predicting cues, which would be a more fitting explanation. Over the three testing sessions there was an increase in correct responding and a significant decrease in omissions during negative trials (Figure 3-22), which suggests a gradual decrease in pain sensitivity over time where the repeated acute doses may have resembled a sub-chronic dosing regimen. The changes in pain sensitivity following LiCl administration are varied and depend on the dose, administration route,

length of administration period, and the method of testing (Hines and Poling, 1983), and so this theory demands further investigation.

3.VI.iv.5.2 Cue salience and attention

Lithium narrows the breadth of attention onto stimuli of high salience at the expense of processing of stimuli of low salience (Cappeliez and Moore, 1988). The disparity in responding on the positive and negative trials (Figure 3-15) suggests that the positive cue was more salient. An attentional shift towards stimuli of high salience would therefore increase rats responding to the positive cue, and reduce that to the negative cue. As the rats consistently responded correctly >95% during the positive trials, there was a ceiling to which responding could be increased, but we did observe a reduction in responding during the negative trials, accordingly.

3.VII Judgement bias experiment 2 - Pharmacological reversal of nausea-induced bias using antiemetics

3.VII.i Rationale

In the first study (Experiment 1) in this section I aimed to identify whether a judgement bias was produced by injection of the emetic drug LiCl. The data from this study indicated a positive biasing of rats' interpretation of ambiguous-cues. The study was repeated (Experiment 2) to identify firstly whether the results could be replicated, and secondly to determine whether a reduction in nausea would reverse the observed effects associated with LiCl. The second experiment involved consecutive administration of an anti-emetic drug, ondansetron, with injection of LiCl, with the view that any psychological effects would be attenuated or reversed.

3.VII.i.1 Anti-emesis and ondansetron

Vomiting is regulated centrally in two separate neural units in the medulla – the vomiting centre (Borison and Wang, 1949) and the chemoreceptor trigger zone (CTZ; Borison and Brizzee (1951)). Toxins are thought to act on the CTZ to induce emesis and nausea (Bernstein et al., 1992, Kosten and Contreras, 1989, Rabin et al., 1983). Ondansetron is a specific antagonist of 5-HT_3 receptors, and produces antiemetic actions via these receptors which have visceral afferents projecting to the CTZ (Borison et al., 1981). Ondansetron is traditionally used to reduce nausea and vomiting associated with the treatment of cancers with chemotherapy, and has helped to revolutionise this area of therapy where these adverse effects can serve to reduce patient compliance (Laszlo, 1983).

The dose of ondansetron used was selected from a review of the literature (see Table 3-7). There does not appear to be much agreement as to what amount of ondansetron should be administered to animals in order to counteract the effects of LiCl, where doses in the range of 0.1 – 0.5 mg/kg ondansetron have been used to attenuate effects of the highest dose of LiCl (127.2 mg/kg). A dose of 0.1mg/kg ondansetron was selected as Balleine et al (1995) demonstrated that this was sufficient to attenuate LiCl-induced rejection of a sucrose solution at a dose similar to that used in my project.

Dose LiCl	Dose Ondansetron	Test outcomes	Reference
63.6mg/kg	0.2mg/kg	Conditioned place avoidance in the rat blocked by ondansetron	Rinaman et al., 2009
31.8mg/kg	0.1mg/kg	Animals drank more of a solution when administered ondansetron with LiCl than when LiCl was paired with a vehicle injection	Balleine etal., 2005
127.2mg/kg	0.5mg/kg	Ondansetron significantly reduced LiCl-induced LOB and also conditioned gaping reactions	Tuerke et al., 2012
127.2mg/kg	0.1mg/kg	Ondansetron reversed LiCls effect in blunting an acoustic startle response	Rana and Parker, 2007
127.2mg/kg	0.5mg/kg	Ondansetron counteracted the effects of LiCl on changes in breathing rate suggested to be a novel index of nausea in the rat	Ngampramuan et al., 2013

Table 3-7 ***A summary of studies investigating the antiemetic effects of ondansetron on LiCl-induced nausea in the rat.***

3.VII.ii Methods

3.VII.ii.1 Animals

Housing conditions were as 3.II.ii.1. Rats were ~500g at the beginning of testing. Due to limitations of equipment availability, only the 8 rats that showed the most consistent responding above criteria (≥70 % correct responses on both positive and negative trials) during the baseline discrimination sessions were included in the analyses. The remaining rats were excluded from experiment 2.

3.VII.ii.2 Drug treatments

Rats were administered either an antiemetic, 0.1mg/kg ondansetron, or saline (0.9% sodium chloride) s.c. at a volume of 1ml/kg in the holding room and replaced in their homecage. 30 min later the rats received the emetic drug LiCl 31.8mg/kg or saline i.p. at a volume of 10ml/kg as in Experiment 1.

3.VII.ii.3 Experimental procedure

Experiment 2 began 10 days after the end of experiment 1. All rats were exposed to all different treatment combinations (Table 3-8), although due to the limited number of rats we were unable to include all of the possible combinations (i.e. for four treatments there are 4 x 3 x 2 x 1 = 24 orders). The rats then underwent 3 days of baseline discrimination retraining immediately before 3 days of ambiguous-cue testing. This was followed by a 1 day wash-out period, and the schedule was repeated over 4 weeks so all treatments would be received.

3.VII.ii.4 Statistical analysis

The analyses were as 3.VI.ii.6.

Treatment orders		Treatment key	
B D C A	**A C D B**	**A:**	**Sal_Sal**
B A D C	**C D A B**	**B:**	**Ond_Sal**
C B A D	**D A B C**	**C:**	**Sal_LiCl**
A C B D	**D B C A**	**D:**	**Ond_LiCl**

Table 3-8 ***Treatment orders for the 8 rats included in experiment 2*** *Treatments were:(saline and saline (Sal_Sal); ondansetron and saline (Ond_Sal); saline and LiCl (Sal_LiCl); and ondansetron and LiCl (Ond_LiCl)). The orders in which the rats received the four treatments were counterbalanced*

3.VII.iii Results

3.VII.iii.1 Was there a biasing of ambiguous-cue interpretation by ondansetron or LiCl treatment?

Positive responding As observed in the previous experiment (Figure 3-17), rats performed fewer positive responses as tone frequencies departed farther from the frequency of the positive tone (RM-ANOVA: $F_{2,14} = 44.99$, $p < 0.001$; Figure 3-23). More rats responded positively during trials with the near-positive tone (NrPos) than in the midpoint and near-negative trials (NrPos vs. Mid: t(7) = 5.37, $p < 0.001$; NrPos vs. NrNeg: t(7) = 24.83, $p < 0.001$), and also during the midpoint trials compared to near-negative trials (t(7) = 7.22, $p < 0.001$). There was no effect of either emetic (RM-ANOVA: $F_{1,7} = 0.13$, p = 0.725) or anti-emetic (RM-ANOVA: $F_{1,7} = 0.78$, $p = 0.407$) on positive responding in the ambiguous-tone trials, nor was there an effect of any of the combinations of treatments (RM-ANOVA: $F_{1,7} = 0.12$, $p = 0.735$). The latencies to make a positive response were similarly unaffected by either emetic (Linear Mixed Model: $F_{1,63} = 2.67$, $p = 0.108$) or anti-emetic treatment (Linear Mixed Model: $F_{1,64} = 0.85$, $p = 0.771$), but were different according to the ambiguous-cue trial frequencies (Linear Mixed Model: $F_{2,65} = 12.8$, $p < 0.001$). Rats were slower to respond to the near-negative tone than the near-positive and midpoint tones (NrNeg vs. NrPos: t(64) = 2.0, $p = 0.036$; NrNeg vs. Mid: t(63) = 2.1, $p = 0.036$).

Negative responding Rats showed increased negative responding during ambiguous trials as they more closely resembled the negative reference tone (RM-ANOVA: $F_{2,14}$ = 19.04, p >0.001), with more rats responding negatively to the near-negative tone (Nr Neg) than the midpoint (Mid: t(7) = 7.16, p < 0.001) and near-positive (Nr Pos: t(7) = 8.24, p < 0.001) tones. Response levels were also greater on the midpoint trials compared to the near-positive trials (t(7) = 2.73, p = 0.010; Figure 3-24). This overall increase in responding was not affected by emetic (RM-ANOVA: $F_{1,7}$ = 0.29, p = 0.608) or antiemetic drugs (RM-ANOVA: $F_{1,7}$ = 1.40, p = 0.276). However, a significant tone x emetic x antiemetic interaction was identified (RM-ANOVA: $F_{2,14}$ = 4.78, p = 0.026), with t-tests showing reduced responding on the midpoint ambiguous-cue trial (Mid) by rats administered LiCl compared to those administered saline after an initial ondansetron injection (Ond_Sal vs. Ond_LiCl: t(7) = 2.77, p = 0.028, Figure 3-25). There was also less responding on the near-negative trials (Nr Neg) by rats after two injections of saline as compared to an injection of ondansetron followed by saline (Sal_Sal vs. Ond_Sal: t(7) = 3.87, p = 0.006, Figure 3-25b). A marginally significant increase in responding on the midpoint trials was obtained when rats were administered saline and LiCl when compared to the control (Sal_LiCl vs. Sal_Sal; t(7) = 2.20, p = 0.064), and also when given an initial injection of ondansetron when compared to the control (Ond_Sal vs. Sal_Sal; t(7) = 2.28, p = 0.057). Latencies to make negative responses to ambiguous-cue trials were affected by the ambiguous-cue type (Linear Mixed Model: $F_{2,42}$ = 5.77, p = 0.006), specifically that rats were quicker to respond to the near-negative probe tone than the midpoint tone (p =0.005; Figure 3-28).

Omissions Omitted responses increased as the frequency of the ambiguous tones neared that of the negative tone (RM-ANOVA: $F_{2,14}$ = 7.08, p = 0.008), with significantly more omissions made during trials with the midpoint (t(7) = 4.72, p < 0.001) and near-negative (t(7) = 5.71, p < 0.001) tones than during the near-positive trials. There was no difference in the number of omissions during near-negative and midpoint trials (t(7) = 1.91, p = 0.066). Whether an emetic treatment or an anti-emetic treatment was administered did not alter the number of response omissions that rats made during

testing sessions (RM-ANOVA: Emetic: $F_{1,7} = 0.31$, $p = 0.598$; Anti-emetic: $F_{1,7} = 0.04$, $p = 0.851$; Figure 3-26).

3.VII.iii.2 Did any treatment affect the rats' responses to learned cues?

Positive responding Rats made significantly more (RM-ANOVA: $F_{1,7} = 9070.18$, $p < 0.001$) and quicker (Linear Mixed Model: $F_{1,49} = 26.4$, $p < 0.001$) positive responses on the positive trials, as compared to positive responding on the negative trials (as can be seen in Figure 3-23 and Figure 3-27 respectively). Rats administered LiCl displayed no difference in the number of positive responses made (RM-ANOVA: $F_{1,7} = 1.32$, $p = 0.289$) or in the latency to respond (Linear Mixed Model: $F_{1,50} = 0.29$, $p = 0.592$) than rats administered saline in the second injection, as was also the case when rats were administered ondansetron in the first (latency: Linear Mixed Model: $F_{1,48} = 0.16$, $p = 0.690$, responses: RM-ANOVA: $F_{1,7} = 0.33$, $p = 0.587$).

Negative responding These data show that rats performed more negative responses on negative trials (RM-ANOVA: $F_{1,7} = 444$, $p < 0.001$; Figure 3-24), but conversely they were quicker to make negative responses to the positive trials ($F1_{,106} = 106$, $p < 0.001$; Figure 3-28). There were no differences in response latencies after treatment with emetic (Linear Mixed Model: $F1_{,32} = 0.70$, $p = 0.409$) or antiemetic (Linear Mixed Model: $F_{1,32} = 0.18$, $p = 0.677$), and similarly no difference in the number of responses (RM-ANOVA: emetic: $F_{1,7} = 0.35$, $p = 0.572$; antiemetic: $F_{1,7} = 0.13$, $p = 0.730$).

Omissions Rats made fewer response omissions in the positive trials compared to the negative (RM-ANOVA: $F_{1,7} = 140.13$, $p < 0.001$), but this was unaffected by the treatments administered (RM-ANOVA: emetic: $F_{1,7} = 0.56$, $p = 0.480$; antiemetic: $F_{1,7} = 0.27$, $p = 0.875$; Figure 3-26).

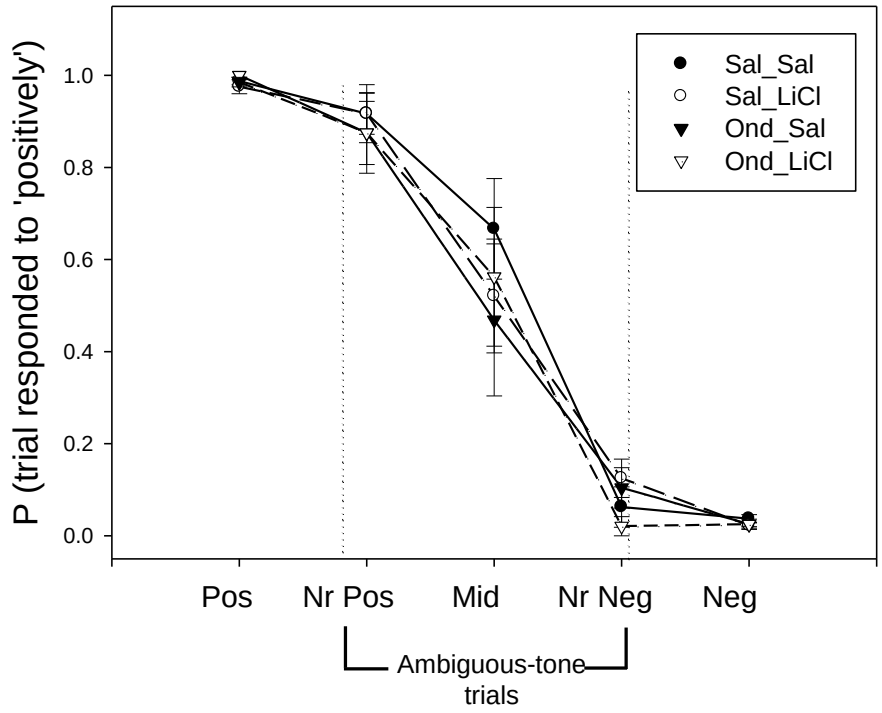


Figure 3-23 ***Proportion of 'positive' responses during the ambiguous probe test sessions*** *(responses made on the lever predicting a food reward) to the trained audio cues (reference cues: Pos and Neg) and three intermediate audio cues (ambiguous-cues: NrPos, Mid, and NrNeg) when administered the emetic LiCl, the antiemetic ondansetron (Ond) or saline vehicle (Sal. Symbols show means +S.E.M. (n = 8).*

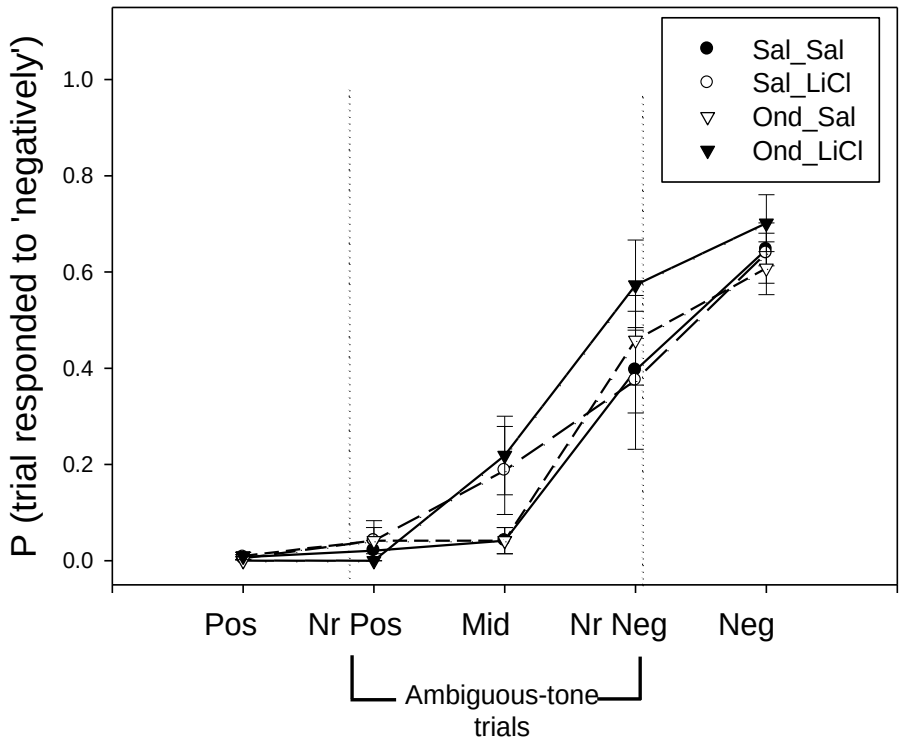


Figure 3-24 ***Proportion of 'negative' responses during the ambiguous probe test sessions*** *As Figure 3-22 but showing negative responses (where a response was made on the lever predicting avoidance of mild foot-shock). Symbols show means +S.E.M. (n = 8.)*

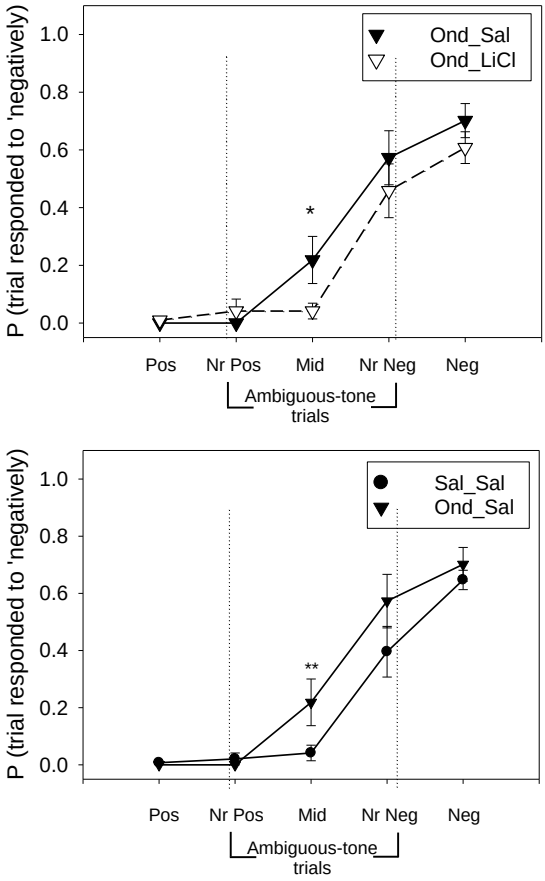


Figure 3-25 ***Significant differences in 'negative' responding during the ambiguous probe test sessions*** *As Figure 3-24 showing negative' responses (where a response was made on the lever predicting avoidance of mild foot-shock, but graphs have been reproduced in a) and b) to highlight significant differences.) Symbols show means +S.E.M. (P values denoted as: * p < 0.05, **p < 0.01).*

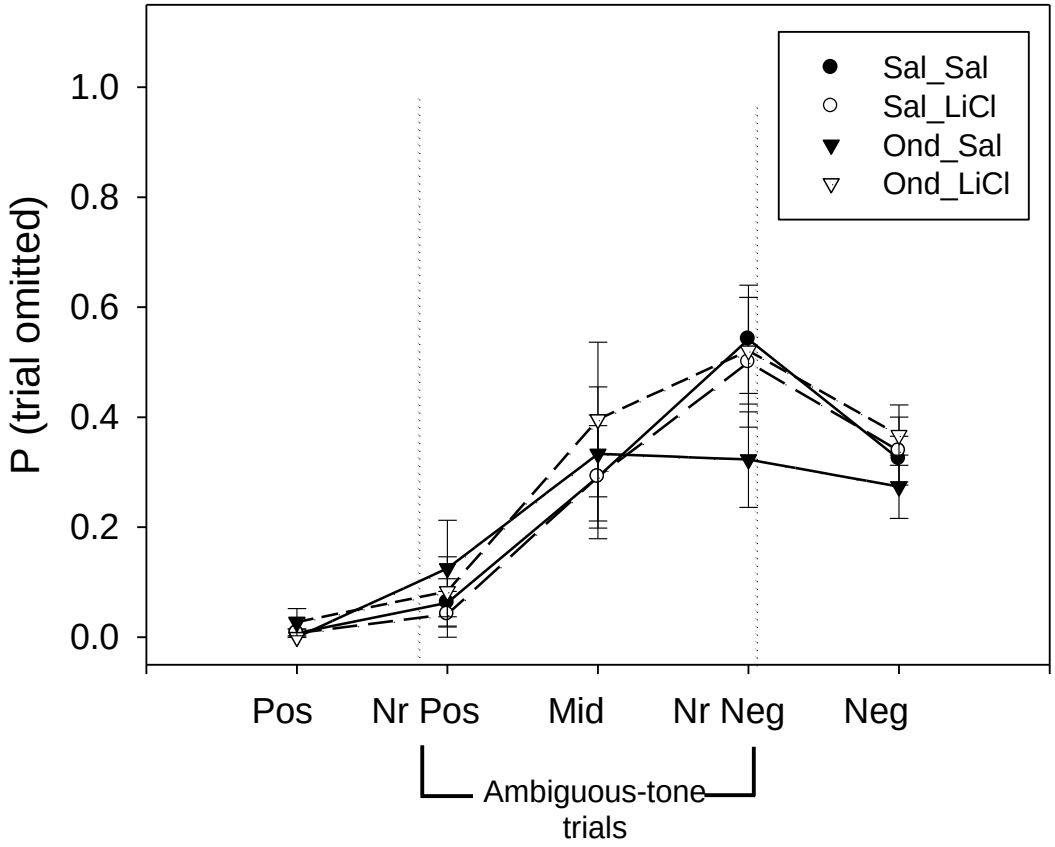


Figure 3-26 ***Proportion of response omissions during the ambiguous probe test sessions*** *As Figure 3-22 but showing the proportion of trials where a response was not made during the 30 s tone presentation. Symbols show means + S.E.M. (n = 8).*

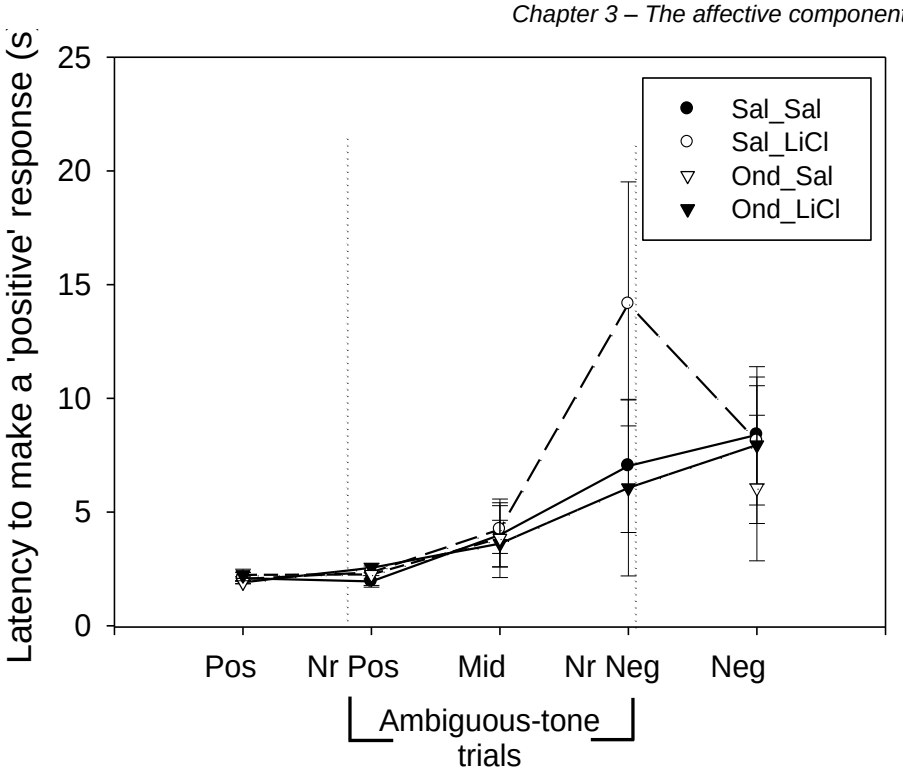


Figure 3-27 ***Mean latencies to respond 'positively' during the ambiguous probe test sessions*** *shows the rats' mean latencies to respond on the lever predicting food reward (positive lever) during the ambiguous probe testing sessions when administered the emetic LiCl, the antiemetic ondansetron (Ond) or saline vehicle (Sal) Symbols show means +S.E.M. (n = 8).*

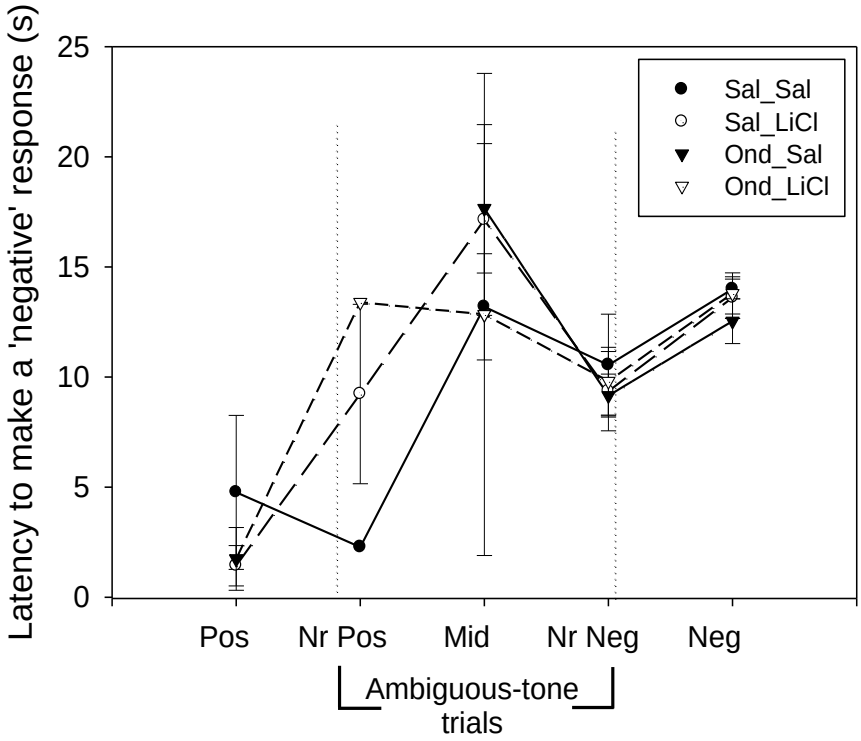


Figure 3-28 ***Mean latencies to respond 'negatively' during the ambiguous probe test sessions*** *shows the rats' latencies to respond on the lever predicting avoidance of a mild foot-shock (negative lever) during the ambiguous probe testing sessions when administered the emetic LiCl, the antiemetic ondansetron (Ond) or saline vehicle (Sal) . Symbols show means +S.E.M. (n = 8).*

3.VII.iii.3 Was escape behaviour altered by drug treatment?

Escape responses and latencies Rats were no more or less likely to escape a shock following its onset with either emetic (RM-ANOVA: $F_{1,7} = 0.58$, $p = 0.470$) or antiemetic (RM-ANOVA: $F_{1,7} = 0.87$, $p = 0.382$) , nor any combination of the drugs (RM-ANOVA: emetic x antiemetic: $F_{1,7} = 0.23$, $p = 0.648$). There was also no difference in the amount of time that rats took to escape a shock (Linear Mixed Model: emetic: $F_{1,7} = 0.62$, $p = 0.459$; antiemetic: $F_{1,7} = 0.12$, $p = 0.738$; emetic x antiemetic: $F_{1,7} = 1.96$, $p = 0.204$).

Antiemetic	Emetic	Proportion of escape responses (n^o escape/n^o shock; % ± s.d.)	Latency to perform escape response (seconds ± s.d.)
Saline	Saline	83.5 ± 10.1	3.94 ± 1.21
	LiCl	82.3 ± 9.8	4.29 ± 1.15
Ondansetron	Saline	79.3 ± 17.3	4.66 ± 2.03
	LiCl	74.5 ± 22.9	3.91 ± 1.22

Table 3-9 ***Proportion of shocks escaped and latencies to perform an escape response.***

3.VII.iv Discussion

3.VII.iv.1 Ondansetron-treated rats display anxiety-like responding on the judgement bias task

Ondansetron appears to mediate anxiety-like behaviour in the rat by increasing the likelihood of responding negatively to ambiguous-cues. This is surprising because, as a potent and highly selective serotonin 5-HT3-receptor antagonist, ondansetron has been reported to have mild anxiolytic effects in rats (Filip et al., 1992, Shenoy et al.), mice (Roychoudhury and Kulkarni, 1997) and humans (Freeman et al., 1997), or no effect on anxiety behaviours at all (Jones et al., 1988, Dunn et al., 1991). There are no publications showing ondansetron to have any anxiogenic properties, although Rana and Parker (2007) showed LiCl to have positive affective properties in blunting an acoustic startle response, which was reversed by ondansetron. Blunting of these responses is related to positive affect, so a reversal could be interpreted as a negative change.

3.VII.iv.2 Ondansetron attenuates the effects of LiCl

There were no differences in positive (Figure 3-23) or negative responding (Figure 3-24), nor response omissions (Figure 3-26) when the rats were injected with a combination of ondansetron and LiCl (Ond-LiCl) or when given two injections of saline (Sal-Sal). This is consistent with findings that pre-treatment with ondansetron attenuates nausea and related behaviours in the literature so that they resemble control animals. As little as 0.5mg/kg ondansetron significantly reduced 127.2mg/kg (i.p.) LiCl-induced LOB and also conditioned gaping reactions, without modifying CTAs (Tuerke et al., 2012), and 0.1mg/kg ondansetron will reverse LiCls effect in blunting an acoustic startle response (Rana and Parker, 2007).

3.VIII General Discussion

At a dosage where sickness behaviours were produced, LiCl induced an increase in positive affect in rats tested in a judgement bias task which was in conflict with *a priori* predictions. This effect was lost on repetition of the judgement bias task. Neither of these outcomes were consistent with a subjective component of sickness, which would manifest as a negative affective state. It was not possible to determine whether pre-treatment with ondansetron reversed nausea in the rat in the judgement bias task as affective signs of nausea were not established overall. However, combining administration of the antiemetic ondansetron and LiCl (Ond_LiCl) left the responding of rats almost indistinguishable to controls (Sal_Sal), suggesting that their additive effects cancelled out.

Substantial variations in the outcomes of the first and second judgement bias experiments in this chapter occurred, with significant results not being confirmed on repetition. The positive biasing of ambiguous information in the first experiment after LiCl injection was not repeated by animals given saline and LiCl in the second experiment. In the first experiment rats injected with LiCl responded more under ambiguity reflecting increased positive affect, whereas in the in the second experiment a marginally significant increase in negative responding during ambiguous trials was observed. It is necessary to note that the power of the second experiment was potentially reduced as the sample size was halved, but the opposing directions of these outcomes do not support an argument that the divergence was due to reduced power, so we must look for explanations elsewhere. The previous treatment and experimental history of the rats, in addition to the increase in age and weight at the start of experiment 2, may have been the contributing factor to the outcomes, although the repeated use of animals in subsequent judgement bias studies has not been reported to impact other experiments (Anderson et al., 2013). The previous drug exposure was also balanced in experiment 2, where half of the rats had received one order of treatments (e.g. saline then LiCl) in experiment 1, and the other half had received the reverse order. That the rats had previously experienced the LiCl treatment before experiment 2 may have prevented the replication of the experimental outcomes if a

positive bias was only produced by an initial administration. I would recommend the use of experimentally-naïve animals to establish the root of this difference.

The phenomenon of increased response omissions by rats in the first experiment following administration of LiCl was also lost in the repetition of the task. Response omissions were rife in earlier stages of the judgement bias training process where the experimental parameters were sub-optimal. It could be argued that rats were continuing to learn to avoid shocks over the course of the first experiment, as a session-by-session analysis of the first study (Figure 3-22) showed that response omissions were reduced on progression of the experiment. However, this was observed only after LiCl but not saline treatment, and as these occurred simultaneously, so it is difficult to argue that learning improved as experimental experience increased. As before, prior drug experience was balanced between groups so it is unlikely that treatment order affected response omissions in the latter experiment.

Finally, the modification of the judgement bias task training schedule had a striking impact on reducing omissions and increasing correct responses in the negative training schedule. Particularly, amending the parameters of the shock and increasing the length of the CS served to improve performance. I would recommend that these revisions be adopted in future training of similar judgement bias tasks to firstly shorten the training requirements, and secondly to reduce the exposure of experimental animals to potentially painful foot-shock.

Chapter 4 - Sickness behaviours in the honeybee

Summary: In this chapter I used the honeybee to test whether toxin-induced changes in behaviour were general features of sickness. The toxins used were LiCl (for comparison with the previous study in this thesis involving rats; Chapter 3), and also two toxins, amygdalin and quinine, that have been previously shown to produce learned aversions in bees. The experiments in this chapter were undertaken to identify the doses of each toxin that produce behavioural changes related to a sickness-induced malaise, so that these could be investigated in a biasing of judgement in the next chapter.

Typically, honeybees encounter toxins through ingestion of nectar and pollen, but for the purpose of our investigation of judgement biases, a more standardised method of toxin injection was used, and so both methods of administration were explored and compared in this chapter.

4.1 Introduction

Eating exposes animals to the risk of ingesting toxic compounds. To avoid poisoning and death when toxins are ingested, the body responds with a suite of physiological detoxification mechanisms such as P450 enzymes and glutathione transferases used to break down toxic molecules for excretion (Jakobi and Ziegler, 1990, Ioannides, 2013). In mammals, infection with pathogens and intoxication are often accompanied by vomiting, nausea and lethargy, and a series of characteristic changes in behaviour that are likely to represent adaptations that improve survival (Hart, 1988). For example, when an animal's immune system is challenged by infection, reducing activity may conserve the resources needed to fight off pathogens (Hart, 1988, Ayres and Schneider, 2009) and reduce exposure to risks such as predation. Sick animals typically spend less time moving, feeding, and grooming and also spend more time huddling and sleeping (Hart, 1988, Millman, 2007). When humans experience toxicosis, they often report generalised discomfort as well as nausea that is often described as 'malaise.' Here, we define sickness-behaviours as the behavioural display of toxicosis,

and nausea as the subjective component as reported by humans.

Invertebrate animals, like the honeybee (*Apis mellifera*) and the fruit fly (*Drosophila melanogaster*), are important model organisms for studying the neural basis of behaviour. Unlike the sub-lethal effects of pesticides that have been well-documented in recent years (Aliouane et al., 2009), we know relatively little about the way that such insect models express toxin-induced sickness. Like other animals, honeybees naturally encounter toxins in their food (nectar and pollen) that could potentially kill them (Holzinger et al., 1992, Alder et al., 2001, London-Shafir et al., 2003, Adler, 2000). Honeybees can learn to avoid odours paired with both the pre-ingestive and the post-ingestive consequences of encountering toxins in food rewards (Wright et al., 2010), implying that ingesting toxins causes them to experience physiological sickness.

Whether or not signalling by the gut is required to produce these behavioural symptoms in animals has been debated since early studies of Garcia on conditioned food aversions in rats (Garcia et al., 1974). Cytokines and/or other peptide signals are produced by gut cells in response to bacterial toxins (Stadnyk, 1994, Guerrant et al., 1999), and their production has been clearly linked to sickness behaviour in animals (Larson and Dunn, 2005, Kelley et al., 2003, Felger and Miller, 2012). While the gut is likely to be directly involved in elicitation of sickness because of peptide signals it produces, other physiological pathways may also signal toxicosis and hence produce sickness in animals. For example, direct injection of LiCl into the blood is the canonical means of producing conditioned food aversions in rats (Hart, 1988, Millman, 2007). Whether or not injection of toxins can also activate pathways involved in signalling toxins and producing sickness behaviours is unknown. To this end, the sickness behaviours displayed following toxin injection and ingestion were compared.

To characterise sickness behaviours in an invertebrate model, and to identify whether toxins had to be ingested to produce a change in behaviour, we used an assay to assess the influence of pharmacological agents on basic motor function in adult worker honeybees (Maze et al., 2006). We also tested whether toxins with different physiological targets produce common behavioural symptoms that would suggest bees experience sickness via a common physiological pathway. We used three different

toxins to test this: amygdalin, a cyanogenic glycoside that binds to cytochrome C disrupting ATP production in mitochondria (Conn, 1969); lithium chloride (LiCl), a salt commonly used in conditioned aversion learning in mammals that affects signal transduction in cells but has otherwise unknown pharmacological targets (Phiel and Klein, 2001); and quinine, an alkaloid with many pharmacological targets that include blockade of sodium channels in nerve and muscle cells (Taylor and White, 2004).

4.I.i Conditioned aversions and sickness

While studies have shown that invertebrates can learn to avoid toxins when associated with foods (Caterpillar: (Dethier, 1980); Army worm*:* (Raffa, 1987); Grasshopper: (Lee and Bernays, 1990)), it remains uncertain whether they also experience a form of physiological malaise as a result of toxin consumption. Arzuffi and colleagues (2000) reported limb trembling, uncontrolled movements and periods of immobility following pericardial injection of LiCl in the crayfish when investigating learning of a conditioned taste aversion. This suggests that there are behavioural correlates of sickness in invertebrates, but to date no further data exists in the literature fully exploring the behavioural constructs of the post-ingestional sickness that is normally held accountable for these aversions. Establishing whether the behavioural symptoms of sickness are shared between phyla would strengthen the hypothesis that these behaviours have an adaptive basis (Bateson, 1991).

We have recently established that honeybees have the ability to learn to avoid food cues associated with both the pre-ingestive and the post-ingestive consequences of encountering toxins in food (Wright et al., 2010). Bees will reject some toxins when they taste them, such as quinine, but appear to be unable to readily detect others like the almond nectar toxin, amygdalin, when such toxins are present in sucrose solutions (Wright et al., 2010). When bees inadvertently ingest amygdalin during associative learning, they learn to avoid odours associated with amygdalin-laced solutions using a post-ingestive signalling mechanism. This mechanism, or the toxin itself, could also manifest itself in other behaviours to produce a state of sickness, but this has not yet been tested. It is suggested that foraging species like the honeybee are more likely to

possess this learning ability to avoid toxic foodstuffs and source nutritional food elsewhere.

4.I.ii Hypothesis

I expected to observe changes in behaviour when sickness was induced in honeybees via the administration of toxins. Specifically I predicted a reduction in behaviours associated with locomotion, as well as an increase in the time spent curled up. Both of which serve to conserve heat energy, which may aid recovery from sickness.

4.II Methods

4.II.i Animals and apparatus

Adult foraging worker honeybees (*Apis mellifera Buckfast*) were collected from an outdoor colony at Newcastle University during the summer 2011 and from an indoor colony during winter 2011/2012. After collection, the bees were harnessed using standardised techniques (Bitterman et al., 1983). Here honeybees were subjected to cooling anaesthesia for 1-3 min (until movement was no longer seen), and were fixed into individual metal harnesses with a strip of tape placed behind the head. These harnesses allow the bees to engage in movement while minimising any damage to the body on removal. Once harnessed each bee was fed to satiation with 1.0 M sucrose and kept at room temperature overnight prior to experimentation. Bees were additionally cold anaesthetised for 1-3 min to allow removal from the harness prior to behavioural observations. The honeybee was allowed 15 min to recover from this cold anaesthesia before observations began.

4.II.ii Treatments

Observations began 18-24 h after harnessing. The aim of the first experiment was to determine whether a dose-dependent malaise response was exhibited to injected toxins. At 1 h before observation, 5 µl of 1.0 M sucrose was fed to each bee. Bees were

cold anaesthetised 3 min prior to injection and injected subcuticularly in the thorax with 1 µl of the treatment solution using a 10 µl Hamilton syringe. Injection treatments were: water (control), 10 mM or 1 mM amygdalin, 1 mM or 0.1 mM quinine, 0.1 mM or 1mM LiCl. All toxins were dissolved in deionized water; water was chosen instead of saline to improve solubility of the toxin. The aim of the second experiment was to determine whether bees exhibited a dose-dependent malaise response to two ingested toxins, quinine and amygdalin, when ingested in high doses (Wright et al., 2010). At 1 h prior to the observation, each bee was fed 5 µl of a 1.0 M sucrose solution. Treatments were: 100 mM, 10 mM or 1 mM amygdalin; 10 mM, 1 mM or 0.1 mM quinine, 0.1 mM or 1mM LiCl or control. (Note: we could not feed bees 100 mM dose of quinine because they would not ingest it and it was difficult to dissolve into solution; the LD50 for amygdalin was 100 mM, whereas the LD50 for quinine was 10 mM, Wright et al. (2010)).

4.II.iii Behavioural Observations

Using an assay for locomotion in honeybees (Maze et al., 2006), we scored the following behaviours: walking, standing still, grooming, upside down, curled up, abdomen dragging and fanning and flying. In a pilot study, we observed that bees exhibited an unusual behaviour where they dragged their abdomens across the surface of the arena after consuming toxins. For this reason, we scored locomotion as two behavioural variables: walking normally (walking) and walking while the abdomen was dragging (abdomen dragging; Table 4-1). Additionally, we observed and scored three types of grooming behaviour during our experiments: proboscis grooming, body grooming and antennal grooming; these behaviours were pooled for the overall analysis because proboscis grooming and antennal grooming were each observed rarely (on average <2% of total time budget). Observational arenas were composed of 100 mm x 15mm plastic Petri dishes. After a 45 min period following treatment solution ingestion or immediately after injection, the subject underwent cooling anaesthesia to allow its removal from the harness, and was placed in the Petri dish and allowed to acclimate for 15 min before the observation began. Observations of 15 min

periods were recorded live using the Observer software (Version 5, Noldus Information Technology).

Behaviour	Description
Walking	Walking and not displaying any other behaviour
Abdomen Dragging	Walking and dragging back legs and abdomen on the floor of the arena
Stopped	Standing still
Upside Down	On ventral surface and attempting to perform righting reflex
Curled Up	Laying on its side and hunched up
Grooming	Rubbing antennae, body or proboscis with legs
Fanning/ Flying	Vigorously beating wings or in flight in arena

Table 4-1 ***Definitions of recorded behavioural categories.***

4.II.iv Quantification of toxins in bee haemolymph

Bee haemolymph samples were obtained from individual honeybees fed 5 μl of one of three doses of quinine (0.1, 1, or 10 mM) or amygdalin (1, 10, 100 mM) in 1.0M sucrose. (Note: the dose of quinine was lower than the dose of amygdalin because honeybees refused to consume solutions containing doses larger than 10 mM quinine.) Each bee was cold euthanised, the abdomen removed, and haemolymph was extracted via centrifugation using the method described in Mayack and Naug (2010). Sample volumes were measured using 5 μl capillary tubes, and samples from individual bees fed the same toxin treatment were pooled to form 10 μl samples to which 10 μl of 50:50 methanol:water was added before the samples were frozen at -80^{o}C. Each sample was analysed for amygdalin or quinine using LC-MS using a Waters Alliance LC

solvent delivery system with a ZQ MS detector on a Phenomenex Luna C18(2) column (150 X 4.0 mm i.d., 5µm particle size) operating under gradient elution conditions, with A = MeOH, B = H_2O, C = 1% HCO_2H in MeCN; A = 0%, B = 90% at t = 0 min; A = 90%, B = 0% at t = 20 min; A = 90%, B = 0% at t = 30 min; A = 0%, B = 90% at t = 31 min; column temperature 30˚C and flow rate of 0.5 ml min^{-1} for amygdalin and A = MeCN, B = H_2O, C = 1% HCO_2H in MeCN; A = 0%, B = 90% at t = 0 min; A = 90%, B = 0% at t = 20 min; A = 90%, B = 0% at t = 30 min; A = 0%, B = 90% at t = 31 min; column temperature 30˚C and flow rate of 0.5 ml min^{-1} for quinine. Prior to LC-MS analysis, 60 µl of HPLC grade water was added to each sample and centrifuged at 12,000rpm for 5 min; the supernatant was used for analysis. Amygdalin eluted at 5.91 minutes while quinine eluted at 5.30 min. Polynomial calibration curves for each compound via quantification of the $[M+H]^+$ molecular ion of commercial standards (Sigma-Aldrich, Dorset, UK) in positive mode with *m/z* = 475.3 (amygdalin) and 325.3 (quinine) were used to quantify the concentrations of each compound in the haemolymph. We were not able to measure the concentrations of LiCl in the haemolymph after ingestion with the equipment available.

4.II.v Statistical Analysis

Analyses of the percentage of the interval that the bees spent performing each behaviour were performed using IBM SPSS software v19.0. The behavioural variables recorded in this analysis were mutually exclusive; therefore, their expression was correlated. To reduce the dimensionality of the data, a factor analysis was performed using the principal components method of factor extraction with a Varimax rotation to increase data fit. The factor scores generated from the factor analysis were entered into a multivariate analysis of variance (MANOVA) to analyse the effect of toxins and route of administration on the performance of the behaviours; the scores represented the correlated behavioural variables and reduced the dimensionality of the data. Pairwise post hoc comparisons were made using a least squares difference and performed against the control group only. For the analysis of dose, the control group was not included in the MANOVA because separate control groups were not

performed for each toxin. Comparisons of haemolymph toxins and behaviours that made up a small portion of the time budget (e.g. proboscis grooming) were carried out using a generalized linear model (GLZM).

4.III Results

4.III.i Characteristics of toxin-induced sickness in bees

Bees spent most of their time walking during the assay (Figure 4-1). Factor analysis revealed the correlations in the behaviours we recorded: time spent walking was positively correlated with fanning/flying and was negatively correlated with time spent stopped and grooming (Table 4-2, Factor 1). Two other behaviours, time spent upside down and time spent dragging the abdomen while walking, were also strongly positively correlated (Table 4-2, Factor 2). Time spent curled up was not strongly correlated to the other behavioural variables (Table 4-2, Factor 3).

If bees had been injected with or had ingested toxins, they spent less time walking, fanning/flying and more time stopped and grooming (Figure 4-1 a-d; MANOVA: toxin main effect, $F_{3,216} = 11.1$, $p < 0.001$). Injection and ingestion of toxins affected these behaviours in a similar way (MANOVA: route-of-administration main effect, $F_{1,216} = 0.028$, $p = 0.867$). Bees experiencing toxicosis also spent more time curled up (Figure 4-1e; MANOVA: toxin main effect, $F_{3,216} = 5.32$, $p < 0.001$) and this was true whether they had been injected with toxins or had ingested them (MANOVA: route-of-administration main effect, $F_{1,216} = 1.68$, $p = 0.196$). Overall, curled up behaviour was seen less than 3% of the time and was specific to intoxication.

	Factor		
	1	2	3
Eigenvalue	1.9	1.5	1.2
% variance explained	27.9%	21.6%	17.3%
Walking	**-0.758**	-0.479	-0.306
Stopped	**0.851**	-0.245	-0.114
Grooming	**0.584**	-0.359	0.419
Fanning/Flying	**-0.560**	-0.280	0.018
Upside Down	-0.004	**0.741**	-0.228
Dragging Abdomen	0.065	**0.686**	0.365
Curled Up	0.006	0.022	**0.865**

Table 4-2 ***Factor analysis of all data****. Fit accomplished using a Varimax rotation. Coefficients for variables with strong contributions (>0.5) are in bold.*

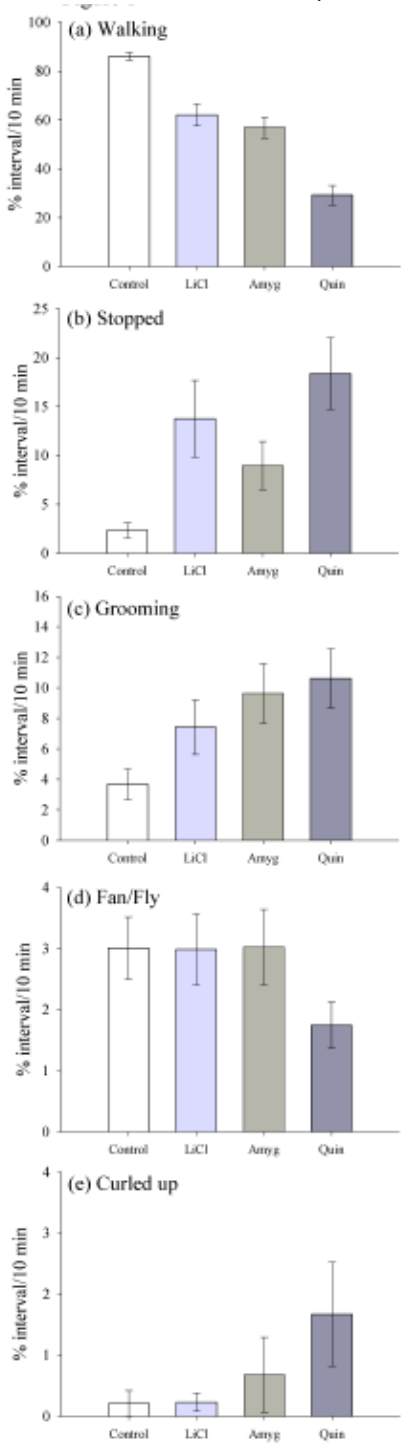


Figure 4-1 ***Behavioural observations of bees during a 15 min period*** *Toxicosis reduced the time spent walking, fanning, and flying and increased the time spent sitting still and grooming. Bees injected with or that ingested lithium chloride (LiCl), amygdalin (Amyg), or quinine (Quin) exhibited less walking (a), more time spent stopped (b), more time spent grooming (c), and less time fanning or flying (d). They were also more likely to exhibit curled up behaviour (e). Error bars represent SE of the mean, control: n = 54, Amyg: n = 58,LiCl: n = 51, Quin: n = 61.*

4.III.iii The righting reflex and abdomen dragging behaviour reveal toxic action

Two variables that were strongly influenced by toxin ingestion or injection were the amount of time spent upside down (the failure to perform the righting reflex) and the amount of time spent dragging the abdomen (Figure 4-2). Upside down behaviour was as much as 20% of the entire interval in some cases of toxicosis, but was never more than 5% of the interval in control bees (Figure 4-2a). Abdomen dragging behaviour was largely peculiar to bees that had ingested toxins (Figure 4-2b).

Whether or not a given toxin influenced either of these behaviours, however, depended on if it was injected or ingested by the bees (MANOVA: toxin x route-of-administration, $F_{3,216} = 7.67$, $p < 0.001$). The effect of LiCl on these two behaviours, for example, depended on how it was administered. Injection with LiCl was more likely to cause a failure to right ($p = 0.001$) whereas ingestion did not ($p = 0.759$). Neither injection ($p = 0.408$) nor ingestion of LiCl ($p = 0.860$) affected abdomen dragging. In contrast, the toxic action of amygdalin depended on whether it had been ingested. Bees that had ingested amygdalin spent up to 20% of their time upside down ($p = 0.005$), but were unaffected when they had been injected with these toxins ($p = 0.993$). They also spent more time dragging the abdomen when they had ingested amygdalin ($p = 0.003$) but did not exhibit this behaviour more often than the control when it had been injected ($p = 0.788$). Quinine caused a higher probability of time spent upside down when ingested ($p = 0.032$) but not when injected ($p = 0.332$). It also elevated time spent dragging the abdomen to over 25% of the interval in both conditions (both $p < 0.001$).

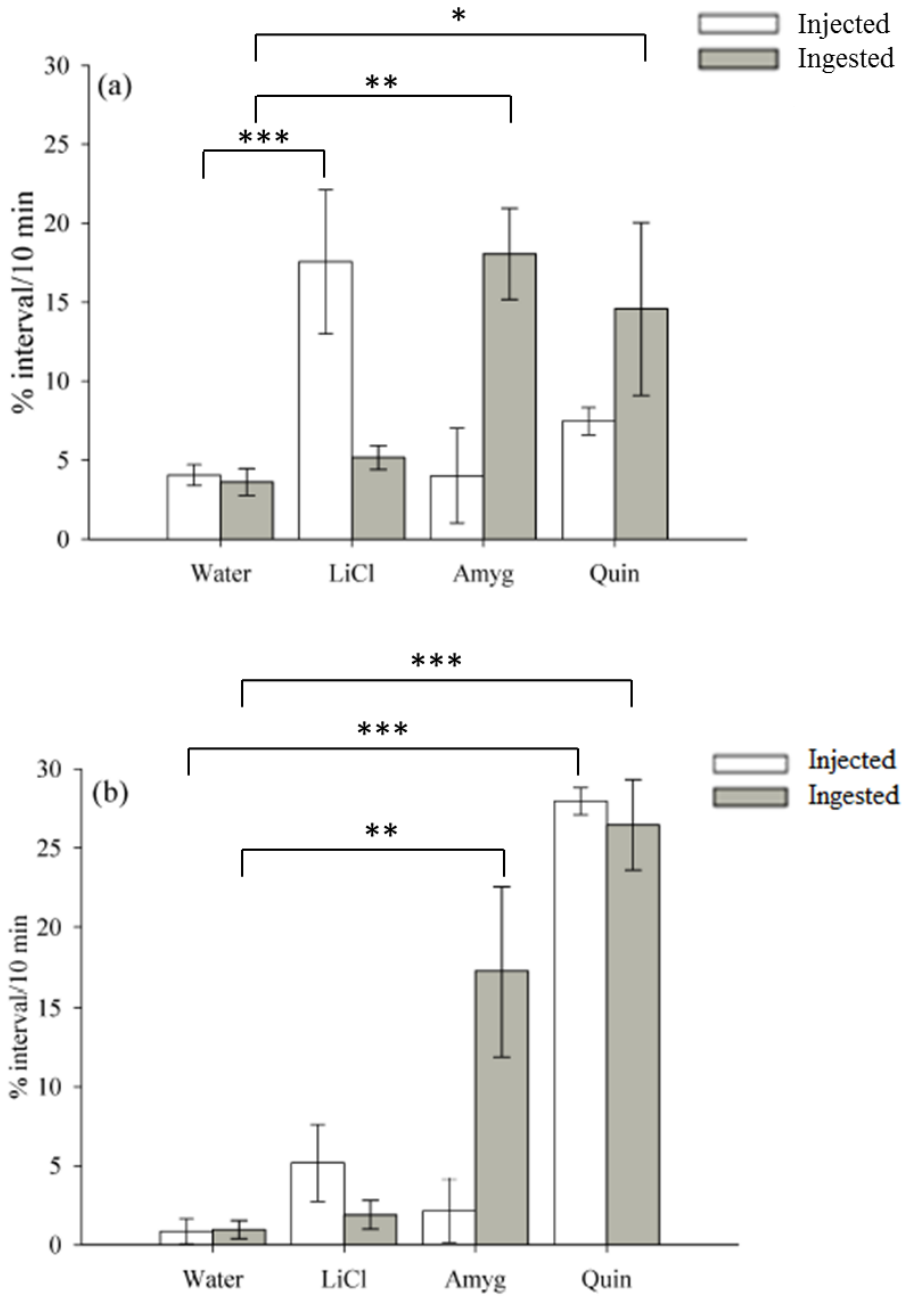


Figure 4-2 ***Sickness behaviours displayed during a 15 min observation.*** *Failure of the righting reflex and abdomen dragging reflect acute sickness caused by injection or ingestion of toxins. (a) Failure of the righting reflex (upside down) depended on whether the toxin had been injected or ingested and the type of toxin administered. Control: n = 24, Amyg: n= 29, LiCl: n = 21, Quin: n = 31. (b) Abdomen dragging behaviour was greatest in bees injected with or that had ingested quinine. Error bars represent SE of the mean. Control: n = 30, Amgy: n = 29, LiCl: n = 30, Quin: n = 30. *: p < 0.05; **: p<0.01; ***: p<0.001.*

4.III.v Grooming

When grooming was being scored during observations, it was split into three behaviours: proboscis grooming, body grooming, and abdomen grooming. In a separate analysis, we also found that each of these behaviours reflected whether a toxin had been injected or ingested (GLZM: toxin x route of administration, $\chi^3_{2=}$ 17.6, p = 0.001). For example, quinine, a toxin that has previously reported to taste bitter to bees, caused an elevation of proboscis grooming (relative to the control) after it had been ingested (and had been in contact with the mouthparts) but not when it was injected (p < 0.001); in contrast, LiCl (p = 0.885) and amygdalin (p = 0.924) had no effect on proboscis grooming when the toxin was eaten. Body grooming and abdomen grooming, on the other hand, were not affected by toxin type (body: GLZM, χ^3_2 = 4.98, p = 0.173; abdomen: GLZM, χ^3_2 = 2.22, p = 0.527) or route of administration (body: GLZM, χ^1_2= 1.64, p = 0.200; abdomen: GLZM, χ^1_2 = 1.30, p = 0.253).

4.III.vi Dose-dependent influences of toxins on the expression of acute sickness

Injection of toxins provides a controlled way of delivering toxins in laboratory conditions; because toxins are almost always acquired by ingestion, injection does not reflect how most animals experience toxins. To identify how the dose fed translated into the dose found in the blood (and hence to relate it to injected toxins), we measured the toxins amygdalin and quinine in the haemolymph of honeybees after feeding them a specific dose (Figure 4-3). Bees fed the highest dose had more toxin in the haemolymph (GLZM: dose main effect, χ^2_2 = 237, p < 0.001). When fed a dose of 10 mM (high) quinine or 100 mM (high) amygdalin, bees had an almost 10 fold lower concentration in haemolymph than the fed dose.

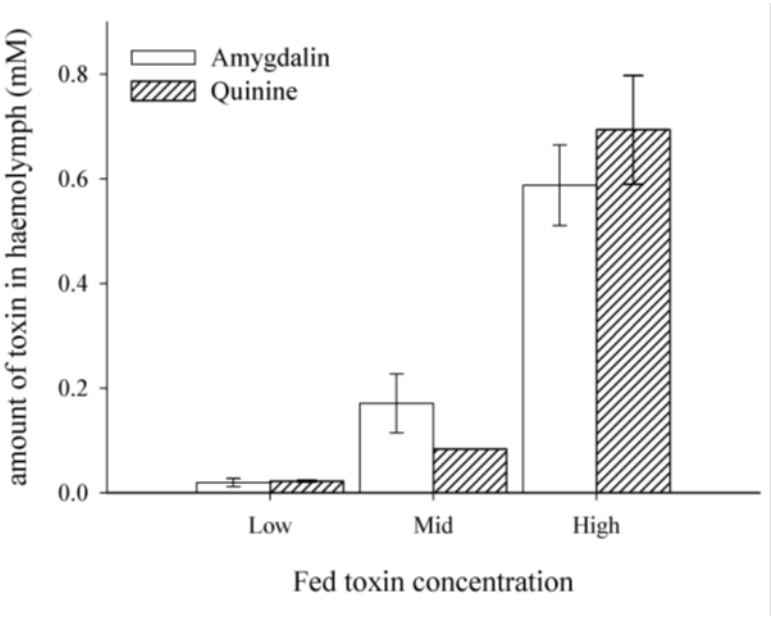


Figure 4-3 ***Concentrations of ingested toxins in haemolymph.*** *Amount of toxin fed to bees was > 10 fold lower than that recovered in haemolymph. Bees were fed amygdalin (low = 1mM, mid = 10mM, high = 100mM) or quinine (low = 0.1mM, mid = 1mM, high = 10mM) at 1 h prior to haemolymph sampling. Values are means of pooled samples, error bars represent SE of the mean. Low: n = 4, Mid: n = 3, High: n = 4.*

To identify the influence of toxin dose on behaviour, we performed separate factor analyses on the two routes of administration. The dose of the toxin in the range we tested (0.1-10mM) did not influence the expression of walking, stopped, grooming or fanning/flying behaviour when injected (Table 4-3; MANOVA: dose main effect, $F_{1,75}$ = 0.260, p = 0.111) or ingested (Table 4-4; MANOVA: dose main effect, $F_{1,112}$ = 0.404, p = 0.526). We also tested whether toxin dose influenced the expression of upside down and abdomen dragging behaviour. When injected, whether or not the toxin caused these behaviours depended on both the toxin dose and the type of toxin (Table 4-3; MANOVA: dose x toxin, $F_{2,75}$ = 4.99, p = 0.009). When ingested, however, the expression of these behaviours did not depend on toxin dose (Table 4-4; MANOVA: dose main effect, $F_{1,112}$ = 0.404, p = 0.526). We also tested how toxin dose influenced the expression of sickness behaviour. As before, we used a factor analysis to first reduce the data to factors that represented the behaviours (Table 4-3 &Table 4-4). (Prior to this analysis, the data was split by route of administration.) Bees that had been injected with the toxins exhibited less time walking and fanning or flying and more time stopped and grooming as in the larger analysis in Table 4-2; these

behaviours were not influenced by toxin dose (MANOVA: dose main effect, $F_{1,75} = 2.60$, $p = 0.111$) or the type of toxin injected (MANOVA: toxin main effect, $F_{2,75} = 1.65$, $p = 0.199$). As before, factor 2 mainly represented the time spent upside down and abdomen dragging, but also included curled up behaviour (Table 4-3). The expression of these behaviours, which we had identified earlier as being specific to each toxin (Figure 4-2) depended on both the toxin dose and the type of toxin as before (MANOVA: dose x toxin, $F_{2,75} = 4.99$, $p = 0.009$).

Bees that had ingested the toxin also spent less time walking and fanning or flying, and more time stopped or grooming (Table 4-4). In this case, however, LiCl had little or no effect at either dose, whereas both quinine and amygdalin ingestion reduced walking and caused an increase in the time spent grooming and stopped (MANOVA: toxin main effect, $F_{2,112} = 10.0$, $p < 0.001$). LiCl also had a limited influence on upside down behaviour and abdomen dragging (factor 2, Table 4-4), whereas the ingestion of quinine and amygdalin both increased the time spent performing these behaviours (MANOVA: toxin main effect, $F_{2,112} = 6.61$, $p = 0.002$). The dose of the toxins did not affect the expression of these behaviours (MANOVA: dose main effect, $F_{1,112} = 0.404$, $p= 0.526$).

	Factor	
	1	*2*
Eigenvalue	2.1	1.5
% variance explained	30.4%	27.7%
Walking	**-0.755**	-0.552
Stopped	**0.81**	-0.174
Grooming	**0.72**	-0.183
Fanning/Flying	**-0.539**	-0.258
Upside Down	-0.252	**0.551**
Dragging Abdomen	0.167	**0.803**
Curled Up	0.038	**0.747**

Table 4-3 ***Factor analysis of injection data.*** *The fit was accomplished using a Varimax rotation. Coefficients for variables with strong contributions (>0.5) are in bold.*

	Factor		
	1	2	3
Eigenvalue	2.1	1.5	1.1
% variance explained	30.7%	21.4%	15.6%
Walking	**-0.898**	0.283	-0.002
Stopped	**0.631**	0.421	-0.486
Grooming	**0.613**	0.513	0.216
Fanning/Flying	**-0.543**	0.212	0.339
Upside Down	0.125	**-0.73**	-0.31
Dragging Abdomen	0.287	**-0.619**	0.465
Curled Up	0.425	0.136	**0.619**

Table 4-4 ***Factor analysis of ingestion data.*** *The fit was not subject to rotation. Coefficients for variables with strong contributions (>0.5) are in bold.*

4.IV Discussion

Our data represent the first complete characterisation of behaviours caused by the feeding or injection of toxins in an invertebrate. These data indicate that sickness is produced directly by toxin interaction with the gut after ingestion, but that it can also be produced as a result of injection. When injected or ingested, all three toxins reduced walking, increased the time spent still, and increased time spent grooming. Both injection and ingestion of toxins caused failure of the righting reflex and caused the expression of abnormal behaviour such as abdomen dragging or curling up that were rarely observed in the control subjects. Some toxins were more effective if injected and others when ingested. We predict that both the gut and the central nervous system can respond to toxins directly and have a shared mechanism for signalling toxicosis that targets the control of motor function.

4.IV.i Toxin-induced malaise is characterised by a reduction in locomotion

Our data agree with previous work in rats (Cappeliez and White, 1981a, Johnson, 1979, Wolthuis et al., 1975) and clearly show that a key characteristic of the change in state caused by toxicosis in animals is an immediate reduction in locomotion. The adult honeybees in the control group of our experiments were very active in our locomotion assay, spending over 80% of their time walking during the 15 min observation period. Insult with toxins reduced this activity by as much as 45% and was accompanied by an increase in time spent still. Spending less time walking could conserve metabolic resources used to neutralise toxicosis, as detoxification commands ATP and amino acids to mobilise the production of enzymes and active transport for the excretion of toxins (Lochmiller and Deerenberg, 2000, Bains and Kennedy, 2004, Cresswell et al., 1992). This idea is supported by the fact that Madagascar hissing cockroaches (*Gromphadorhina portentosa*) exposed to pesticides reduce their metabolic rate (Sawczyn et al., 2012), and energy reserves in the earthworms (*Enchytraeus albidus*) are depleted during a recovery from metal toxicosis (Novais et al., 2013). We predict that because metabolic resources are required for detoxification, the ingestion of

toxins could be particularly harmful to foraging bees. Foragers require foods high in carbohydrates to produce enough ATP to fly (for review see Rothe and Nachtigall (1989), Harrison and Roberts (2000)). If they are forced to use carbohydrates to detoxify ingested toxins, they are likely to face a trade-off between detoxification and foraging for the colony that depends critically on how much ATP is required for detoxification. In addition, bees and other animals may avoid dangers posed by predators or other hazards by remaining still while recovering from toxicosis (Hart, 1988, Aubert, 1999) if detoxification commands physiological resources required to elicit the appropriate escape response.

4.IV.ii Malaise-specific behaviours identified in the honeybee

Bees administered toxins exhibited specific behaviours such as assuming a curled-up posture. Interestingly, we observed these behaviours in bees that had been injected with toxins as well as those that ingested them. While this posture may simply be a reaction to nociception or simply due to a breakdown of posture control and general weakness, curling up behaviour is described as a hallmark of sickness with an adaptive value to conserve body heat and potentially provide comfort in vertebrates (Hart, 1988). Additionally, another unusual behaviour observed following toxicosis in bees, abdomen dragging, also has correlates with mammalian sickness behaviour. Like the bees in our study, rats injected with LiCl display 'body dragging' where the body is elongated and the belly dragged along the floor by the front paws, or writhing caused by muscular contractions in the abdomen (Parker, 1982, Parker et al., 1984, Ohmura et al., 2012) or 'lying on belly' (Parker, 1982, Parker et al., 1984, Meachum and Bernstein, 1990). This behaviour in rats, in particular, is characterised by a 'flattened torso, limp limbs and the head on the floor' and has been previously interpreted to indicate that rats experience malaise-like symptoms associated with toxicosis (Meachum and Bernstein, 1990); however, this behaviour is also often observed in response to procedures expected to cause abdominal pain (Roughan and Flecknell, 2003). It is interesting to note that we observed this behaviour in bees that had been injected with the toxins as well as those that had ingested quinine.

The absence of a dose-dependent effect on sickness behaviours may be explained by the degree of toxicosis experienced. For example, we propose that mild toxicosis presents with generalised symptoms like reduced locomotion, and more severe toxicosis causes acute symptoms like a curled up postures and a loss of the righting reflex. We might therefore observe a transition from one state of toxicosis to another, rather than a linear relationship in the display of sickness behaviours.

4.IV.iii Honeybees increase grooming in response to toxic insult

Our data indicate that bees spent more time grooming during toxicosis, and that this was one of the key characteristics of a sickness response. In insects grooming is also observed in response to insult with pesticides and other toxins (Neuman-Lee et al., 2013, Williamson et al., 2013). The fact that vertebrate animals often stop or reduce grooming in response to toxicosis or pathogen-induced illness (Meachum and Bernstein, 1990, Parker et al., 1984, Hart, 1988, Tikhonova et al., 2011, Kulikov et al., 2010, Ritter and Epstein, 1974, Bassi et al., 2012) indicates a specific difference in the sickness behaviours of insects and vertebrates. In insects, self-grooming rids the body of parasites (Currie and Tahmasbi, 2008), but antennal and mouthparts grooming also enhances the performance of sense organs (Jacquet et al., 2012). In vertebrates, grooming may reduce anxiety after stressful events (Iliadi, 2009, Spruijt et al., 1992), as well as being a way of ridding the body of parasites and keeping feathers and fur in good condition. It also is a means of reducing corporeal temperature (Thiessen et al., 1977). Why bees spend more time grooming when they experience toxicosis is unclear.

4.IV.iv Malaise behaviours are modified by the route of toxin administration

In general, toxin-induced sickness – whether it was produced by injection or ingestion – resulted in a suite of behavioural changes in bees, but there were subtle differences in expression that depended both on the toxin and the way it was administered. When injected directly into the haemolymph, a toxin gains direct contact with tissues and organs within an animal. Even after ingestion, our data show that toxins pass over the

gut into the bee's haemolymph. While there is no direct evidence provided in this study, we expect that toxins can cross the blood-brain-barrier to act directly on central nervous system circuits that regulate behaviour, based on studies of other toxic or pharmacologically active substances such as caffeine ingested by bees (Mustard et al., 2012, Wright et al., 2013). Identification of the extent to which these toxins directly act on the bee's brain, and whether there are specific mechanisms for directly detecting toxins in this neuropil will be the subject of future investigations.

Based on our measurements of toxins in the haemolymph after the consumption of toxins in food, we suggest that ingestion could potentially lead to a slower rate of toxin dose administration than injection because bees can regulate the rate of passage of the food from crop to midgut (Blatt and Roces, 2001). Post-ingestive feedback mechanisms that detect toxins in food exist in the insect crop and the gut (Park and Kwon, 2011). For example, gustatory receptors in enteroendocrine cells in the gut (Park and Kwon, 2011) may mediate nutrient absorption (Miguel-Aliaga, 2012, Miyamoto et al., 2013) and could also detect toxins. These cells also signal the presence of nutrients and toxins to other tissues via peptidergic signals including cytokinins (Behrens and Meyerhof, 2011). Such signals have the potential to be the primary means by which the gut signals a state of toxicosis to the rest of the body.

Our data also showed that a toxin's influence on other behaviours that may characterise sickness (the righting reflex and abdomen dragging) depended on whether it had been ingested or injected. We propose that the expression of these two behaviours indicates an acute state of sickness in insects. In our study, LiCl did not significantly affect these behaviours when it was ingested, perhaps indicating that its uptake into the haemolymph, like that of salts in other insects, is strongly restricted by the gut (Trumper and Simpson, 1993). In contrast, amygdalin was more likely to cause more time spent upside down and abdomen dragging when ingested but not injected. Amygdalin may not be as toxic when injected because its mode of action depends on contact with beta-glucosidase enzymes mainly present in the gut and crop that break it down into cyanide (Conn, 1969, Pontoh and Low, 2002). Although the toxins would gain some contact with the gut when injected into the haemolymph, the extent to which would be less focussed. Amygdalin also activates bitter taste receptors in bees

and could be activating the same pathways as quinine in the gut (Wright et al., 2010). Quinine, on the other hand, produced abdomen dragging and upside down behaviour whether it had been injected or ingested. Quinine blocks sodium channels, and these channels are present in the gut and also in nerve and muscle cells throughout the body, so its targets are not restricted to the gut. Quinine also activates bitter taste receptors in bees (Wright et al., 2010) that are likely to be expressed in the gut (Park and Kwon, 2011) and could also be expressed in the brain.

4.IV.v Concluding comments

Our study is the first to characterise the change in behaviour caused by toxin consumption and injection in the honeybee. All three toxins each had different pharmacological targets but still produced a similar suite of behaviours in bees. That some of these behaviours such as the reduction in locomotion and an increase in sickness-specific behaviours are common to both vertebrate animals and bees implies that this is an evolved adaptation that increases survival.

Furthermore, the toxins used in our experiments are bitter tasting to bees (Wright et al., 2010). When bees consumed toxins, we observed an increase in the time spent grooming the proboscis, a specific behaviour that was not seen when the toxins were administered by injection. The increase in proboscis grooming mirrors a chin-rubbing behaviour observed in rats subjected to orally administered toxins or toxin-paired solutions. For example, chin rubbing is consistent with LiCl-paired conditioned taste responses, but not LiCl injections (Parker, 1982, Parker et al., 1984, Meachum and Bernstein, 1990), indicating that the rats associated LiCl with an aversive taste (Spector, 2000). Thus, proboscis grooming is likely to be a reaction of the bees to a distasteful substance on their mouthparts resulting from consuming the toxins.

Based on our experiments, we propose that changes in behaviour that accompany sickness depend on whether animals experience extreme illness arising from a large dose of toxin or mild illness arising from a smaller toxin dose. This idea was supported by the fact that bees that experienced the highest dose of what we predict to be the most aversive toxin (injection with quinine) were also the most likely to exhibit specific

behaviours like curling up that were not observed in the control bees. When injected directly into the haemolymph, the toxin gains direct contact with tissues and organs within the honeybee, including the gut and the brain. In fact, subcuticular injection in the thorax are said to have similar effects on behaviour as those delivered directly to the brain and other tissues by microinjection (Barron et al., 2007). Injection allows for more contact of the toxin with the brain and other organs, whereas a comparatively small amount of the toxin passes through the gut into the haemolymph when it is eaten.

Mild sickness was accompanied more by time spent grooming and less time walking, as observed in animals fed both quinine and amygdalin. We predicted that ingestion would lead to a slower rate of toxin dose administration than injection. Bees regulate the rate of passage of the food from crop to midgut (Blatt and Roces, 2001) and might be able to reduce the toxin dose they experience if they had post-ingestive feedback mechanisms that detected toxins in food, preventing a large dose from entering the midgut. Like locusts that are more likely to regurgitate when fed the toxin nicotine hydrogen tartrate (Simoes et al., 2012), we also observed that bees vomited the solutions they were fed (data not shown), suggesting they too have a mechanism for detecting toxins and regurgitating food.

As mentioned previously, conditioned taste aversions have been observed in many invertebrates (Simões et al., 2011, Dethier, 1980, Lee and Bernays, 1990, Arzuffi et al., 2000) but ours is the first to characterize the behaviours associated with the change in state caused by toxin consumption. Also, as all three toxins, each with different pharmacological targets, produced a similar suite of behaviours in bees, this strongly suggests that there is a common behavioural syndrome that is activated by toxicosis. Our study adds weight to arguments that sickness behaviour in response to food poisoning is conserved throughout phyla and that it could be an adaptive response.

It should, however, be noted that there were limitations of the methodology employed in this study. Bees were collected in both summer and winter periods, and there is evidence that this effects a bee's foraging behaviour along with altered tolerance to toxins (London-Shafir et al., 2003). Additionally, the cohort of bees used during the

winter was retrieved from an indoor colony with restricted nutrition and foraging opportunities, which may further account for differences in sensitivity to toxins.

Chapter 5 - Emotional characterisation of sickness in the honeybee

Summary: In this chapter, I investigated the effects of malaise on honeybee cognition as measured by the judgement bias task. The cognitive effects of three different toxins that had been shown to provoke sickness behaviours in the previous chapter were studied to determine whether they affected the emotionality of the animals. Inclusion of subjects in the judgement bias analysis was also considered, where different criteria were set regarding their levels of responding during training sessions.

5.1 Introduction

The necessity of reducing the numbers of animals used, or replacing them with a lower-order species is a societal aim in scientific research. The honeybee is a desirable candidate for high-throughput screening of novel compounds in drug development for many reasons. First, a large number of closely related individuals (up to 20,000) reside in the same colony, making it possible to inexpensively rear a large number of genetically-related animals for experiments. Secondly, honeybees possess distinct neurotransmitters that mediate reward and punishment (Hammer, 1997, Hammer and Menzel, 1998, Vergoz et al., 2007), and have also been demonstrated to have the abilities to learn tasks, including the judgement bias task (Bateson et al., 2011b, Perry and Barron, 2013a, Giurfa, 2013). To this end, I ran a set of correlative judgement bias studies with the honeybee as were performed with the rat, to explore the potential for the honeybee to take the place of rodents in this research area.

As discussed in Chapter 1, emotions guide animals' decisions to perform a particular behaviour in response to environmental stimuli, and serve to optimise the Darwinian-fitness consequences of events that arise from them (Nettle and Bateson, 2012). Animals that frequently encounter positive events when exposed to a set of stimuli assign them a positive valence and subsequently increase their expectation of these positive events when re-exposed to these stimuli (Mendl et al., 2010). Equally, exposure to punishing events are thought to cause animals to be more expectant of

further punishing events and may cause them to avoid responding to cues similar to those predicting punishers (Mendl et al., 2010). Expectations of these events cause animals to perform appropriate behavioural responses with presented with the same stimuli in future (e.g. pressing a lever for food reward when played a salient tone in associative conditioning, Harding et al. (2004)). These behavioural responses can be complex, or they can be as simple as basic approach and avoidance responses towards rewarding or aversive stimuli (LeDoux, 2012).

As existing research of emotion in invertebrates is relatively scant, we do not know how malaise affects cognition in these animals. I have previously hypothesised that animals experiencing sickness are likely to experience emotion correlative with low mood (see xref to chapter 3), as is observed in humans (Schiepers et al., 2005, Maes et al., 2012). With a negative affective state resulting from a compromised well-being, an animal could presumably become more hesitant to perform approach or avoidance under uncertain conditions due to a biased perception of caloric cost or risk of predation (Nettle and Bateson (2012); see Chapter 1). Here they will be less likely to perform behavioural responses when presented with ambiguous stimuli. As such, when animals are presented with cues of an ambiguous nature, whether they actively perform or avoid performing a behavioural response can indicate their underlying affective or emotional state, where fewer approach responses reflects pessimism, and fewer avoidance responses reflects optimism (Harding et al., 2004, Paul et al., 2005, Mendl et al., 2009). For this reason, I hypothesised that bees experiencing sickness would also experience a negative affective state and hence would make fewer optimistic responses towards ambiguous stimuli than healthy bees.

5.I.i Cognitive abilities of the honeybee

Honeybees, like vertebrates, possess neural pathways associated with reward (octopamine-mediated; Hammer (1997)) and punishment (dopamine-mediated; Vergoz et al. (2007)), which offers the tantalising possibility that positive and negative affective states can be induced in a similar manner to vertebrate animals. However, they lack the complex brain regions associated with emotional processing in humans

(the amygdala), but they may instead possess a simpler system that produces an analogous form of primitive emotion.

Honeybees have shown evidence of task learning and cognitive processing; their ability to associate reward with scent stimuli is biologically relevant, allowing more efficient foraging, and as such associative conditioning can be performed (Bitterman et al., 1983). In addition to a developed olfactory sense, honeybees have spatial awareness (Dyer et al., 2008) and are able to differentiate colours (Giurfa, 2004); all of which could be exploited in discrimination tasks to measure cognitive processing. Honeybees have been demonstrated to learn an avoidance of the consumption of sickness-inducing agents via associative conditioning (Wright et al., 2010), which is presumably an adaptive response (Rozin and Kalat, 1971) and it is considered to encompass a negative association (Garcia and Koelling, 1967) and a reduction in hedonic states (Berridge, 2000). More recently, honeybees have been shown to appear to monitor uncertainty in decision-making by adapting their decision strategy in response to task difficulty (Perry and Barron, 2013a), and an ability to learn a variety of spatial and relational rules (see Giurfa (2013)).

5.I.i.1 Processing of reward and punishment

Information regarding the processing of reward and punishment in the honeybee can be accessed in a similar way to mammals – via their memory, attention and decision-making processes elicited by exposure to salient stimuli.

Reward processing in honeybees is generally measured by their responsiveness to a sucrose stimulus. Adult foraging honeybees will extend their mouthparts, a behaviour known as the proboscis extension response (PER), when their sensory organs (antennae) come into contact with sucrose. This is the mechanism by which they extract nectar from flowers, and is an easily quantifiable behaviour in laboratory settings. Gil et al. (2008) demonstrated that honeybees were capable of learning and forming memories about the relative value of reward via a simple assay that measured the time taken to perform the PER when stimulated with sucrose. Honeybees were

conditioned either with a constant or increasing concentration of sucrose following antennal stimulation, and the authors subsequently tested their PER times 24 h later where antennal stimulation was given but not paired with reward. The bees that were given increasing levels of sucrose reward showed quicker PER times than bees given a constant level of reward, indicating that they were able to learn, and form memories of, reward valuation. This is functionally beneficial given that bees must learn about the quality of nectar in different food sources when foraging (Hammer, 1997). The authors interpreted this anticipatory response as a behavioural adaptation that reflects incentive salience. This expectation of reward involves the formation and activation of memories of the specific properties of the reward (here it was the magnitude), where the memory can be recalled by exposure to predictive cues (antennal stimulation) even in the absence of reward (Gil et al., 2008).

Attentional processes have also been implicated in associative learning of reward. Honeybees can rapidly learn to associate a sucrose reward with a coloured target (Giurfa, 2004), where time taken to reach the target decreases with repeated exposure. In both absolute conditioning (where bees are presented with one coloured target) and differential conditioning (where bees are presented with two coloured targets, but only a single colour is rewarded), bees can differentiate between the rewarded target and an additional novel, distinctly coloured target, but only bees trained on the differential conditioning protocol can learn to differentiate between the rewarded target and one that is less perceptually different (Giurfa, 2004). The training procedures provoke different cognitive requirements in the bees, and evidence for attentional processes is demonstrated via the suppression of responsiveness to competing stimuli (Miller et al., 2011, Giurfa, 2004). This has been shown in further experiments where honeybees are trained to choose a target disc from a number of distractors (Spaethe et al., 2006).

More commonly, the processing of conditioned responses to reward are investigated using an olfactory conditioning protocol developed by Takeda (1961), which is explained further in section 5.I.iii.2. In this protocol, a neutral odour is forward-paired with a salient stimulus, and after conditioning the odour takes on the affective valence

of the stimulus. In section 5.I.iii I will discuss how it can be used to probe biases in honeybee judgement of ambiguous stimuli.

When we come to review the processing of punishment in honeybees this can be difficult, as many of the approaches studied also include an appetitive component (Tedjakumala and Giurfa, 2013), for example, visits to artificial flowers are punished with the administration of quinine (Chittka et al., 2003, Avarguès-Weber et al., 2010). The presence of both appetitive and aversive components might compound the study of associative learning (Tedjakumala and Giurfa, 2013). This problem has been circumvented by Vergoz et al. (2007) who developed a method based on aversive learning in drosophila (Tully and Quinn, 1985), which involves exposing the honeybee to a mild electric shock. When bees are stimulated with a shock, they perform a defensive response known as the sting extension response (SER; Breed et al. (2004), Núñez et al. (1997)). Vergoz et al. (2007) demonstrated that dopamine was crucial for aversive olfactory learning in the honeybee by blocking dopaminergic signalling in the honeybee brain, which attenuated olfactory conditioning of the SER. Free-flying honeybees show altered decision-making when exposed to shock, and will choose to avoid conditioning odours associated with shock on a y-maze test (Carcaud et al., 2009). The memory of a shock can be recalled as much as 3 days after initial conditioning, where the SER is elicited by presentation of the conditioned odour (Giurfa et al., 2009). This paradigm has not been as extensively developed as PER conditioning, but it would be interesting to address similar questions regarding the processing of punishment, as has been done with processing of reward with the PER.

5.I.i.1.1 Neural correlates

The processing of rewards and punishments in insects in not as fully understood as it is in mammals and humans. Almost all of what we know about reward learning in insects is derived from reward learning paradigms (Perry and Barron, 2013b), and the basis of our knowledge of the neural correlates of cognitive processing stems from investigations of olfaction-based associative conditioning (Giurfa, 2013, Roussel et al., 2012, Tedjakumala and Giurfa, 2013, Vergoz et al., 2007). As in rodents, some of the

neurotransmitters involved in reward and punishment have been identified in insects. Reward and punisher learning is localised to the mushroom bodies and antennal lobes of insects (Perry and Barron, 2013b), and it has been recognised that the monoamines 5-HT, octopamine and dopamine act as neurotransmitters to transmit this information, with the most prominent in appetitive learning being dopamine and octopamine (Burke et al., 2012). In honeybees, octopamine mediates the reinforcing capacity of sucrose (Hammer and Menzel, 1998) whereas dopamine mediates aversive learning of punishment, be it in the form of distasteful food (Wright et al., 2010) or electric shock (Vergoz et al., 2007). These neurotransmitters are also found to mediate analogous reward and punishment pathways in other insects (*Drosophila Melanogaster:* Schwaerzel et al. (2003); *Gryllus bimaculatus:* Unoki et al. (2005)).

5.I.ii Performance in a judgement bias task

Of particular relevance is the 2011 paper by Bateson et al describing a novel judgement bias task for use in the honeybee. The authors here used a go/no-go task, where a simple extension of the mouthparts serves as the 'go' response. The bees responded to an olfactory stimulus associated with sucrose reward by extending the mouthparts which resulted in its delivery, and did not respond ('no-go') to an olfactory cue associated with a bitter quinine punisher, preventing its feeding. Honeybees were tested for a biased judgement of novel odours following a predator-like threat which was predicted to produce a negative affective state. The experimental outcomes confirmed these predictions, with fewer responses made to the ambiguous-cues by shaken honeybees. These behavioural responses are functionally comparable to those seen in humans and other animals in the judgement bias task (e.g. (Harding et al., 2004), (Mendl et al., 2006)). Furthermore, there was a reduction in the neurotransmitter 5-HT (serotonin) in the shaken honeybees, which correlates with a depletion of 5-HT in the brains of depressed humans (Owens and Nemeroff, 1994). The discovery of shared physiological mechanisms underlying pessimism in humans and bees may aid the understanding of the evolutionary origins of emotion. To date there

have been no further published studies attempting to measure biases in judgement of ambiguous information in honeybees or other invertebrate species.

In summary, honeybees have been shown to demonstrate the presence of some similar behavioural and cognitive outcomes to vertebrates in emotion research and as such there is a need to investigate further to determine their potential use as a reliable model. In the remainder of this chapter I investigate the affective changes induced by sickness in honeybees as was done in rats (Chapter 3), to allow a comparison of their performance on judgement bias tasks.

5.I.iii Honeybees and judgement bias

Detection of these emotional changes may be possible using a judgement bias paradigm, where an animal's affective state is measured by analysing their responses to ambiguous stimuli (see Chapter 1). A judgement bias task was developed for use with honeybees by Bateson et al. (2011b), who developed a 'go-no-go' method whereby honeybees discriminated between two odours, one associated with a rewarding sucrose droplet, and the other associated with a distasteful quinine punisher (see Figure 5-1). The unconditioned response observed was the extension of the proboscis (mouthparts), which occurred when bees were presented with sucrose (a 'go' response), and not when presented with quinine ('no-go' response).

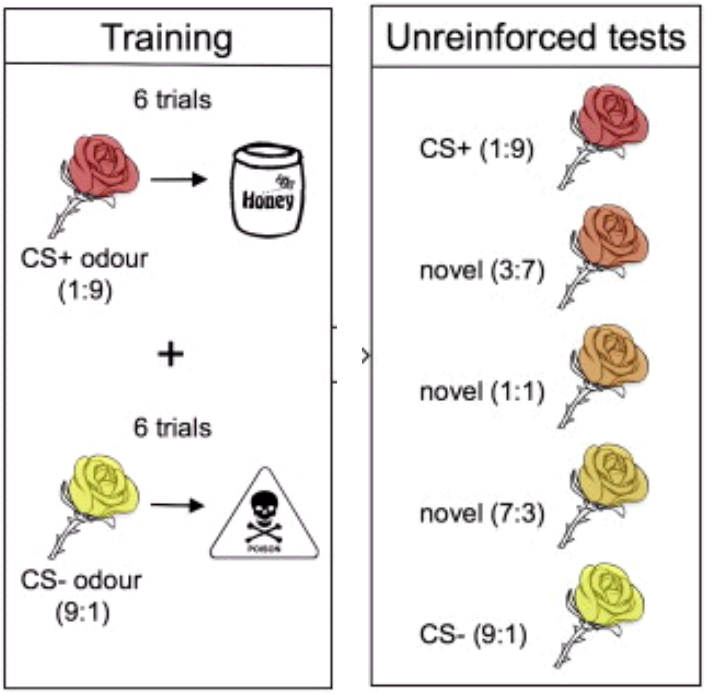


*Figure 5-1**Training and testing honeybees in a judgement bias task.** Honeybees were trained for six trials with each stimulus (CS) in a pseudorandomised sequence. The CS+ odour was a ratio of 1 part 1-hexanol to 9 parts 2-octanone; the CS− was a 9:1 ratio of the same two odours. After conditioning, bees were placed either in a group that was exposed to 60 s of shaking or in a control group. They were then tested with each CS and three novel, intermediate ratios of the same two odours. All test trials were unreinforced, and the order of test odours was randomized across subjects. Reproduced from Bateson et al 2011.*

Following the training of a discrimination of the two response outcomes related to the scent stimuli, the researchers were able to observe the responses of honeybees that were exposed to ambiguous combinations of these scents in unreinforced tests (Figure 5-1). The selections of these methodological parameters are discussed in the following sections.

5.I.iii.1 Rewards and punishers for the judgement bias task

Quinine is distasteful to the honeybee (Wright et al., 2010) and its rejection is stimulated by pre-ingestive feedback mechanisms facilitated by dopamine (Wright et al., 2010) implying that it acts as a punisher. To this end, quinine was selected for use as a punisher to stimulate 'no-go' responses in a judgement bias task (Bateson et al.,

2011b). Sucrose is an inherently reinforcing substrate for animals, including the honeybee (Hammer, 1997) and as such was selected as the rewarding outcome in this paradigm.

5.I.iii.2 Conditioning of the proboscis extension reflex (PER) in the honeybee

Conditioning honeybees to extend their proboscis in response to an odour associated with an odour cue is not a new phenomenon. It was established over 50 years ago by Takeda (1961) and has been subsequently adopted in hundreds of olfactory protocols in laboratories worldwide (Giurfa and Sandoz, 2012). The substantial olfactory capabilities of the honeybee were studied by Vareschi (1971), who identified that bees could differentiate 1816 odour pairs at a rate of >95%. Guerrieri et al. (2005) further demonstrated the generalisation of odour cues by honeybees; ecologically, this is relevant as bees are required to identify different floral odour mixtures to discern their biological importance (i.e. the content and quality of floral nectars). Thus, olfactory stimuli can be discriminable and generalised and are suitable to be used in a judgement bias paradigm.

The PER itself is shown by bees when their tarsi, mouthparts or antennae come into contact with sucrose. When this contact is presented in temporal association with an odour, associative conditioning takes place (Bitterman et al., 1983). Following a period of training, the odour provokes the PER in the absence of sucrose reinforcement (Figure 5-2). The PER is a simple and quantifiable response that reflects a positive salience of a stimulus (i.e. it is performed in anticipation of rewarding events), and is therefore a suitable measure to be used in behavioural paradigms where measurement of stimuli salience is desired, such as in the judgement bias task.

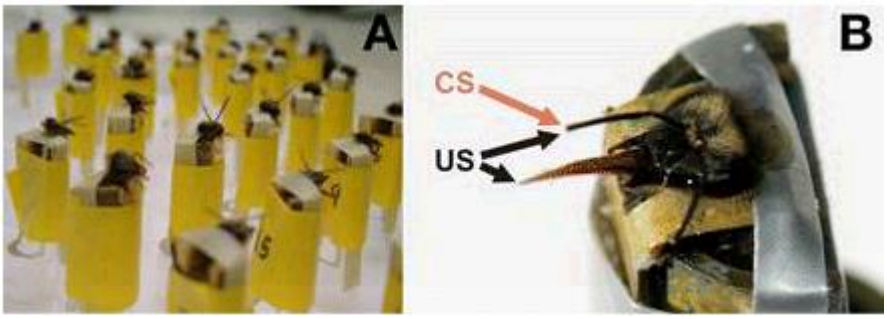


Figure 5-2 ***Conditioning of the proboscis extension response in restrained honeybees.*** *(A) Honeybees placed individually in metal holders are awaiting conditioning. Small pieces of tape restrain the bees without harming them, so that only the antennae and mouthparts can freely move. (B) Conditioning of the proboscis extension response on restrained bees. The PER is a response shown by bees when their antennae, tarsi, or mouthparts are contacted with sucrose solution. During conditioning, an odour (conditioned stimulus) is presented in temporal association with sucrose solution to the antennae and to the proboscis (unconditioned stimulus). After conditioning, the odour CS, which initially did not evoke any response, triggers the PER. Reproduced from Giurfa and Sandoz 2012.*

Development of the task in Bateson et al. (2011b) also accounted for the temporal learning abilities of the honeybee. Maintenance of olfactory memory in honeybees is facilitated by salient cues when they are presented with spacing (spaced learning), rather than in bulk (massed learning), where longer ITIs between odour presentations have been demonstrated to result in enhanced memory (see Menzel et al. (2001) for review). In Bateson et al. (2011b), they adopted spaced learning while training the olfactory discrimination (5 min between odours), and tested the bees' responses to the non-reinforced test odours in closer temporal proximity (30 sec between odours) to avoid extinction of the PER.

5.I.iii.3 Experimental outcomes of judgement bias tasks

Bateson et al. (2011b) demonstrated that honeybees made fewer optimistic responses to ambiguous stimuli in a judgement bias task when exposed to a predator-like affective manipulation (Figure 5-3); presumably this was due to an increased expectation of threat, and a correlative increase in the perceived cost of falsely interpreting stimuli as signalling reward. Giurfa (2013) critiqued the task outcomes of shaken bees in Bateson et al's study, suggesting that the reduction of bees responding

to the negative and near negative odours was evidence of improved discrimination of the task due to increased attention, rather than of a pessimistic bias. I would argue that an increased attentional bias to negative stimuli is also a hallmark of anxiety, as is explained in Chapter 1.

One element of this judgement bias task that sets it apart from others is that each honeybee was exposed only once to each test stimulus as compared to multiple exposures in the majority of judgement bias tasks. This was done in order to reduce the chance that they would learn about the non-reinforced outcomes, as has been exposed as an experimental disadvantage in a number of other tasks (e.g. Brilot et al. (2010), see Chapter 1). The presentation of the test odours in the test session was pseudorandomised to offset this effect.

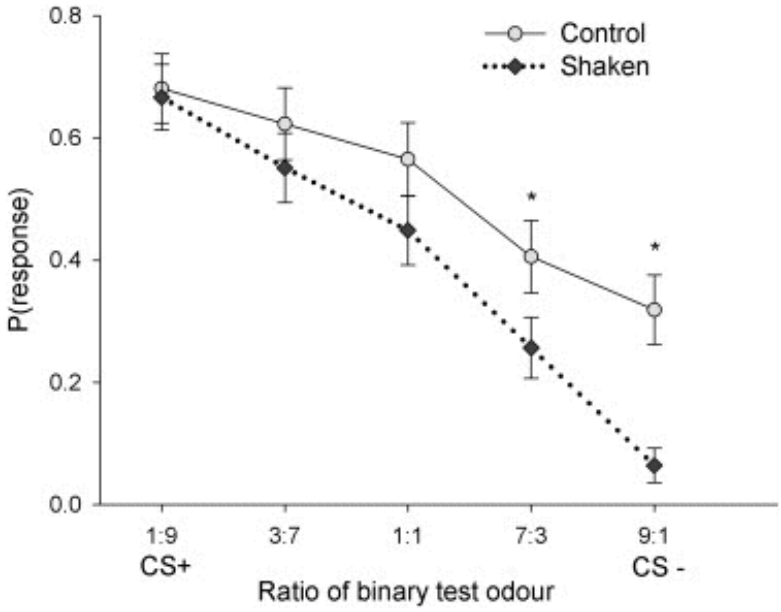


Figure 5-3 ***Shaken honeybees display a pessimistic bias.*** *When honeybees were subjected to shaking and then tested with the CS+, the CS−, and three novel odours, the slope of the gradient of the line indicating the proportion of bees that extended their proboscis [i.e., P(response)] became steeper.. The bees were significantly less likely to respond to the CS− and its adjacent novel odor (*$*p < 0.05$*). Reproduced from Bateson et al 2011*

Demonstration of associative learning in the judgement bias task

Bees can show conditioned responding following just one trial (Menzel, 2001) and were shown to discriminate between two floral odour combinations after six training trials of each type (i.e. CS+ and CS-; Bateson et al. (2011b)). The training requirements for associative learning in bees is substantially less compared to that in mammals, where hundreds or even thousands of training trials are required. In mammalian tasks, the animals must meet some criterion of accurate discrimination responses in training sessions before they undergo testing sessions. Typically they undergo many training sessions and have to display consistent and high response accuracy (e.g. >75% accuracy over five consecutive training sessions (Brydges et al., 2012)). With such few trials in the honeybee, it is difficult to gauge where the discrimination has been acquired and to set an appropriate criterion for differential responding. In Bateson et al. (2011b) bees were not excluded from the analyses in accordance with their performance during training or testing. During training, all of the honeybees were exposed to the same conditioning procedures, and it was argued that their learning of the associations between the learned odours and their appetitive outcomes was not necessarily reflected in whether they responded correctly or not. In this chapter I compare the outcomes of analyses of the honeybees' responses on the judgement bias task where moderate, stringent or no inclusion criteria were set.

5.I.iv Hypothesis

In humans, emotion related to sickness manifests as a reduced expectation of positive events occurring, and it is hypothesised that this will be similarly exhibited by the honeybees in this experiment in the form of fewer responses to ambiguous cues. In chapter 4, I observed pronounced sickness behaviour when honeybees were injected with toxins. In line with the view that emotional changes comprise of behaviour, physiology and cognition, we expected that honeybees would display a cognitive bias when administered toxins that produced behavioural sickness. Although a general suite of behaviours was identified to correspond to sickness behaviours in the honeybee, we found that each toxin contributed differentially to the behavioural

displays. For example, amygdalin-malaise manifested with a reduction in walking and increase in grooming behaviours, whereas LiCl caused bees to spend more time upside down and curled up and quinine injection produced a considerable amount of abdomen dragging. How these behaviours relate to specific negative emotions (i.e. anxiety or depression) is unclear, so no differential predictions were made with regards to the manner by which the ambiguous stimuli might be biased in the task (i.e. whether changes in responding occurred at the rewarding or the punishing end of the stimulus-spectrum).

5.II Methods

5.II.i Induction of sickness in the honeybee

Toxins were injected rather than fed to the bees in this experiment to allow for more precise control of the dosages received. The doses were selected by a small pilot study (data not shown) to ascertain the mortality of the bees administered each dose. Doses of the toxins which did not cause the mortality of bees were used and are outlined in 5.II.ii, but do not mirror the doses used in the behavioural study. The behavioural and judgement bias experiments took place during different times of year, and there are some seasonal variations in the bees' tolerance to toxins (London-Shafir et al., 2003). A cohort of bees used in the behavioural experiments (Chapter 4) during the winter was retrieved from an indoor colony with restricted nutrition and foraging opportunities, which may further account for differences in sensitivity to toxins.

5.II.ii Animals and apparatus

Adult foraging worker honeybees (*Apis mellifera Buckfast*) were collected from an outdoor colony at Newcastle University during the summer 2012. A screen was placed over the entrance to the hive and bees were collected on their return. After collection, the bees were subjected to cooling anaesthesia and harnessed using standardised techniques as in 4.II.i, ensuring that the thorax was accessible for injection. Once

harnessed each bee was fed to satiation with 1.0 M sucrose and kept at RT overnight prior to experimentation.

5.II.iii Treatments

Amygdalin (Sigma - Aldrich, Dorset, UK), quinine (Sigma - Aldrich, Dorset, UK), and LiCl (Sigma - Aldrich, Dorset, UK) were diluted in deionised water. Bees were cold anaesthetised 3 min prior to injection and a small hole was punctured in the thorax with a 19G DB Microlance syringe tip. One µl of a treatment solution was injected subcuticularly in the thorax with a Hamilton syringe. Treatments were: surgery (puncturing the thorax with no administration of fluid), deionised water, 10 mM amygdalin, 10 mM quinine, 100 mM quinine and 0.1mM LiCl. Treatments were administered in a pseudorandomised order so that a similar number of each were given throughout a test day so that bees from all of the treatment groups were equally exposed to any potential confounding effects of day or time.

5.II.iv Training and testing odours

1-Hexanol (hex, 99.8% purity, Sigma-Aldrich, Dorset, UK) and 2-Octanone (oct, 99.8% purity, Sigma-Aldrich, Dorset, UK) were diluted to 2.0M stock solutions with mineral oil and combined to produce the training and testing odour mixtures as outlined below. Odours were delivered in an air-puff at a two inch distance from the bee (Bateson et al., 2011b).

5.II.v The judgement bias task- immediate recall

Training of the judgement bias task began 18-24 h after harnessing. Feeding motivation was determined prior to training sessions by touching the antennae with a sucrose solution and observing extension of the proboscis. Bees that did not extend their proboscis were put aside for later training sessions and either retested or discarded, dependent on whether feeding motivation was later exhibited. Ten bees

pretested for feeding motivation were used in each conditioning session, where they were exposed to two CS-US combinations.

The CS consisted of mixtures of two odours: 9:1 Hex:Oct or 1:9 Hex:Oct (see Table 5-1). The CS+ was paired with a small droplet of 1M sucrose solution, and the CS- was paired with a small droplet of 0.01M quinine solution. For half of the bees the 9:1 Hex:Oct odour acted as the CS+ and 1:9 Hex:Oct acted as the CS-. This was reversed for the other half of the bees (Wright et al., 2007).

Odour combinations	Stimulus type	Group 1	Group 2
9:1 Hex:Oct	Conditioned	1M sucrose (CS_+)	0.01M quinine (CS_-)
7:3 Hex:Oct			
5:5 Hex:Oct	Tested	No outcome	
3:7 Hex:Oct			
1:9 Hex:Oct	Conditioned	0.01M quinine (CS_-)	1M sucrose (CS_+)

Table 5-1 ***Training and testing odour combinations.*** *The table shows the combinations of 2.0 M 1-hexanol (Hex)and 2.0M 2-Octanone (Oct) used as conditioned or tested stimuli in the judgement bias task. For group 1 9:1 Hex:Oct acted as the conditioned stimulus for reward (1.0M sucrose; CS+) and 1:9 Hex:Oct acted as the conditioned stimulus for punishment (0.01M quinine; CS-). For group 2 these stimulus pairings were reversed. For both groups the tested odours acted as intermediate, ambiguous stimuli.*

The CS-US combinations were presented 6 times each in a pseudorandomised order, (A B B A B A A B A B B A where A = CS+ and B= CS-) with a 5 min interval separating them. A bee was exposed to an odour for 4 s. During the first two trials (A and B) the antennae were stimulated during exposure to the odour with a small amount of the relevant solution (0.01M quinine or 1M sucrose) and this was fed to the bee if its proboscis was extended. This continued in subsequent trials unless the bee extended its proboscis in response to the odour before antennal stimulation, in which case the solution was directly fed to the bee. If the bee extended its proboscis with no antennal

stimulation in response to an odour this was recorded as a 'response'. If the proboscis was not extended in response to the odour this was recorded as 'no response'.

Following the 12 training trials the bees were immediately treated as in 5.II.iii. They were left to recover for 15 min before testing for the proboscis extension response with 5 odour mixtures including the two learned odour cues (1:9 and 9:1 Hex:Oct) and three previously unencountered 'ambiguous' odour cues (3:7, 1:1, 7:3 Hex:Oct; Table 5-1). Whether or not the bee extended its proboscis in response to each odour cue was recorded.

5.II.vi The judgement bias task- 24 hour recall

Bees have been shown to retain olfactory memory in conditioning tasks for 24-72 h post-training (Stollhoff et al., 2005), and we were interested determining whether this may influence the outcomes of the judgement bias task. A separate study was performed where bees were trained in an identical manner to 5.II.v, but the bees were not treated and tested until 24 h after training. The bees were fed 18µl 1.0M sucrose 1 h following training in order to facilitate survival over the 24 h period.

5.II.vii The effect of inclusion criteria on experimental outcomes

As there is currently no standardised criterion for including honeybees in analyses of the judgement bias task, the experimental outcomes of this task were compared when different levels of responding were displayed during the training sessions. Bees were included under three conditions; in the first, all bees were included regardless of their performance ('inclusive'); in the second, bees were included if they showed at least one response during the final three positive training trials ('moderate'); and in the third, they were included only if they responded positively to all three of the final CS+ training trials ('exclusive').

The choice to use the moderate criterion in the main analysis arose from the indication that it was sufficient in eliminating non-responders, as the honeybees continued to

respond to the positive and negative stimuli as they did in training sessions. The more stringent criterion eliminated too many of the data from the analysis and was deemed too exclusive.

5.II.viii Statistical analysis

Training data were measured as binary outcomes ('response' or 'no response') so a binary logistic Generalised Estimating Equations (GEE) was used with bee ID as a repeated subject variable, and trial type (positive or negative) and trial number as within-subjects variables. Trial type and trial number were included as factors. GEE's models are used to analyse binary data in preference to Generalised Linear Models when repeated measures need to be accounted for. The judgement bias test data were analysed with a binary logistic Generalised Estimating Equations, where bee ID was included as a repeated subject variable and test odour as a within-subjects variable. Test odour and treatment were included as factors. For all analyses pairwise comparisons were adjusted for multiple comparisons by least significant difference (lsd).

5.IV Results

5.IV.i Training data

The data were screened to probe responding on the final three positive trials in the training session. Data were excluded from subsequent analyses if the bee responded less than once during these trials, indicating that the discrimination was not learned. Additionally, bees were excluded from analyses due to mortality or experimental fault. A total of 238 bees (45.2%) were excluded from the original cohort of bees collected for experimentation.

Bees were significantly more likely to respond to positive trials than negative trials during the training sessions (GEE, main effect: $\chi^2 = 15.84$, df = 1, $p < 0.001$). They responded more during the first negative trial than the first positive trial (presumably generalisation of conditioned responding), but after these initial trials bees made more responses to the positive trials. Responding increased on successive trials (GEE, main effect: $\chi^2 = 169.07$, df = 5, $p < 0.0001$), and occurred significantly more on positive trials (GEE, trial type x number interaction: $\chi^2 = 73.02$, df = 5, $p < 0.001$) indicating that the bees had learned the discrimination.

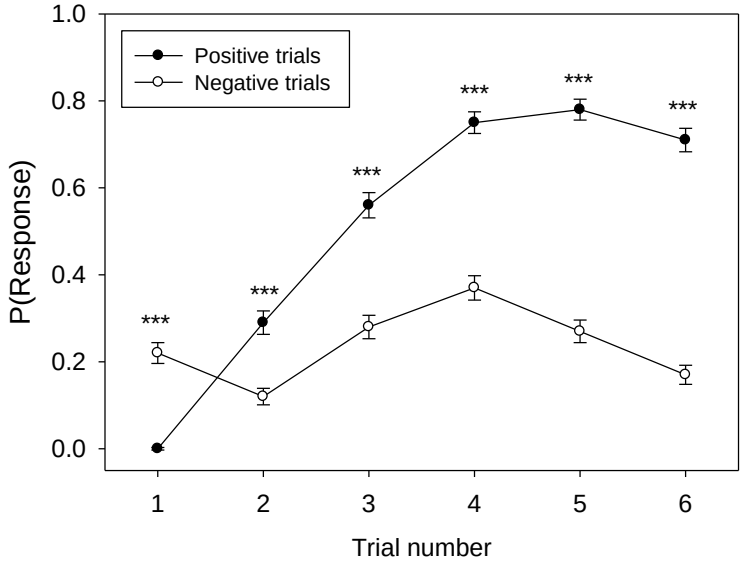


Figure 5-4 ***Honeybees learned to discriminate the training odours.*** *The graph shows the mean proportion of trials responded to during training sessions consisting of twelve positive and negative trials. The CS-US combinations were presented 6 times each in a pseudorandomised order (A B B A B A A B A B B A where A = CS+ and B= CS-). Symbols show means ±S.E.M. (***: p<0.001; n = 288).*

5.IV.ii Was generalisation of the odour cues affected by the injection of water following 'surgery'?

Bees' responding following water or surgery was compared to identify whether the injection of fluid itself caused a change in responding on the judgement bias task. The analysis indicated that there was no difference in responding following either treatment (GEE, main effect: $\chi^2 = 0.03$, df = 1, p =0.869), and no interaction of the treatments and test odours (GEE, treatment x test odour interaction: $X^2 = 1.12$, df= 4, p = 0.891).

The data from the two groups were subsequently consolidated in order to form a larger control group to improve the statistical robustness of subsequent analyses.

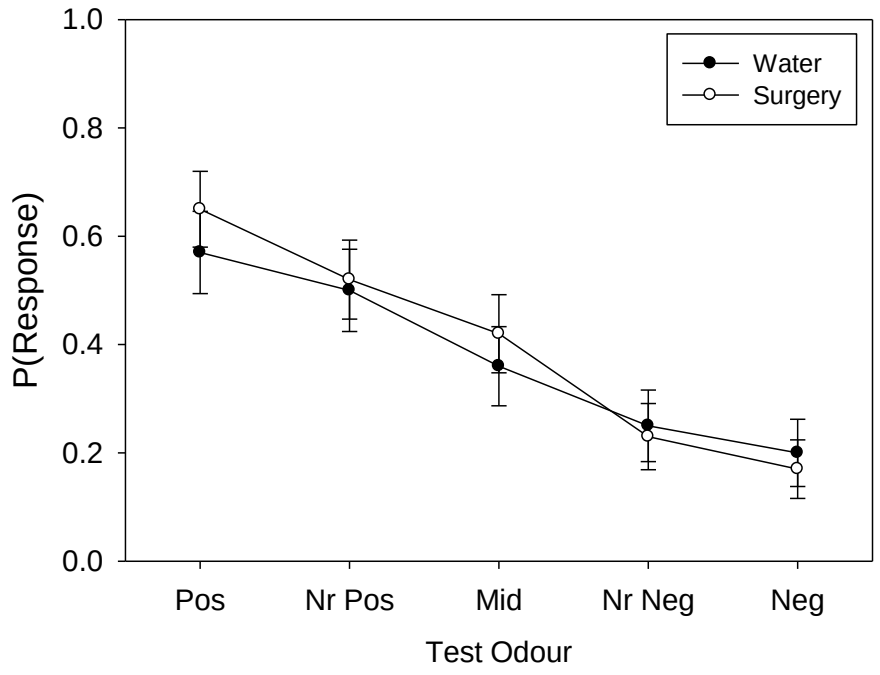


Figure 5-5 ***Proportion of trials responded to during the ambiguous cue testing session: water vs. surgery*** *Water (n = 44); surgery (n = 48). No significant differences were found by pairwise comparisons.*

5.IV.iiiDo honeybees generalise odour cues in the judgement bias task?

Analysis of the bees' responses during the judgement bias task showed that the combinations of the odours significantly affected whether or not the bees under control conditions performed a response (GEE, main effect: $\chi^2 = 23.92$, df = 4, p <0.001). As predicted, the bees generalised conditioned responding from the sucrose-rewarded odour, and responded no differently to the Nr Pos and Pos odour mixtures (pairwise comparisons: df = 1, $p = 0.124$). Also, the bees generalised conditioned responding from the odour associated with quinine, and responded with PER less when presented with the Mid, Nr Neg and Neg odours than the positive odour (pairwise comparisons, all df = 1; Pos:Mid: $p = 0.024$; Pos:Nr Neg: p <0.001; Pos:Neg: p < 0.001). In addition, there was no difference in the proportion of responses performed by bees elicited by the Nr Neg odour mixture and the Neg odour associated with quinine (pairwise comparisons: df = 1, $p = 0.724$). This indicates a generalisation of the odour cues.

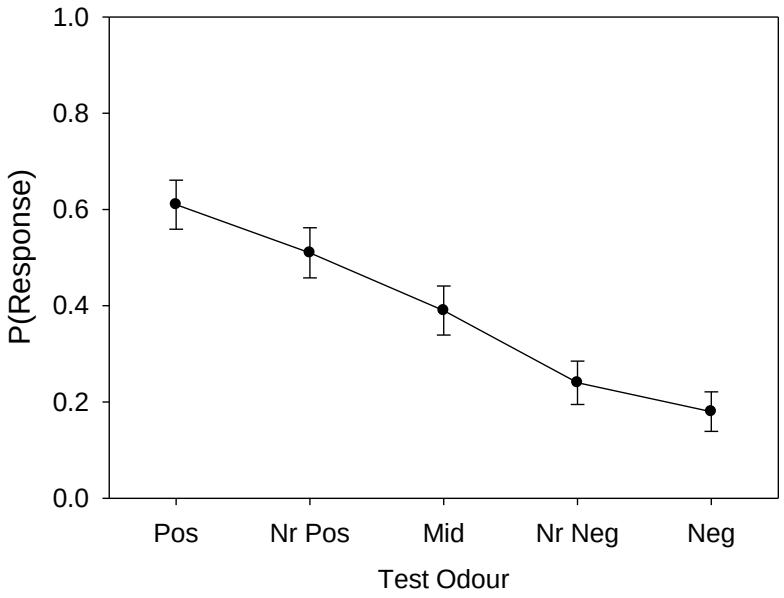


Figure 5-6 ***Proportion of trials responded to during the ambiguous cue testing session: cue generalisation*** *The graph shows the proportion of trials responded in ambiguous cue testing sessions. Bees showed generalisation of the cues, where cues with odour mixtures intermediate of the learned Pos and Neg cues were responded to proportionally less in a correlative manner to their decreasing similarity to the Pos cue.(n = 92).*

5.IV.iv Can bees generalise odour cues 24 h after training?

When bees were treated and tested 24 h after learning the task, they were unable to discriminate the two learned odour cues or generalise the intermediate ambiguous cues. Instead, responding was 'flattened', and was significantly different to that seen after immediate testing (GEE, main effect: $\chi^2 = 8.96$, df = 1, $p = 0.003$). Although there was no interaction between the test odour and the time period between training and testing (GEE, test odour x time period interaction: $\chi^2 = 7.77$, df = 1, $p = 0.100$), pairwise comparisons indicated that the greatest divergence in responding between the Pos, Nr Pos and Mid odour cues after 15 min and 24 h testing (Figure 5-7).

Due to the stunted responding observed in control bees after 24 h instead of the predicted improvement in memory recall, only the responses from the 15 min test period were analysed for all subsequent experiments.

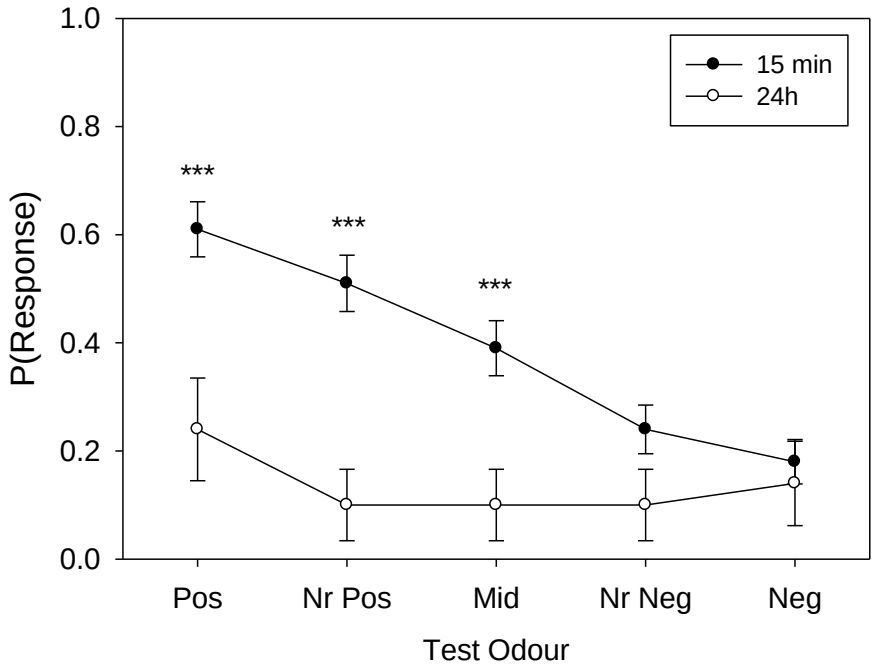


Figure 5-7 ***Proportion of trials responded to during the ambiguous cue testing session: 15 min vs. 24 h*** *The graph shows the proportion of trials responded in ambiguous cue testing sessions by bees treated with water either 15 min or 24 h after training. Symbols show means ± S.E.M. (*:p < 0.05; **: p<0.01; ***: p<0.001; 15 min: n = 92, 24 h: n =21).*

5.IV.v Does injection of toxins bias bees' judgements of ambiguous odour cues?

Similarly to the control bees, bees administered malaise-inducing toxins generalised the odour cues (GEE, main effect, odours: $\chi^2 = 150.14$, df = 4, $p < 0.0001$) where more responses were elicited by the Pos and Nr Pos odours, than by the Neg and Nr Neg odours. The injection of toxins had a significant influence on the average rate of response during the test (GEE, main effects, treatment: $\chi^2 = 10.55$, df = 4, $p = 0.032$). Although no interaction was specifically found between the treatments and the test odours (GEE, treatment x test odour interaction: $\chi^2 = 16.61$, df = 16, $p = 0.411$), pairwise comparisons were performed for each test odour to identify whether responding towards ambiguous odours differed between control and malaise treatment.

5.IV.v.1 Amygdalin

Bees injected with amygdalin were less likely to respond during the judgement bias task than control bees throughout the test sessions (pairwise comparisons; df = 1, $p = 0.001$). This reduction in responding occurred when bees were presented with the Nr Pos and Mid odours, as well as the odour associated with a quinine punisher (Neg, Figure 5-8).

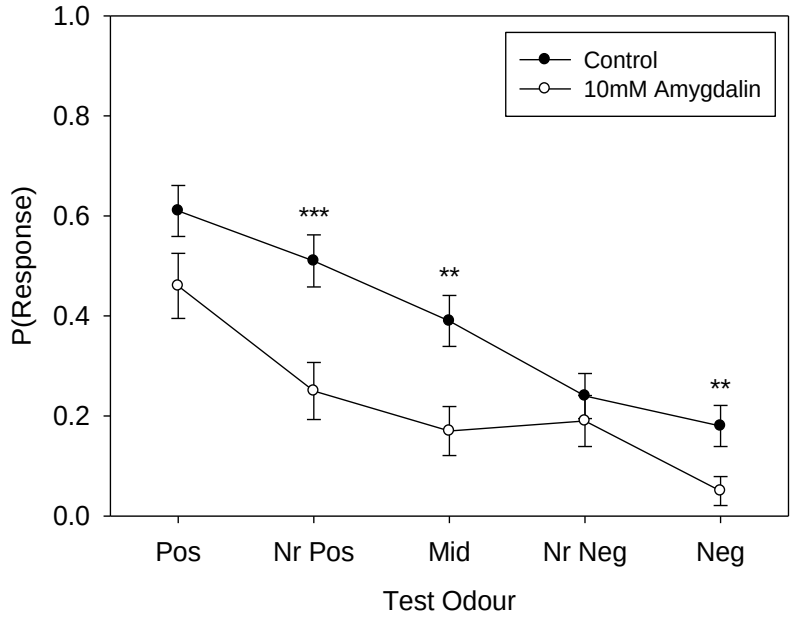


Figure 5-8 ***Proportion of trials responded to during the ambiguous cue testing session: control vs. amygdalin*** *The graph shows the proportion of trials responded to during the ambiguous cue testing sessions. Symbols show means +S.E.M. Significant differences were identified in pairwise comparisons (*:p < 0.05; **: p<0.01; ***: p<0.001).Control (n = 92), 10mM Amygdalin (n = 59).*

5.IV.v.1 Quinine

Although responding as a whole was not different when bees were injected with 100mM quinine (pairwise comparisons: df = 1, p = 0.132), pairwise comparisons showed a reduction in responding to the Mid odour (Figure 5-9, top panel). Unlike bees that had been injected with amygdalin, responding to the learned odours was retained. No difference in responding as compared to the control bees was observed when bees were injected with the lower dose (10mm) of quinine (pairwise comparisons: df = 1, p = 0.203; Figure 5-9, bottom panel).

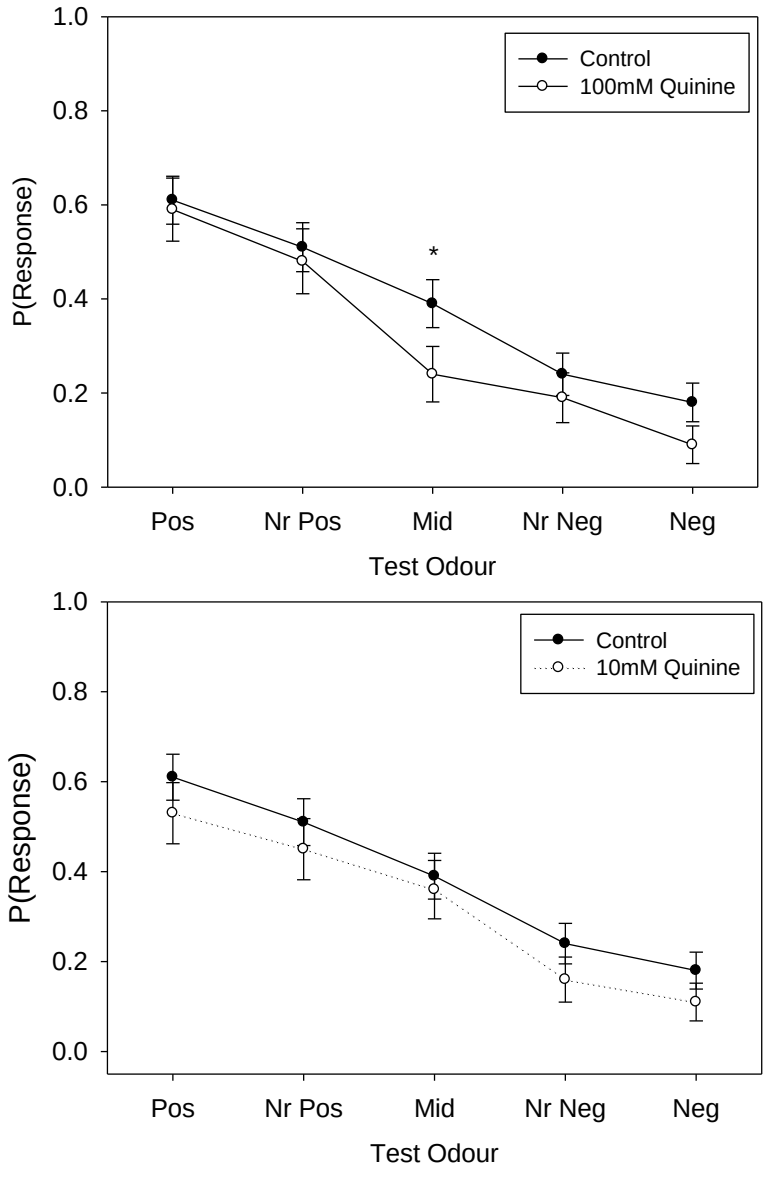


Figure 5-9 ***Proportion of trials responded to during the ambiguous cue testing session: control vs. quinine*** *The graphs show the proportion of trials responded to during the ambiguous cue testing sessions. Symbols show means +S.E.M. Significant differences were identified in pairwise comparisons (*:p < 0.05; **: p<0.01; ***: p<0.001).Control (n = 92), top panel) 100mM Quinine (n = 54); bottom panel) 10mM Quinine (n= 55).*

5.IV.v.2 Lithium chloride

Bees' responding following LiCl injection was not altered as compared to the control bees (pairwise comparisons: df = 1, p = 0.945; Figure 5-10).

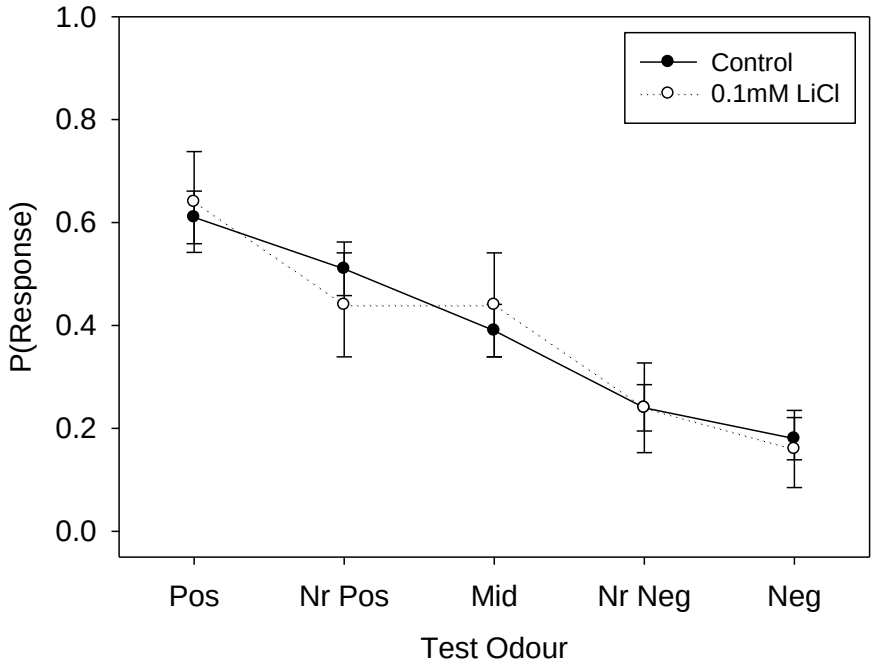


Figure 5-10 ***Proportion of trials responded to during the ambiguous cue testing session: control vs. LiCl*** *The graph shows the proportion of trials responded to during the ambiguous cue testing sessions. Symbols show means +S.E.M. No significant differences were identified by pairwise comparisons. Control (n = 92), 0.1mM LiCl (n = 25).*

5.IV.vi Does performance during training affect interpretation of the judgement bias task?

In this section the effect of inclusion criterion on the outcomes of the task was investigated. We observed a generalisation of the odour cues in a progressively downward manner throughout these analyses, which showed that this effect was robust and independent of these criteria (Inclusive: GEE, main effect, odours: χ^2 = 150.14, df = 4, p <0.0001; Moderate: GEE, main effect, odours: χ^2 = 130.56, df = 4, p <0.0001; Exclusive: GEE, main effect, odours: χ^2 = 89.73, df = 4, p <0.0001). The slope of the generalisation curve is however shallower when all of the bees were included

(Figure 5-11), which indicates that a proportion of the bees that did not respond during training also did not respond throughout the test session. These bees were consequently excluded from the other analyses.

Treatment did not affect the responses to the test odours when all bees were included in the analysis (inclusive: GEE, main effect, treatment: $\chi^2 = 2.34$, df = 4, $p = 0.673$), or when the most restrictive criterion was set (exclusive: GEE, main effect, treatment: $\chi^2 = 4.53$, df = 4, $p = 0.339$). This is contrary to a main effect detected in the analysis where a moderate criterion was used (GEE, main effects, treatment: $\chi^2 = 10.55$, df = 4, $p = 0.032$).

When all bees were included, there was a significant interaction of odours and treatments, so although there was no overall effect, the treatment influenced responding on specific odour cues (inclusive: GEE, treatment x odour interaction: $\chi^2 = 26.93$, df = 16, $p = 0.043$). A significant interaction of odours and treatments was not observed when the exclusive criterion (exclusive: GEE, treatment x odour interaction: $\chi^2 = 20.16$, df = 16, $p = 0.213$), or moderate criterion was used (moderate: GEE, treatment x test odour interaction: $\chi^2 = 16.61$, df = 16, $p = 0.411$), but the pairwise comparisons were mined for significant effects to allow a contrast of response outcomes between the 3 criterion contingencies.

In the 'inclusive' analysis, pairwise comparisons of these interactions showed that responding was reduced when bees were exposed to the Mid odour stimulus when injected with amygdalin (Figure 5-11), which is indicative of a negative bias. In contrast to this, we found that when the stringent criterion was used, responding to both the Pos and Neg stimuli was reduced following amygdalin injection, but there was no difference to those deemed ambiguous. Interestingly, when the moderate criterion was applied, we observed a combination of these two outcomes, where there was a reduction in responding to ambiguous stimuli as well as the learned Neg stimulus.

Following administration of 100 mM quinine, bees demonstrated increased responding towards the positive odour when all bees were included (Figure 5-12). This is in contrast to a negative biasing towards ambiguous cues that was observed when the

moderate criterion was used, and in contrast to a null effect when the exclusive criterion was employed.

There was no difference in the performance of bees injected with 10mM quinine or 0.1mM LiCl as compared to the control bees, regardless of the inclusion criterion (data not presented).

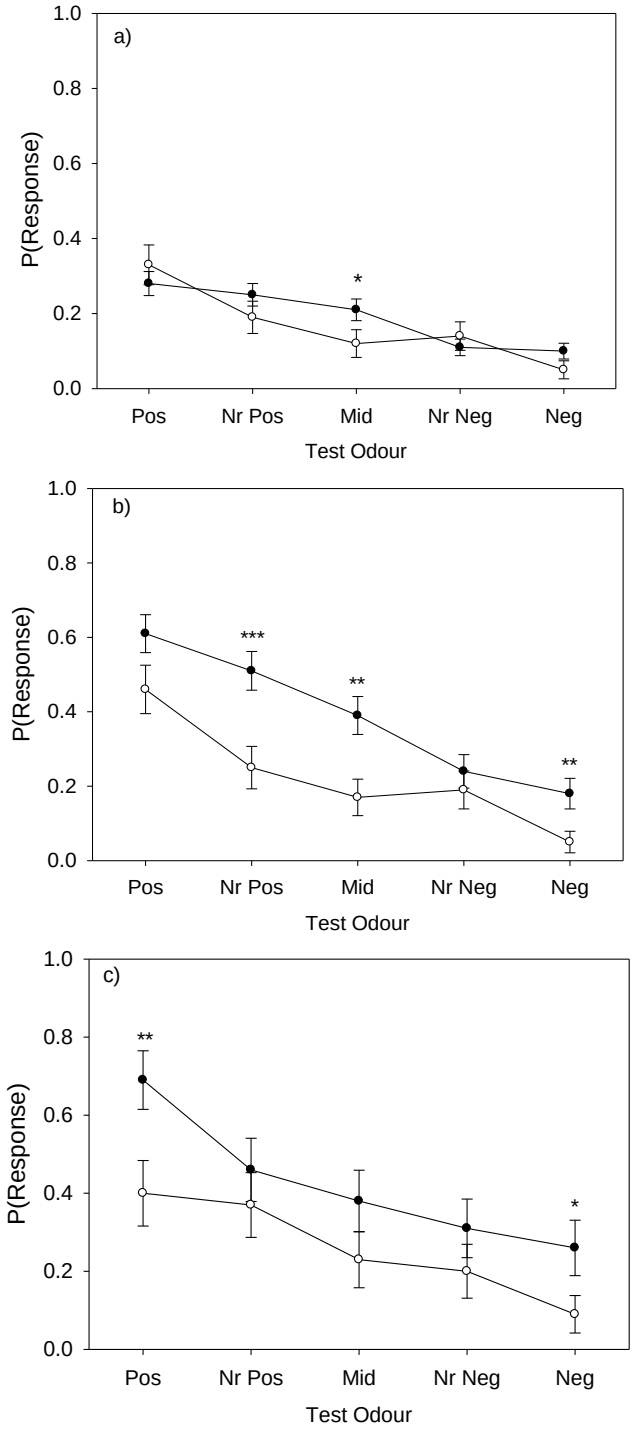


Figure 5-11 ***Effect of Inclusion criteria on the proportion of trials responded to during the ambiguous cue testing session: amygdalin*** *The graphs show the outcomes of the judgement bias task when bees were administered 10mM amygdalin (open circles) as compared to the controls (closed circles). The inclusion criteria were a) all bees; b) bees that performed the PER at least once during the final three positive trials in training; and c) performed the PER on all of the final three positive trials during training. (*:p < 0.05; **: p<0.01; ***: p<0.001).a) Control (n = 205), 10mM Amygdalin (n = 81); b Control (n = 92), 10mM Amygdalin (n = 59; c) Control (n = 39), 10mM Amygdalin (n = 35).*

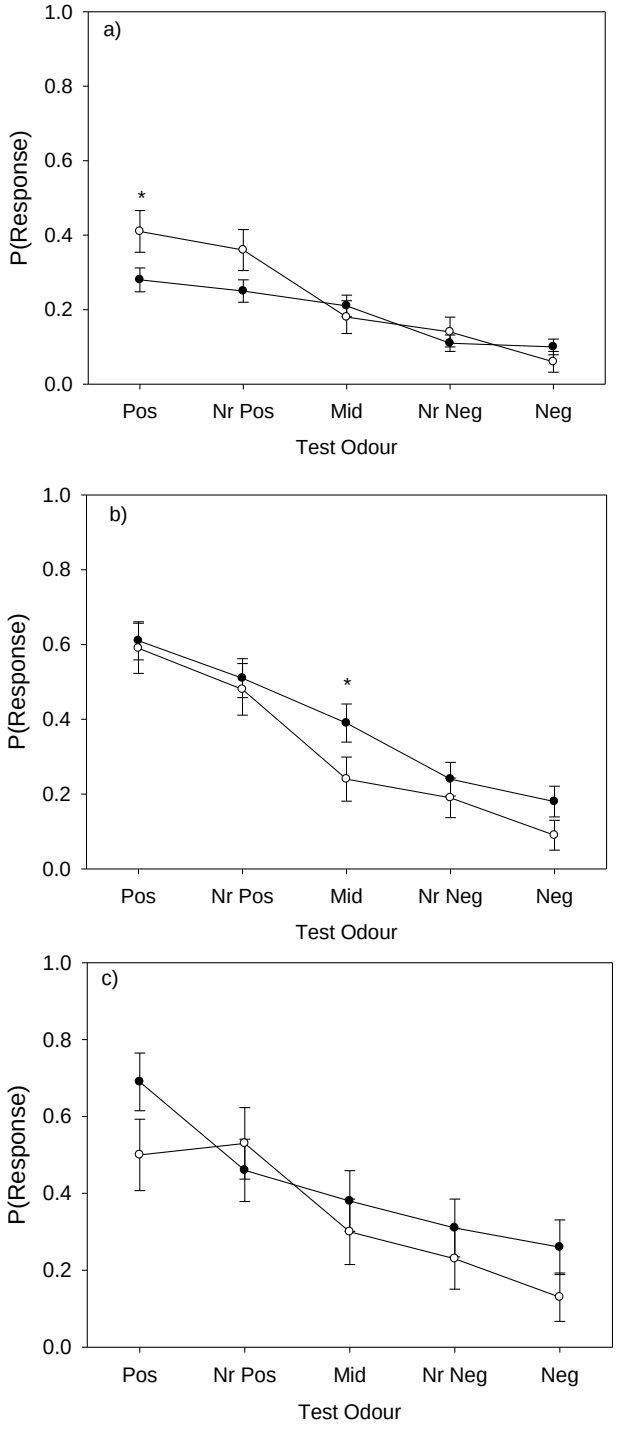


Figure 5-12 ***Effect of inclusion criteria on the proportion of trials responded to during the ambiguous cue testing session: quinine*** *The graphs show the outcomes of the judgement bias task when bees were administered 100mM quinine (open circles) as compared to the controls (closed circles). The inclusion criteria were a) all bees; b) bees that performed the PER at least once during the final three positive trials in training; and c) performed the PER on all of the final three positive trials during training. (*:p < 0.05; **: p<0.01; ***: p<0.001).a) Control (n = 205), 100mM quinine (n = 78); b Control (n = 92), 100mM quinine (n = 54; c) Control (n = 39), 100mM quinine (n = 30).*

5.V Discussion

5.V.i Honeybees generalised the ambiguous cues in the judgement bias task

This study is the first replication of the honeybee judgement bias task originally developed by Bateson et al in 2011. Their methods of training and testing the honeybees, as well as general husbandry practices, were followed. As in Bateson et al. (2011b), the honeybees were able to discriminate between the two odours, learning that one predicted a sucrose reward and the other a quinine punishment. The proportion of responses that bees made to these training stimuli were comparable, where bees under the control conditions responded to the Pos odour ~61 % in this study, and ~67% in Bateson et al; and ~19% to the Neg odour in this study, and ~35% in Bateson et al (see Figure 5-3 and Figure 5-6). The intermediate ambiguous cues were also generalised to a similar extent. The variance in responding to the Neg odour between our studies may have resulted from the different exclusion criteria used in these two studies. A number of bees that did not display sufficient discrimination during learning were excluded from this experiment, whereas Bateson et al. (2011b) do not explicitly state any exclusion criteria. Stricter or less stringent criteria affects the interpretation of experimental outcomes (see 5.V.iii), showing the importance of excluding bees that had not learned the task to avoid confounding results. It also demonstrated the vulnerability of this data to type II errors, as when the sample sizes were further reduced with the exclusive criterion no significant effects of treatment or interactions of treatments with particular cues were identified. It would be preferable for standardised criteria to be included in future studies. Below I discuss the outcomes of the analysis where the moderate criterion was employed.

The stress of surgery and injection did not impair the retention of the bees' memory of the trained stimuli during the test sessions. A comparison of Figure 5-4 and Figure 5-6 shows the initial discrimination of the odours by the bees injected with the toxin vehicle (water) or that underwent surgery only. During training bees responded 70% of the time to the positive odour stimulus and 20% to the Neg stimulus, and during testing responses were 61% to the positive stimulus and 19% to the negative stimulus. This implies that the value of the reward and punisher was unaltered as the

discrimination of the two was retained. The bees also demonstrated generalisation towards the ambiguous odour mixtures (Wright et al., 2007, Wright and Schiestl, 2009). The progressive and orderly gradient of the response curve reflects the bees' relative perception of the ambiguous odours, where levels of responding to the ambiguous odour mixtures most resembled that of the learned odour cue that it was closest to on the stimulus spectrum (Chapter 1). As the honeybees showed retained memory of the task and generalisation of the intermediate cues, we can confidently go further to assess the changes in cue generalisation by toxin administration.

5.V.ii Responding was altered following injection of toxins

Our data showed that the injection of toxins caused the honeybees to alter their generalisation towards ambiguous cues. We can also conclude that these effects on responding were catalysed by the contents of the toxin solutions and not by the injection of fluid (Figure 5-5), as no changes in bees' responding were observed when they were injected with deionised water compared to bees that had solely undergone the process of surgery.

5.V.ii.1 Amygdalin

When honeybees were injected with amygdalin we observed a parallel shift in responding, where responses were reduced during the presentation of odour stimuli at both the positive and negative ends of the scale. If we interpret this in terms of the judgement bias model (Chapter 1), it could be theorised that bees experienced a decrease in expectation of the occurrence of positive events and also an increased expectation of negative events. This is in line with the idea that sickness can produce both symptoms of depression and anxiety (Schiepers et al., 2005), and, if confirmed, could prove the honeybee to be a potential model for exploring the neural causes of the psychological symptoms of sickness.

However, the data could also be interpreted as showing that bees undergo a general attenuation of the proboscis extension response (i.e. feeding) following amygdalin

administration. This is different to an absence of responding, where we would see a horizontal flattening of the generalisation curve (as in Figure 5-7 where honeybees no longer responded following a 24 h gap between training and testing).

A previous study showed that 5-HT was the neurotransmitter governing learned aversions towards foods containing amygdalin, and that consumption of amygdalin inhibited the PER (Wright, 2011). In preference tests bees have been shown to demonstrably reduce their intake of amygdalin-containing foodstuffs when alternatives are available (London-Shafir et al., 2003), indicating that there is some level of aversion to this toxin.

5.V.ii.2 Quinine

Bees injected with the highest dose of quinine exhibited a shift in the generalisation gradient that is consistent with our interpretation of the expression of a judgement bias (Chapter 1). The bees demonstrated a reduced anticipation of the sucrose reward when presented with the most ambiguous (Mid) odour. Like amygdalin, this might indicate that toxin-induced sickness produces emotional effects in the honeybee, or more specifically a decrease in emotional valence. Here this result is more robust as bees responded in a similar manner to the Pos and Neg odours whether injected with quinine or acting as a control showing that there was no impairment of the discrimination, or deficits in responding related to motivation. Thus we can more confidently conclude that these bees displayed a bias in their judgement of ambiguous information rather than a general attenuation of the PER. Quinine injection therefore might provide a comparatively more reliable model of reduced affect produced by sickness.

5.V.ii.3 LiCl

Following injection of LiCl at a dose where sickness related behaviours are observed (see Chapter 4), we observed no change in the bees' abilities to discriminate between the two learned cues and also no alteration in the generalisation of the ambiguous cues. This would indicate that LiCl-induced sickness produces no change in a honeybee's affective state. It might be that although LiCl produces an observable, physical malaise, it is not be perceived by the bee and cognition is unaffected. Salts may not inhibit feeding like the other toxins, and perhaps cannot be internally detected by the same neural circuits. However it is also possible that honeybees may consume or avoid toxins to a different extent due to seasonal variations (London-Shafir et al., 2003), and we could also surmise that perception of toxic effects may also be subject to this variability.

5.V.iii Interpretation of the judgement bias task outcomes was affected by inclusion criteria

As is displayed in Figure 5-11 and Figure 5-12, our data demonstrate the sensitivity of interpretation of the judgement bias task to alterations of the inclusion criteria. The gradients of the generalisation curves were much shallower when the entire cohort of bees was included in the analyses. For instance, if we compare the responding of the control bees presented with the Pos stimulus during testing, the proportion of bees responding ranges from just 28% when we include all of the bees ('inclusive'), to 69% when only the most trained bees are included ('exclusive'), which was just marginally higher than the 61% that responded when the moderate criterion was adopted ('moderate'). It was apparent that a number of the bees that did not respond during training also failed to respond during testing, so the use of inclusion criteria minimises the effects of non-responders on the overall analyses.

Additionally, the selection of bees for inclusion in the analyses affected the explanation of the toxins effects on judgement bias. When we interpret the outcomes of injecting honeybees with 100mM quinine, we will either deduce that it produced motivational effects that increased their responding to the learned Pos stimulus

('inclusive'); that it produced a reduction in the expectation of reward synonymous to a more negative affective state ('moderate'); or that it had no effect whatsoever ('exclusive'), depending on the criterion used. Equally, when we come to describe the effects of 100mM amygdalin on judgement bias in the honeybee, it could be interpreted to produce a negative bias if all subjects were included; to affect the motivation for the bees to respond if we choose to only analyse the most trained bees; or a combination of both if we use the moderate criterion. This standardised criterion for subject inclusion is therefore recommended for future studies.

5.V.iv Concluding comments

When we compare our experimental outcomes with those of the experiment in Bateson et al. (2011b) it is apparent that the changes in response patterns observed following toxin administration do not mirror that seen when bees were shaken. The shaken bees were described as displaying an anxiety-like state as reflected by reduced responding to the more negative cues (Bateson et al., 2011b). The bees administered amygdalin in this study showed reduced responding to both the Pos and Neg cues, whereas the bees administered quinine displayed a bias only to the Mid cue. This suggests that bees in different emotional states may alter their responding to different cue types; where less responding to positive cues reflects depression-like states and less responding to negative cues reflects anxiety-like states. We have therefore demonstrated further potential for the bee judgement bias task to identify a variety of emotional states, and it would be interesting to replicate this task with additional affective manipulations to confirm this.

This judgement bias task does however fall victim to the difficulty in interpretation of go/no-go tasks, where emotionally-provoked reductions in responding towards stimuli cannot be distinguished from a general reduction of the motivation to respond (see Chapter 1). Groups that have employed judgement bias tasks with other species tend to use a choice task where both positive and negative cues have to be responded to, so they can more confidently interpret activity on negative trials. There has not yet been such a task developed for the honeybee, which would be the next logical step in

pursuing this investigation. Unfortunately, in the absence of knowing whether reductions in optimistic responding are accompanied by increases in pessimistic responding, we cannot conclude as to whether honeybees display a judgement bias following amygdalin-induced sickness.

The outcomes of the judgement bias task in this chapter were specific to the toxin type and also the dose administered. Counter-indicatively, where we saw malaise-induced behaviours, we did not see a malaise-induced judgement bias; a behavioural malaise was clearly indicated by LiCl injection, but it had no effect on bees' responding on the judgement bias task. Additionally, although we observed minimal alterations of behaviour in bees injected with amygdalin, there was a pronounced reduction in the PER in the judgement bias task. Quinine, on the other hand, produced both behavioural changes and changes in responding which were synonymous with a judgement bias, which suggests that bees experienced a more negative affective state when undergoing quinine-induced sickness. The disagreement between these experimental outcomes might suggest that our data are therefore not sufficiently conclusive for us to determine whether a general toxin-induced sickness produces a judgement bias, but do not rule out the potential to identify judgement biases associated with specific treatments. That the bees displayed behavioural and cognitive changes in association with quinine-sickness is a step towards development of a model of sickness. Bees possess 85,000-fold fewer neurons to humans (Giurfa, 2013), which leads to the tantalising prospect of a simplified model with which we can deduce neural pathways active in emotional response.

Chapter 6 - Discussion

In this thesis my aim was to explore the use of judgement bias tasks in emotion research. I was particularly interested in how judgement bias paradigms have been designed and implemented in the ten years since their introduction; to what extent sickness affected emotional responses; and whether an invertebrate model of sickness was comparable to a vertebrate model in a judgement bias task.

The opening chapter in this thesis discussed the measurement of affect in humans and non-human animals. There is growing circumstantial evidence pointing to the existence of affect in non-human species, which has in turn increased concerns regarding the degree that animals are capable of suffering emotionally (Broom, 1998, Broom, 2007, Boissy et al., 2007a). The use of novel non-verbal tests to measure emotional states of animals arose from difficulties that exist in the assessment of affective valence (Paul et al., 2005). One of these tests, the judgement bias task has been gaining popularity since its introduction in 2004 (Harding et al.).

The first chapter included the first complete critical evaluation of judgement bias studies to date (but see Hales et al. (2014) for a review of modelling judgement biases in MDD using rodents), where I described the parameters currently used to train discriminations in animals and to examine biases in their assessment of ambiguous information. Although these judgement bias tasks are based on the same principles of measuring emotional valence under ambiguity, there is little agreement as to how exactly these experiments should be performed. I reviewed the methodology and research outcomes of existing tasks to identify opportunities for their design to be streamlined.

The first research question that I considered was whether exposure to sickness-inducing toxins changed the affective states of animals, and the remaining chapters touched on the behaviour and cognition of the rat and the honeybee when administered LiCl. This compound is used to model states of sickness and nausea in experimental animals, and can cause them to find neutral or normally rewarding situations aversive (Garcia and Koelling, 1967, Hernandez et al., 2011, Parker et al.,

2008). I performed experiments to determine whether the negative aspects of sickness were also accompanied by a negative affective state.

Secondly, I asked whether any emotional response to sickness would manifest in a similar manner in a vertebrate and an invertebrate animal. I performed measurements of spontaneous behaviour and judgement bias tasks with a rat model and a honeybee model of LiCl-sickness.

6.I The judgement bias paradigm

My primary finding from the judgement bias tasks review was the prevalence of an incompatibility of the task design to answer research questions related to particular affective states. This occurred in one of every three studies, and exclusively where the affective manipulations were predicted to produce anxiogenic or anxiolytic states. These states are expected to influence an animal's expectation of aversive events, but the tasks did not include aversive outcomes. The judgement bias paradigm has been predominantly used to test hypotheses in the animal welfare domain (83% of studies used it to investigate affective manipulations associated with welfare concerns), so perhaps these researchers are less willing to expose animals to aversive stimuli. However, in attempting to measure the emotional state of anxiety without relevant anxiogenic stimuli, the original research question – whether animals are more or less anxious - cannot be answered. In the experiments in this thesis, I predicted that we might observe changes in cognition during sickness that were indicative of anxiety- or depression-like states (or both), and to this end I used judgement bias tasks that measured the anticipation of both reward and punishment.

6.I.i 'Go/no-go' vs. choice tasks

Possibly the second-most pressing issue that I identified was the prevalence of the 'go/no-go' approach to measure biases in judgement (30% of all studies reviewed). These tasks are quite popular due to the ease of training; and, when measuring an avoidance response, can identify the anticipation of negative events without excessive

exposure to the aversive stimuli as compared to choice tasks. However, as discussed in Chapter 1, these tasks are subject to ambiguities in their interpretation, where changes in motivational or affective states cannot be disentangled. In fact, Harding (2002), who created the judgement bias paradigm, recommended that two-choice tasks be used in preference to 'go/no-go' tasks. In such tasks, affect can be separated from motivational deficits by allowing the animal to perform an alternative response that can be easily distinguished from a response omission. This line of reasoning has been adopted by the majority of authors of judgement bias studies since this first task (e.g. Burman et al. (2008), Parker (2008) etc.).

In my 'go/no-go' experiments (Chapter 5), there were instances where honeybee performance was altered. Specifically, injection of quinine caused bees to respond less to an ambiguous scent cue. However, I was unable to conclude whether this was due to a decreased anticipation of reward (indicative of negative affect), or whether the bees had learned that test odours were not reinforced and attenuated their responding accordingly. Whilst it is possible that the injection of this toxin caused negative affect in the bee, this selection of a 'go/no-go' judgement bias paradigm was not the most suitable to uncover it. Development of a choice task for invertebrates would be a logical progression to probe this further, and already we have some methodological options to aid our pursuit of this goal. For instance, harnessed honeybees have demonstrated discriminated learning by differential responding by turning their heads left or right in response to odour stimuli (Buckbee and Abramson, 1997), and free-flying bees have been trained to perform in a y-maze where they take a route based on visual stimuli (Giurfa et al., 1996). The flexible nature of the judgement bias paradigm means that tasks can be developed from any discrimination task where stimuli exist on a continuous scale, so it is likely that the these simple discriminations could be modified to more closely resemble the choice tasks used with other animals.

The difficulty encountered in interpreting 'go/no-go' tasks explains my use of choice tasks with rats in chapters 2 and 3. Here a reduction of responding was correlated with an increase of the opposite response, or with an increase in response omissions,

mitigating the ambiguity of involvement of non-emotive factors involved with changes in responding.

An important extension of the investigation of the differences between 'go/no-go' and choice methodologies would be implementing these tasks in parallel and determining whether the selection of task influences experimental outcomes.

The review also uncovered disagreement in interpretation of altered responding to reference cues during test sessions. A judgement bias is defined as a bias in the cognitive processing of ambiguous information (Burman et al., 2009, Tsetsenis et al., 2007), but in the judgement bias studies reviewed, some authors inferred that a change in responding to both reference cues and ambiguous cues indicated the display of a bias (e.g. Enkel et al. (2009), Boleij et al. (2012)). I propose that altered responding to reference stimuli results from inconsistent task performance, where a decline correlates with an impaired memory of the discrimination and/or a decreased motivation to respond. This illustrates a conceptual misinterpretation of what constitutes a judgement bias, and we perhaps need to be clearer in our definitions. A relevant example of reduced responding to reference cues occurred in chapter 5 where the cognitive effects of toxin administration in honeybees were examined. Here I found that injection with amygdalin caused a reduction in responding to ambiguous cues, which is predictive of a negative affective state. However, the bees also responded fewer times to the negative cue, which might indicate a general weakening of the discrimination. In these circumstances, I therefore could not conclude that a reduction in responding to ambiguous cues was the result of negative affect as it could also reflect an artefact of diminished responding on the task.

It is important to clarify our definition of biases and to agree how altered responding could be explained in 'non-emotional' terms so that we can continue to make consistent and objective assumptions of our data.

6.I.ii Methodological considerations

The experiments performed within this thesis clearly identified instances where minor changes to methodologies had profound effects on the conclusions drawn. For example, where animals are tested over multiple sessions, additional training parameters must be considered when designing these studies such as partial reinforcement and additional retraining. The consequence of the failure to do so was clear in this thesis. Without partial reinforcement of trained end points in Chapter 2, just a third of the rats that had met requirements to partake in testing were included in the final analysis of our judgement bias task, as the performance of the remaining two-thirds dropped below the criterion. Partial reinforcement attenuated response extinction in Chapter 3 where no rats were excluded from repeated test sessions.

Another influence of task design on the outcomes of judgement bias tasks was identified in this thesis, but is perhaps specific to only few studies. Here, honeybees were not trained to meet a particular criterion, and assessments were made *post hoc* of their learning abilities. Inclusion criteria for subjects in the analysis of this task greatly influenced our interpretation of the treatment effects (Chapter 5). The extent of an animal's discrimination abilities clearly has an effect on the task outcomes, and so criteria for displaying this discrimination should be set at a level sufficient to ensure consistent performance throughout testing.

I summarised the critical review with an outline of these and other factors that may affect the interpretation of judgement bias tasks, and concluded the chapter with a list of key questions that should be considered prior to their design. It is my view that researchers should be able to tailor the design of their experiment to answer their specific research questions, and I would recommend that a future project included further dissection of these training and testing methods and for guidelines to be published regarding the optimisation of task designs for a range of species.

6.II Sickness and negative affect in animals

The first research question addressed experimentally in this thesis was whether affective states were altered in a rat and a honeybee model of sickness. The aversive conditioning agent LiCl produced a behavioural component of sickness in both rats and bees (Chapters 3 and 4). However, no correlative changes indicative of negative affective state were measured by the judgement bias paradigm in either of these species (Chapters 3 and 5). LiCl injection instead produced a positive bias in rats and no bias in bees. These outcomes were inconsistent with *a priori* predictions of a negative bias developing. This illustrates that the assessment of affective state during sickness is not as straightforward as hypothesised. There are at least two explanations for this outcome: either the judgement bias task was not sensitive enough to assess this aversion, or the aversion does not materialise in the form of more negative affect.

6.II.i LiCl aversion as an emotional response

An aversion produced by LiCl administration was demonstrably present in the form of sickness behaviours and the rejection of a novel foodstuff, but perhaps these responses do not necessarily involve an emotional component. This is counterintuitive as there is a functional argument for emotional involvement, where a negative association of sickness would reduce an animal's future contact with toxic substances, and enhance survival (Rozin and Kalat, 1971).

The classification of LiCl-induced aversion (here implicated as one of many inducers of 'disgust') as an emotion has recently been the subject of debate (Toronchuk and Ellis, 2007, Panksepp, 1998). Toronchuk and Ellis (2007) presented their case for disgust to be categorised as an emotion, where they explained how it fulfilled a number of criteria set out by the prominent emotion researcher Panksepp (1998) to qualify as an emotional response. They argued that disgust, like other emotions, is accessed by certain unconditional emotional stimuli, and these can be in the form of taste, olfactory, auditory, tactile, and/or visual cues (Curtis and Biran, 2001). They also described how disgust is capable of activating and regulating complex cognitive strategies and can influence the hedonic value of normally pleasurable substances or

activities (in this case – taste), which is also a key feature of emotion (Panksepp, 1998). Disgust also shows similarities with other primary emotions like being accompanied by autonomic changes that are longer lasting than the sensory stimuli that caused its production, and it can be also be generated without the presence of external stimuli by the recall of salient situations (Levenson et al., 1990, Fitzgerald et al., 2004).

Panksepp (2007) countered these ideas by Toronchuk and Ellis (2007), and in a retort argued that disgust cannot be classified as an emotional response for a number of reasons. Firstly, disgust cannot be experienced in an abnormally excessive manner that leads to an emotional disorder. For example, while we might describe anxiety as a normal response to aversive events, anxiety disorders materialise when this system is hyperactive (Johnson-Laird et al., 2006, Bateson et al., 2011a). There are, however, no disorders related to individuals over-experiencing disgust. Obsessive compulsive disorders related to cleaning rituals could arguably be classified as a hyperactivity of the disgust system, but are more readily accepted as excessive fear (of bacteria and sickness) and so represent an anxiety disorder (American Psychiatric, 2013). Panksepp also argued that the feeling of disgust does not long outlast the precipitating circumstances that induce it, as was argued by Toronchuk and Ellis (2007), but is instead a stimulus-bound reflexive response. Finally, he argued that there are no discrete brain areas that can be stimulated to precipitate disgust, and that disgust exists only as a construct of learning, thus leaving many of his own criteria of emotional constructs unfulfilled. We could therefore argue that the lack of effect following LiCl administration is evidence that there is no emotional component of LiCl-induced sickness in animals.

However, the responding of honeybees administered other toxins did in fact match patterns of responding observed with reduced affect, so whether negative affect is selectively induced by some forms of toxicosis, but not others, requires further investigation.

6.II.ii Cognitive enrichment overshadows negative affect

This brings us to revisit the studies in the critical review that, like mine, reported counterintuitive judgement bias tasks outcomes. We might explain them as a consequence of inadequate task design as described earlier, or they might lead us to delve further into the assessment of manipulated emotional states. For example, it has been shown repeatedly that participation in the judgement bias task itself can be inherently rewarding for animals when their affective states have been compromised, where conversely a negative biasing of ambiguity had been anticipated (Keen et al., 2013, Sanger et al., 2011, Doyle et al., 2010a). There is a growing body of literature that suggests that performance of discrimination tasks can in fact generate positive affect in animals, and this is termed 'cognitive enrichment' (Milgram, 2003). Cognitive enrichment and other problem-solving activities have been implicated in the processing of rewarding activities via activation of the mesolimbic brain axis (Schultz, 2001). It is thought that positive affect arises in animals performing these tasks because they are stimulated to acquire strategies to cope with environmental demands, and this results in a greater control of their environments (Manteuffel et al., 2009). In addition, the anticipation of reward in itself can be gratifying (Bindra, 1978). Cognitive enrichment is even considered to have stronger rewarding effects than more traditional means of environmental enrichment (Manteuffel et al., 2009), where animals can habituate to novelty and lose interest in enrichment items over a period of days (Platt and Novak, 1997, Wells, 2004, Tarou and Bashaw, 2007).

Enrichment is thought to be more valuable for animals with more negative affective states, where the contrast of circumstances from 'bad to good' is substantially greater than from 'good to better'. In this case, anticipation of the task itself and/or the rewarding outcomes produces a greater state of arousal in animals with anxious- or depressive-like states. This may explain why these animals respond in a manner demonstrative of a greater expectation of reward during the judgement bias task. It would therefore be plausible to surmise that the positive affect observed in LiCl-treated rats was generated by an effect of cognitive enrichment; LiCl coloured the animals' perception of the task, and overshadowed the negative affect produced by

the treatment. It is unclear why this cognitive training improves affect in some judgement bias tasks and not in others, and this requires further attention.

Additionally, affective manipulations strongly hypothesised to alter affective states in animals were found to have no effect on judgement biases (Mueller et al., 2012, Keen et al., 2013, Titulaer et al., 2013, Verbeek et al., 2014). This phenomenon was encountered in Chapter 5 where animals were administered an aversive compound but showed no negative biasing of ambiguous stimuli.

It is of continuing importance to highlight experiments which do not meet hypotheses as much as it is to share instances where they do. These counterintuitive or null results contribute to the development of theoretical approaches of the measurement of animal emotion. Unfortunately, there tends to be a publication bias where data like these don't go further than individual laboratories, and it is hard to know how many other judgement bias studies have resulted in confusing data like my own. The availability of these studies would help us to build a more rounded perspective of how judgement bias tasks can add to our measures of affect in animals.

6.III Invertebrates in emotion research

The final question asked in this thesis was whether we could build on evidence for or against the replacement of some vertebrate animals in emotion research. The emotion system evolved from a system of survival circuits (LeDoux, 2012) and these circuits are thought to have originated in the most basic of life-forms (Macnab and Koshland, 1972). Homologies have been established in some of the basic mechanisms of emotions in vertebrates and invertebrates, for example the control of reward and aversion is similarly controlled by neurotransmitters (Hammer and Menzel, 1998, Wright et al., 2010, Vergoz et al., 2007). Interest in invertebrate models of human psychological disorders is building, but these models are mostly centred on Drosophila and in the investigation of anxiety (Iliadi, 2009), which is thought to be a hyperactivity of anti-predation mechanisms (Bateson et al., 2011a).

6.III.i Sickness behaviours in invertebrates

In this thesis, the majority of evidence was favourable to an argument of homology with vertebrate and invertebrate sickness. I found that bees displayed behavioural correlates of sickness that matched those of vertebrate animals (Hart, 1988), which adds weight to an already well-established argument for an evolutionary basis of sickness behaviours (Aubert, 1999). I expected that an accompanying negative affect might be present, as it is in humans.

6.III.ii Biased judgement in invertebrates

With administration of certain toxins, we observed changes in responding on the judgement bias task consistent with a negative bias. These cognitive changes were comparable to the previous honeybee judgement bias task which used entirely different affective manipulations (Bateson et al., 2011b), demonstrating the breadth of affective states measurable with this approach.

This bias in judgement was also akin to those seen in vertebrate animals, where aversive affective manipulations caused the honeybees to make fewer positive responses to ambiguous stimuli (Harding et al., 2004, Enkel et al., 2009, Murphy et al., 2013, Neave et al., 2013). This also parallels with judgement biases that are observed in humans (Mendl et al., 2006), which is favourable of the viewpoint that the honeybee might be developed further to model some human psychological disorders. We must, however, be cautious in our speculations; in the rat and the honeybee model, the same pharmacological manipulations did not produce parallel effects on cognition. Bees did not show a biasing in judgement with LiCl administration, and conversely rats displayed a positive bias. This calls into focus areas where this data may not be consistently translatable.

I tentatively conclude that the content of the final two chapters in this thesis adds to the existing evidence that invertebrate animals could be used to explore the behavioural and cognitive effects of sickness. A great deal more work is necessary to implement a judgement bias task synonymous with those used with vertebrate animals, but this progression should be pursued.

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