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Reward Learning Deficits as a Cross-Species Cognitive Biomarker of Negative Affective States

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Abstract

Animal welfare is an important topic of societal interest. Despite this, measuring animal affective states objectively and reliably is challenging. Animals are not able to directly tell us how they feel, so numerous methods have been developed to monitor affective state indirectly. Traditional physiological and behavioural welfare assessment methods have often been challenging to interpret. More recently, there has been interest in using affect-induced cognitive biases to examine affective state more specifically. However, these methods have also faced difficulties with reliable protocol design across species. This thesis investigates reward learning as novel method of assessing affect-induced cognitive biases. Reward learning deficits are a feature of human depression and may serve as a reliable cross-species biomarker of negative affect.

Previous work has demonstrated reward learning deficits in mice in putative negative states, using a novel associative learning task conducted in a T-maze. We progressed this by assessing reward learning in mice subjected to further affective state manipulations. Group-housed mice showed signs of reward learning deficits consistent with negative states, unlike singly-housed mice. However, when using repeated restraint as a stressor, neither control nor restraint animals could learn the task. Further experimental protocol optimisation and refinement is needed. To initiate investigation of reward learning and affective states in other species, a novel reward learning testing arena was also developed in chickens. Whether chickens can discriminate between different reward magnitudes in the arena still needs to be validated, before we can test whether reward learning deficits associate with negative affective states, in this species.

While previous work suggested that reward learning deficits could be used as a “cognitive” biomarker of negative affective states, this thesis highlighted some challenges with protocol design practicality and reliability. Future work needs to address this, if reward learning is to be widely applied for welfare assessment in practice.

Dedication and Acknowledgements

I would like to thank my supervisors Professor Emma Robinson and Dr Jennifer Davies for giving me the opportunity to undertake this project. I am very grateful for being able to become part of the Robinson group and pursue my interests in animal welfare. Particular thanks go to Emma for support and guidance while giving me the freedom to explore my own ideas. I would also like to thank Dr Megan Jackson for her encouragement and general enthusiasm throughout my time in the group.

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Another thank you goes to everyone in the Robinson lab for their friendliness, good humour, and cakes, which helped get me through this Master’s degree.

Lastly, a big thank you to my husband George, who wasn’t phased when I decided on a dramatic career change and has subsequently endured my worrying and lack of free time while writing up!

Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: DATE: 03/08/2023

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List of abbreviations

ACTH	Adrenocorticotrophic Releasing Hormone
ANOVA	Analysis of Variance
AUC	Area Under Curve
CCTM	Complex Cue T-Maze
CNS	Central Nervous System
CRH	Corticotrophin Releasing Hormone
GC	Glucocorticoid
HPA	Hypothalamic Pituitary Adrenal axis
PVN	Paraventricular Nucleus
QBA	Qualitative Behavioural Assessment
RFID	Radio Frequency Identification
RM ANOVA	Repeated Measures Analysis of Variance
SEM	Standard Error of the Mean
SNS	Sympathetic Nervous System
UCMS	Unpredictable Chronic Mild Stress

Chapter 1: General Introduction

1.1 Animal Welfare

Animal welfare is a broad concept concerning the physical and psychological wellbeing of non-human animals (hereafter animals). It is a significant area of societal interest, and the UK commonly views itself as a "nation of animal lovers" with high animal welfare standards (*Action Plan for Animal Welfare*, 2021). As such, a strong understanding of what constitutes good animal welfare is an area of significant research importance.

Concern for animal welfare typically derives from the understanding that animals are sentient beings with the capacity to experience different emotional states. These emotions, and the capacity to suffer, are an increasingly key component of determining what constitutes good animal welfare (Webb et al., 2019). However, the concept of animal "emotion" remains controversial. Colloquially, our understanding of human emotions is focused on conscious experience, and the extent to which this is present in other species is not known (Paul & Mendl, 2018). Nonetheless, from a scientific perspective, emotion can also be considered more broadly as an "an internal Central Nervous System (CNS) state that gives rise to physiological, behavioural, cognitive (& subjective) responses" (D. J. Anderson & Adolphs, 2014). Under this definition, emotions consist of four key components; valence, scalability, persistence, and generalisation. Briefly, valence refers to the inherent pleasantness/positivity or aversiveness/negativity of emotional states. Scalability reflects how the intensity of the emotional state, and its effects, can vary depending on the situation. Persistence refers to how emotional states can continue to be influential for some time even after a triggering stimulus has ceased. Finally, Generalisation is the phenomenon whereby an emotional state induced by one stimulus can subsequently also influence responses to other stimuli in different contexts (D. J. Anderson & Adolphs, 2014; Paul & Mendl, 2018). There is evidence for these components in both humans and other animal species (for further elaboration see (Paul & Mendl, 2018)). In this thesis, the term "emotional states" should be considered in this context. The related term "affective states" is also used; this just refers to valenced states, encompassing both emotions and sensations. Sensations have a valenced aspect (e.g. the pleasantness of an optimal temperature), alongside a physical sensory aspect (e.g. body temperature detection) (Mendl & Paul, 2020). As with emotional states, affective states are often assumed to be consciously experienced, but this may not necessarily be the case, and non-conscious components (e.g. neural changes) will also be involved (Mendl & Paul, 2020).

While the way animals experience their emotions remains unclear, the potential suffering that could be allowed by delaying efforts to promote animal welfare until we have a greater understanding of animal consciousness could be significant. Utilising these broader terms for animal emotion allows us to set aside this difficult issue and examine other aspects of the emotional response that can be empirically studied. These may serve as biomarkers of an animal's welfare. Nonetheless, so far, robust biomarkers of emotional or affective states in animals have remained elusive.

1.1.1 Dimensions of good animal welfare

Good animal welfare is a multidimensional concept. The factors considered have developed to increasingly emphasise a positive mental state, alongside physical needs. An important early concept was the "Five Freedoms", which defined good welfare as freedom: 1) from hunger and thirst, 2) from discomfort, 3) from pain, injury or disease, 4) to express (most) normal behaviour and 5) from fear and distress (*FAWC Report on Farm Animal Welfare in Great Britain*, 2009). This construct focuses on meeting physiological needs and minimising negative experiences. While a good starting point for recognising what is important to animal welfare, it doesn't encompass how physical factors integrate with affective states to reveal how an animal's overall welfare state arises (D. J. Mellor, 2016). Some states mentioned (e.g. hunger) are also somewhat normal and necessary to initiate important behaviours, creating ambiguity over how achievable each "freedom" is. The lack of clarity over the importance and interactions of each "freedom" has limited this concept's ability to assess animals' overall welfare states.

Reflecting increasing knowledge of animal mental states, the Five Freedoms concept has been revised into the Five Domains model (D. J. Mellor & Reid, 1994). The model comprises four physical domains (nutrition, environment, health, and behaviour) and a fifth "mental" domain that represents the affective consequences of the physical domains. The animal's experience of the fifth, "mental", domain is what determines the animal's welfare status (D. Mellor & Beausoleil, 2015). This model integrates a range of positive and negative affective experiences across the physical domains and suggests measuring an animal's overall affective state is key to measuring its overall welfare status (**see Figure 1.1**).

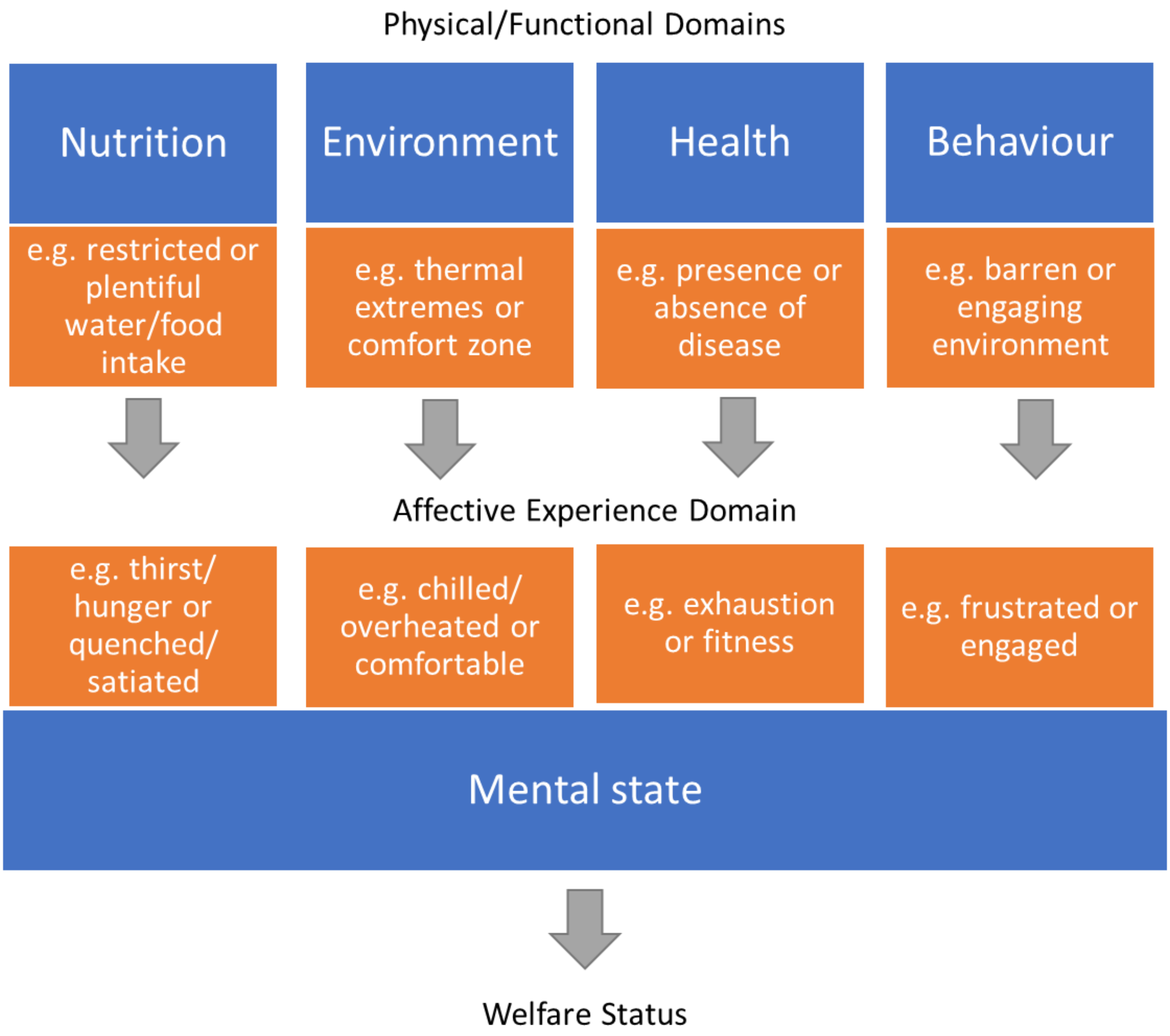


Fig 1. 1 The Five Domains model

Blue elements represent the five domains defined by Mellor and Reid. The four physical domains have positive and/or negative influences on the fifth mental state domain, which determines an animal's overall welfare status. Orange elements show examples of how the physical domains can influence the mental state. Adapted from (D. J. Mellor, 2017).

1.1.2 Why objective measures of overall welfare status are needed

Animal use remains an important part of human society, primarily due to consumption of animal products. To a lesser extent, pet-keeping, scientific testing, and interactions with captive and free-living wildlife also occur. All these aspects present challenges to animal welfare, as human and animal needs may conflict. Where animals are kept for a specific use, certain aspects of their physiology related to that use are prioritised, potentially at the expense of other areas that also influence welfare. For example, livestock reared for meat will likely have excellent nutrition as their purpose is to grow well but may be behaviourally restricted due to confined and/or barren housing conditions which minimise costs. Economic constraints also exist in laboratory environments, where animals are often housed in similarly behaviourally restrictive environments.

Understanding how different factors influence an animal's overall welfare is important for prioritising viable improvements in husbandry systems. Without accurate animal-centric welfare measures, changes may be made from an anthropomorphic and/or aesthetic viewpoint. This may not lead to the most effective improvements and may even worsen other problems. For example, outdoor access is typically perceived as enabling good farm animal welfare (YouGov, 2020), and consequentially UK free-range egg production has grown. Free-range production offers greater opportunities for behavioural expression than caged systems, but a similar level of welfare may also be achievable in loose-housed barn systems, which are favourable from a biosecurity perspective. Questions over how welfare compares in barn and free-range systems should be answered from the hen's perspective. This highlights the need for objective biomarkers of animal affective states.

1.2 Current approaches to assessing animal welfare; strengths and limitations

Traditionally, animal welfare was largely treated as synonymous with physical health. There are many definitions of veterinary health (Gunnarsson, 2006), but it broadly refers to normal physiological functioning without pain, injury or disease. Indicators of disease are commonly included in welfare assessments (Cockram & Hughes, 2018) (e.g. dairy cattle (Coignard et al., 2013)), likely in part because they are relatively easily measured. Good physical health is undoubtedly an important part of animal welfare and forms a component of the five domains model (D. J. Mellor & Reid, 1994). Nonetheless, a healthy animal will not necessarily experience a positive state overall, and equally, poor health alone might not lead to negative welfare (I. J. Duncan & Petherick, 1991). A pig might be physically healthy, but experiencing poor welfare due to behavioural frustration while confined to a farrowing crate (Baxter et al., 2018). Conversely, subclinical cancerous growth may not present a welfare problem, as there may be no physiological symptoms causing suffering (Keeling et al., 2018). This highlights the need for broader welfare measures that encompass affective state.

Reproductive success has also been used as an animal health and welfare measure (Gunnarsson, 2006; Keeling et al., 2018). There are factors that underlie both reproductive success and welfare, as poor health and nutrition may compromise both. However, animals may still reproduce normally under poor conditions. Conversely, high fertility could be welfare negative due to compromise on other aspects of animal physiology (Ritter et al., 2019).

Physical health and fertility can be important aspects of animal welfare. However, they only consider part of the overall welfare status. We want to develop an objective method of assessing affective state, which should reflect an animal's overall welfare status. Different approaches to this, and their strengths and weaknesses, are considered below.

1.2.1 Physiological animal welfare assessment methods

Physiology has been used to assess animal welfare outside the context of health and disease. Measures aiming to quantify the "stress response" have been widely used as overall welfare state indicators. Stress can be defined as a state of threatened homeostasis, where an organism activates a specific adaptive compensatory response to sustain homeostasis (the stress response) (Pacák & Palkovits, 2001). A stress response can be activated in response to challenges in a range of the domains influencing an animal's affective and welfare state. Examples include restraint (behaviour), injury (health), thermal stress (environment) or hypoglycaemia (nutrition).

The stress response comprises activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis (**Figure 1.2**). Glucocorticoid (GC) secretion is therefore a commonly used measure of stress and ultimately welfare (Mormède et al., 2007), with GC sampled from body fluids such as blood, urine or saliva. This approach was used to examine the effect of tail vs cup handling in laboratory mice. Cup handling led to lower corticosterone production, indicating less stressed mice and a more welfare-friendly handling technique (Ono et al., 2016). However, there are challenges associated with this method. Importantly, positively valenced, high arousal, situations can also increase GC secretion, including sexual stimulation and increased environmental enrichment (Iyasere et al., 2022). In addition, factors unrelated to stress influence HPA activity (e.g. age and sex, (Gaffey et al., 2016; Goel et al., 2014)), and response level can vary between individuals and species, making interpretation difficult. GC secretion exhibits circadian and circannual variation in most species, which complicates collection and data interpretation (Mormède et al., 2007). For some methods, sampling body fluids from animals may also confound the results if the collection process itself is stressful.

There are other physiological methods of assessing the animal stress response (see (Blache et al., 2018)). These typically face similar challenges regarding difficulties with interpretation and sampling methods. Heart rate and blood pressure may be used to indicate Autonomic Nervous System activation (Kovács et al., 2014), but it may be difficult to determine whether an elevated reading is due to stress or other factors. General arousal can again induce similar changes, but may be in response to positive events such as feeding (Bloom et al., 1975) or sexual activity. Alternatively, adrenaline levels may be measured to indicate sympathetic-adrenal axis activation (Lefcourt & Elsasser, 1995). However, sampling this from body fluids can be difficult due to its short half-life, and may be invasive (Blache et al., 2018), potentially influencing the hormone levels recorded.

While physiological methods can provide a good indication of the animal stress response, results may not easily map onto affective state. Physiological measures do not show the valence of the animal affective state, merely that there has been some kind of stress response activation or in some cases, arousal. These methods are limited as welfare measures unless interpreted alongside behavioural data for context. Behavioural measures will now be considered, to ascertain whether these show more potential to be robust biomarkers of animal welfare states.

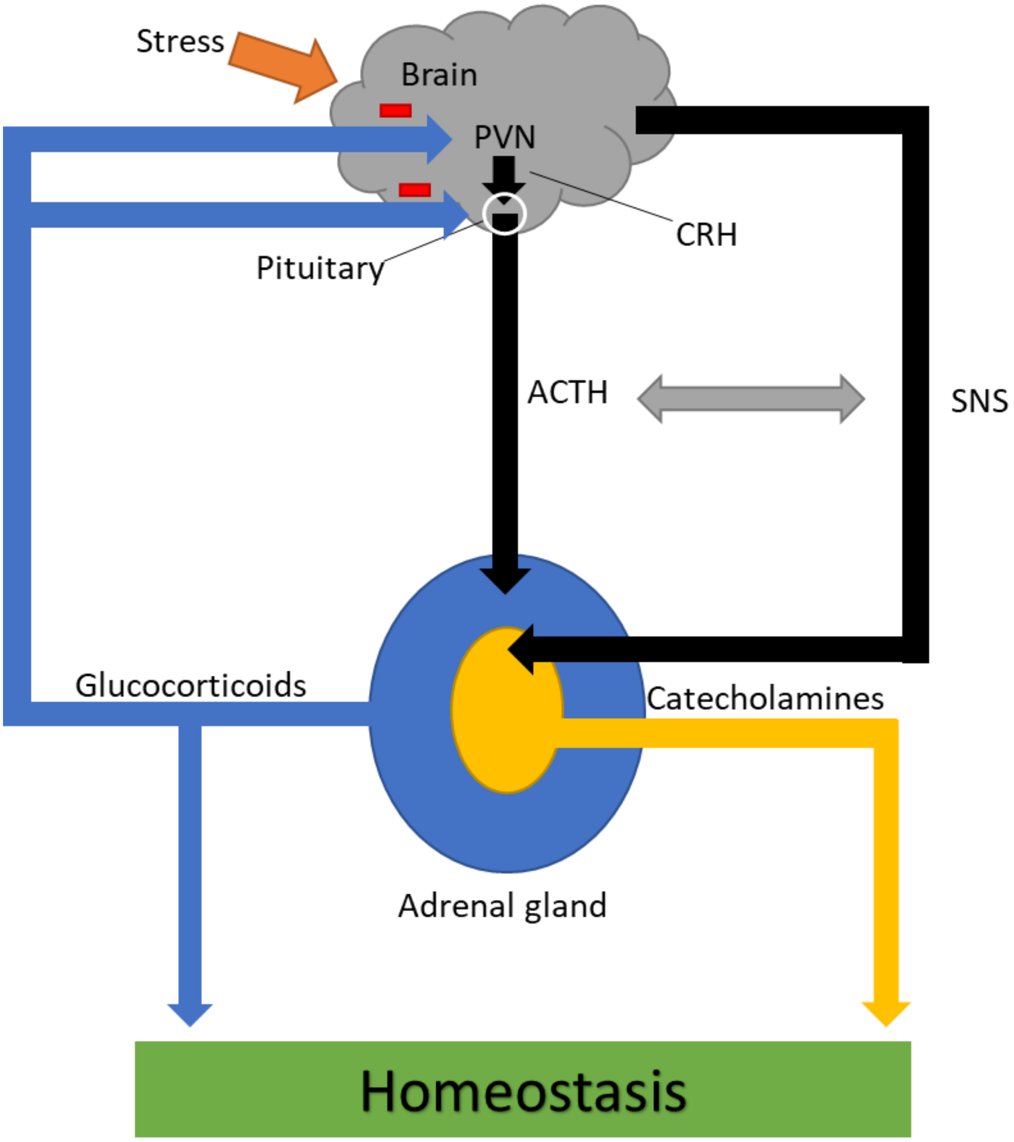


Fig 1. 2 Classical representation of the hypothalamic-pituitary-adrenal-axis and sympathetic-adrenal axis.

The HPA axis and SNS are activated in response to stress. On activation, the PVN of the hypothalamus secretes CRH, which stimulates the release of ACTH by the anterior pituitary gland. ACTH is released into the bloodstream and stimulates glucocorticoid production and release by the adrenal cortex. Activation of the sympathetic nervous system also stimulates the release of catecholamines (adrenaline) from the adrenal medulla. There is interaction between the two systems and glucocorticoid release is regulated by negative feedback. PVN = paraventricular nucleus; CRH = corticotrophin-releasing hormone; ACTH = adrenocorticotrophic releasing hormone; SNS = sympathetic nervous system. Adapted from (Blache et al., 2018).

1.2.2 Behavioural monitoring

Observation of spontaneous animal behaviours has also been commonly used in animal welfare assessment. Behavioural responses are a component of animal emotional states (D. J. Anderson & Adolphs, 2014), with emotion thought to be evolutionarily important in guiding decision-making across species (Mendl & Paul, 2020). Some behaviours may also serve as social signals of emotional states between conspecifics (Seyfarth & Cheney, 2003). Practically, behaviour is also relatively easy to monitor. To use specific animal behaviours as a biomarker of welfare, careful validation is needed. This can be achieved by observation of behaviours in contexts reliably thought to induce specific states.

Animal vocalisations have been studied in many species. The ability to recognise different vocalisations and the contexts in which they are produced has been increasing (see (Laurijs et al., 2021)). Nonetheless, the picture remains complicated; e.g., both lower and higher frequency vocalisations have been associated with positive states in pigs (Imfeld-Mueller et al., 2011; Leliveld et al., 2016). Vocalisations need careful validation to be effective affective state biomarkers (Manteuffel et al., 2004), but understanding different contexts' significance from the animal's perspective may be challenging and make interpretation difficult. Acute vocalisations also appear to be primarily influenced by short-term emotions, which may limit their suitability for assessing overall welfare (Laurijs et al., 2021; Mendl et al., 2010). Furthermore, vocalisations are species-specific, requiring extensive research to understand each species, and some appear to show limited communication this way (e.g. most reptiles). Other areas of animal signalling have also been studied, such as posture (e.g. (Proctor & Carder, 2014)) and facial expression (Descovich et al., 2017). While these areas may also show some promise, they face similar limitations to vocalisations as elaborated previously.

Animal welfare can also be inferred based on the presence/absence of behaviours other than animal signals. Play behaviour is a potential indicator of positive affective states, and not only because the behaviour itself is likely rewarding. Animals are thought to only invest time and energy in play when other needs are well met (Boissy et al., 2007). Therefore, play may be an indicator of an overall positive welfare state. However, play behaviour is primarily seen in young mammals, limiting applicability to older animals, and potentially across wider species where play behaviour is less well-studied (Oliveira et al., 2010).

Conversely, abnormal behaviours are often used as indicators of poor welfare. Abnormal behaviours differ significantly from those seen in free-living animals, in form, intensity, variability or orientation (Olsson et al., 2018). Examples include barbering (Vieira et al., 2017) or repetitive stereotypic somersaulting (Tanimura et al., 2008) in rodents, and injurious feather pecking in chickens (Daigle, 2017). These tend to associate with poor environmental conditions where a highly motivated behaviour is frustrated, and correlate with other poor welfare indicators (Mason & Latham, 2004). Interpretation can nonetheless still be difficult; expression of abnormal behaviours varies between individuals in a manner that may not reflect variation in welfare state (Mason & Latham, 2004). Animals performing stereotypic behaviours may experience a more positive state than others in similar conditions without the behaviours, as this may help them cope with the environment (Mason & Latham, 2004). Also, stereotypies may remain even if environmental conditions are improved, which could reflect a long-term welfare impairment or simply behavioural establishment (Tilly et al., 2010). Observation of abnormal behaviours is therefore useful but limited with regards to informing us about the welfare status of individuals.

Another approach to assessing animal welfare by observing spontaneous animal behaviours is Qualitative Behavioural Assessment (QBA). Observers characterise how animals perform certain behaviours with adjectives such as “bored” or “excited”, and trends are quantified. Theoretically, QBA provides an integrated assessment of whole animal welfare, demonstrates good inter-observer consistency, and can align with other animal welfare measures (Wemelsfelder, 2008). In an on-farm welfare assessment context, QBA is also seen as advantageous as it is non-invasive and does not require significant time/labour investment (Grosso et al., 2016). Nonetheless, there are concerns regarding how well a method reliant on human interpretation can truly capture affective state from an animal’s perspective. There are risks that anthropomorphic and aesthetic factors could confound the results, particularly for non-mammalian species which may be less relatable. Overall, approaches minimising human influence and opinion are still more likely to accurately represent the animal's affective state.

1.2.3 Behavioural tests of preference and motivation

Tests designed to measure animals’ preferences for certain factors (e.g., environmental conditions) and motivation to perform particular behaviours have become a core part of animal welfare research.

In preference testing, animals are given the opportunity to choose or interact with different resources or move between areas with different environmental conditions. By observing animal choices, researchers can make inferences about what is important to them, what they prefer, and presumably, what is good for their welfare. In an early example of this method (Hughes & Black,

1973) hens were housed in battery cages with sections of different types of flooring. The hens demonstrated a preference for both standing and oviposition on a very light, fine hexagonal mesh, compared to coarse rectangular mesh and perforated steel sheet. The mesh was previously assumed to be less comfortable than a heavier floor for the hens' feet. This highlights a strength of this approach in enabling researchers to gain insight from the animals' perspective, which may differ from our own. Many variations of these tasks have been used to tackle welfare challenges (see (Fraser & Nicol, 2018)).

Motivation tests have also been designed to assess the extent to which animals are willing to work to access their preferred option or perform key behaviours. Some minimal motivation tests simply assess whether a resource can serve as a reward for instrumental training (e.g. hens could be trained to break a photobeam to access litter (Dawkins & Beardsley, 1986)). More typically, the level of effort animals are willing to exert to obtain the reward is compared to other rewards. For example, sows are almost as motivated to access straw as feed in the 24 hours before farrowing, when nest-building would normally take place (Arey, 1992). Such tests are valuable for gaining insight into what is important from animals' perspectives.

There are nonetheless still challenges with interpreting the results of these experiments. If animal affect is key to drive appropriate decision-making, animals should make choices supporting positive affective states and good animal welfare, at least in the short-term (Bechara & Damasio, 2005; Mendl & Paul, 2020). However, this may not always be the case. Prior experience may influence choices, with animals initially preferring environments they are more accustomed to (Dawkins, 1977). Choice context can also be important, with preference contingent on factors that may not be understood and controlled for (e.g., time or temperature) (Kirkden & Pajor, 2006). Understanding the motivation behind choices can be difficult. Male mice choose to sleep next to other males in an adjacent cage (Van Loo et al., 2001), but this may be due to investigative, thermal, and/or social needs. Animals, especially domestic breeds selected for "unnatural" characteristics, may also prefer scenarios that are detrimental to long-term health. Broiler breeders, for example, become obese if given free access to food, compromising their long-term welfare (I. J. H. Duncan, 1992). Animals within a population can also show variation in their preferences (e.g. (Nicol et al., 2009)), which needs to be taken account of when interpreting study results. Only analysing population level results may disguise subgroups that prefer environments with different characteristics, and these also need to be considered when making welfare recommendations.

We want to know not just what animals will work for and are drawn towards, but whether different scenarios induce more positive or negative states. Behavioural tests have been developed to assess overall affective state more directly and these are considered in more detail below.

1.3 Affective state-induced cognitive biases as objective welfare state measures

Researchers are interested in assaying the affective state of animals under different circumstances as directly as possible. We want to reduce ambiguity in the interpretation of animal welfare measures, which can be challenging in the measures described previously. More direct affective state measures would be beneficial in themselves, but secondarily, could also help validate other methods, including those mentioned earlier where interpretation has been unclear.

Human psychology research indicates that cognitive information processes interact with affective state (Robinson & Roiser, 2016). Emotional valence affects decision-making, learning and memory, and attention (Robinson & Roiser, 2016). Depressed patients showed impaired risk adjustment in the Cambridge Gamble task, being unlikely to place large bets even with a high likelihood of winning (Roiser et al., 2009). Depressed patients also show a reduced ability to learn cues predicting reward (Henriques et al., 1994), an attentional deficit towards words associated with positive emotions (Erickson et al., 2005), and increased intrusion and working memory interruption when presented with negative words (Joormann & Gotlib, 2008). Overall, humans in negative states show poorer reward learning, biased attention to and recall of negative events and make more negative judgements regarding future outcomes. This is affective state-induced cognitive bias. Whether similar characteristic cognitive changes are seen in other species is an area of research interest. If we can measure similar cognitive changes in response to similar emotional states across species, these measures could provide a robust and objective cross-species biomarker of affective states and animal welfare.

Established and developing assays to evaluate animal affective state-induced cognitive biases are considered below (see **Figure 1.3**).

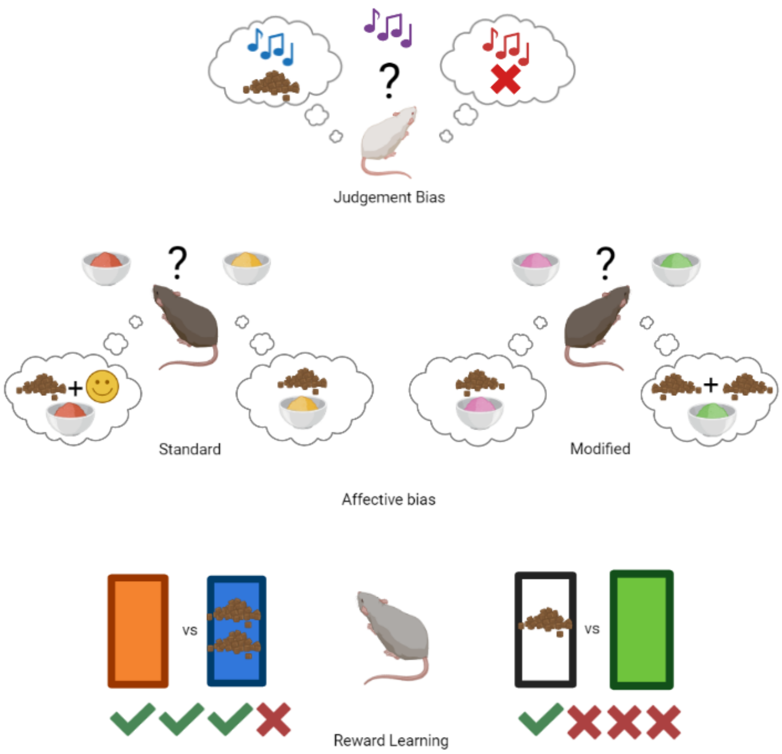


Fig 1. 3 Approaches to testing affective state-induced cognitive bias in animals.

Affective state-induced cognitive bias was originally tested in animals using the **Judgement Bias** task. Animals are trained to perform different responses to positively rewarded and less/unrewarded/punished stimuli (e.g., approach a positive cue for a reward and refrain from approaching a negative cue to avoid punishment, or press one lever following a positive cue for a large reward and press another lever following a different cue for a smaller reward). Animals are then tested with ambiguous stimuli and the response recorded. Animals in a more positive state should interpret the ambiguous stimuli more “optimistically” than those in a negative state and respond accordingly. In the standard **Affective Bias** task, rats are trained to associate a digging substrate with a reward in two different substrate pairs. One of the pairs is associated with an extra experimental affective state manipulation. The rats are then tested by presenting both rewarded substrates simultaneously. A substrate associated with an additional positive affective state manipulation should be favoured over the other substrate. Conversely, a substrate associated with an additional negative affective state manipulation should be less favoured than the other substrate. In the **modified Affective Bias** task, rats are again trained to associate a digging substrate with a reward in two different substrate pairs. One of the rewards is larger than the other. Rats are tested by presenting the two rewarded substrates simultaneously; rats in a positive affective state show a bias towards the substrate previously associated with a higher reward, whereas rats in a negative affective state do not. **Reward Learning** tasks present animals with two pairs of reward cues interchangeably, one pair is associated with a higher reward and one pair is associated with a lower reward. The learning rate of each reward pair is monitored. Animals with normal reward learning should learn the higher reward pair cue more quickly than the lower reward pair cue. Animals with impaired reward learning and in negative affective states should not show this reward learning bias. Created with BioRender.com

1.3.1 Judgement bias tasks

The main assay currently used to assess affective state-induced cognitive bias in animals is the judgement bias task (**Figure 1.3**). In the first study developing this task (Harding et al., 2004), rats were trained to associate one tone with a positive event (food reward) and another tone with a negative event (white noise). Rats were then presented with unreinforced ambiguous tones, with frequencies between the positive and negative tones. Rats subjected to unpredictable housing were more likely to respond to the ambiguous tones as if they predicted a negative event compared to unstressed rats. This was the first demonstration that affective state appears to affect animal cognition, and induce a “pessimistic” judgement bias similar to humans (Wright & Bower, 1992). Subsequent work shows a variety of affective state manipulations can influence judgement bias in many species (see (Lagisz et al., 2020)). Studies support welfare-related conclusions including the benefits of enriched cages for laboratory rats (Brydges et al., 2011) and the negative effect of maternal separation on dairy calves (Daros et al., 2014).

There are still uncertainties and challenges regarding judgement bias as an effective animal welfare assay. Some tasks are designed with the two trained stimuli both requiring active responses (e.g., pressing a left or right lever). In others, the positive option requires an active response, and the negative option requires no response (e.g., pressing or not pressing a lever). The latter design (“go/no go” tasks) is vulnerable to potential confounds with mobility and motivation that may be separable from affective state (Hales et al., 2014). Another design variation is whether the two trained stimuli indicate reward and punishment, or reward and lack of reward/a lower reward. Repeatedly exposing animals to punishments during welfare assessment raises ethical questions and risks worsening affective state during training. The drive for punishment avoidance may also induce a more negative bias even without a negative affective state (M. H. Anderson et al., 2013). When assessing judgement bias, active choice tasks that train animals without punishers should be used.

Judgement bias tasks require lengthy training to establish the positive and less positive/neutral/negative stimuli responses. Training rats in operant active choice tasks takes around 3 months (Robinson & Roiser, 2016). This is time consuming and impractical in animal welfare assessment, particularly if studying animals in commercial environments. Also, while results have generally shown animals in putative positive conditions perform more “optimistically” in judgement bias tests (Lagisz et al., 2020), this is not universal. For example, enrichment levels did not affect pessimistic responses in starlings or laying hens (Brilot et al., 2010; Wichman et al., 2012). Equally, judgement bias and other measures of animal welfare do not always align (Paul et al., 2022). There may be difficulties with developing task design across species. For example, it has proven difficult to demonstrate judgement bias in mice (Resasco et al., 2021). Furthermore, with long training protocols, animals may behave procedurally during testing, meaning the expected cognitive effects on choice bias cannot be seen. The training itself may also act as an enrichment, and over a prolonged period, could confound results by influencing affective state. Despite these challenges, the mechanisms underlying cognitive affective bias and its influence on decision-making could be highly conserved across species (Mendl et al., 2009). So, it is desirable to develop more ethologically relevant tasks that are easier to train, but still assay the effect of affective state on animal cognition.

1.3.2 Affective bias tasks

The affective bias task also assesses animal affective state-induced cognitive bias (**Figure 1.3**). Again based on human depression research, this task draws on the observation that reward associated memories appear to be biased by affective state at the time of learning (Hales et al., 2014; Roiser et al., 2012). An individual's affective state while experiencing reward influences learning, such that subsequent recall is coloured by a positive or negative bias.

The affective bias task was developed as a rat bowl digging task (Stuart et al., 2013). Rats are trained in two independent discrimination learning tasks, each consisting of two pairs of bowls containing different digging substrates. One substrate per pair is associated with the same food reward. Training sessions are performed on different days, either under neutral conditions or with affective state manipulations. A subsequent preference test assesses affective bias, with both previously rewarded substrates presented simultaneously. The affective state manipulations associated with the digging substrate influence preference. Substrates associated with positive manipulations are favoured over neutral substrates and vice versa. Rats have shown a preference for substrates associated with antidepressant administration, as well as highly enriched social play (Stuart et al., 2013). Equally, pro-depressant drugs and exposure to combined restraint stress and social isolation have induced a negative bias (Stuart et al., 2013). So far, affective bias testing has focused on rats, and research is less extensive than for judgment bias. Nonetheless, rats in multiple studies show the expected substrate biases following pharmacological and psycho-social manipulation, validating the procedure (Hinchcliffe et al., 2017, 2020a). Much affective bias literature focuses on rats as animal models for human depression. However, the test has also been used to demonstrate that specific rat vocalisations indicate a positive affective state during human-rat "tickling" interactions. (Hinchcliffe et al., 2020b). This could support appropriate use of "tickling" to benefit laboratory rat welfare. Similarly, the affective bias test could be used to determine how animals perceive specific husbandry practices if protocols were developed for more species.

A limitation of the original affective bias task is that the protocol can only be applied to acute affective state manipulations. To overcome this, a modified version of the task was developed to assess the influence of chronic manipulations (and therefore long-term affective state) (Stuart et al., 2017). In this protocol, one of the rewarded digging substrates is associated with a higher reward than the other. With normal reward processing, a preference for the higher reward substrate should show during the preference test. If reward processing is impaired then this preference may not be present, as is the case in human depression (Pizzagalli, 2014). As expected, rats chronically treated with pro-depressant drug treatments did not demonstrate a bias for the higher reward substrate, unlike controls (Stuart et al., 2017). A similar lack of larger reward bias has been shown in rats subjected to early life adversity (Stuart et al., 2019). The modified affective bias task has only been used in rat-based depression studies so far but could be applied to animal welfare assessment, if protocols were developed for more species. For example, the affective bias of animals kept under different husbandry conditions could be compared.

Like judgement bias tasks, affective bias also relies on pre-training discriminative learning. However, the bowl digging protocol is quicker than some operant tasks used for judgement bias (five days for training and testing (Stuart et al., 2013, 2017)). Without the constraints of needing an intermediate cue, it is perhaps easier to develop a task ethologically relevant to rats. Developing ethologically relevant tasks for other species may help overcome the unexpected results sometimes seen with judgement bias (e.g. (Brilot et al., 2010; Paul et al., 2022; Wichman et al., 2012)). Aside from efficiency, faster learning and relevant tasks help ensure animals are responding cognitively rather than procedurally to the cues. This maximises the likelihood of affective state-induced cognitive biases being detectable by the task.

Nonetheless, challenges remain. When affective bias was attempted in mice, the expected bias towards a digging substrate associated with enrichment was not seen (Graulich et al., 2016). Note, however, that the mice were exposed to the enriched environment *after* the affective bias procedure each day. Positive affect is unlikely to be induced immediately upon exposure to an enriched environment, especially in mice which may find handling aversive. Any positive affect induced by the enrichment later may have been too dissociated from the digging substrate to induce an affective state-induced cognitive bias. Similarly, contact with humans is necessary to run the affective bias tasks, but is a limitation as this may influence the subject's affective state. This laboratory has also been unable to consistently demonstrate the affective bias in mice using bowl digging protocols (Robinson group unpublished observations). Challenges with task design and complexity for more cognitively simple or nervous animal species remain. Even simpler methods of assessing reward learning deficits with reduced human contact are desirable.

1.3.3 Reward learning tasks

Judgement and affective bias tasks demonstrate reward processing deficits in animals in negative affective states when recalling and making decisions about reward-related information. Negative affective states associate with “pessimistic” choices when presented with ambiguous information (judgement bias). The ability to recognise and select a higher reward cue when two differently rewarded stimuli are presented together for the first time is also worse (modified affective bias task). Depression literature also suggests that reward related impairments appear at the level of reward learning itself, not just in its application.

Probabilistic reward learning tasks demonstrate how depressed human patients, and associated rodent models, show a blunted reward feedback response which impairs reward learning. Humans with few depressive symptoms develop a response bias towards a cue associated with a higher probability of reward, unlike those with more depressive symptoms (Pizzagalli et al., 2005). Similarly in rats, pharmacological manipulation to induce a negative affective state blunted a response bias towards a more frequently rewarded stimulus compared to untreated rats (Der-Avakian et al., 2013). Here, negative states seemingly influence the initial reward learning stage.

Therefore, the learning phase may be a suitable affective state assay. Neutral/positive animals should learn a larger associative reward pairing more quickly than a smaller reward (Clayton, 1964; Reynolds, 1950). Conversely, if feedback to reward is impaired during learning in negative affective states, different reward levels would be learnt at similar rates. Protocols focused on monitoring learning rate may be simpler than other affective state assays. The training becomes the assay, potentially reducing testing time. Reward learning protocols may also be more accessible for less cognitively complex organisms (e.g. mice) because the reward pairs presented remain consistent. There is no testing stage where animals decide between both previously rewarded stimuli or new ambiguous stimuli. This stage risks confusion in less cognitively complex species, with responses potentially defaulting to random chance.

The Robinson group have developed a novel reward learning assessment method for mice and rats using a T-maze with interchangeable textured floors. Floor texture reward cues tap into natural rodent investigatory behaviour (Pacchiarini et al., 2020), and so are hypothesised to facilitate associative reward learning. Basic discrimination can be taught within 5 days, considerably fewer than some operant tasks. The task is also semi-automated (with potential for full automation) and does not require animal handling during the procedure. This mitigates the risk of human interference confounding task results, a potential difficulty with previous affective bias tasks.

In the task, subjects are interchangeably presented with two pairs of reward cues. One pair is associated with a higher reward than the other. The learning rate of each reward pair is monitored over successive sessions (**Figure 1.3**). Animals with normal reward learning should show a reward learning bias towards the higher reward cue as sessions progress (Clayton, 1964; Reynolds, 1950). Those with impaired reward learning and in negative affective states should not show this bias. Mice and rats in a neutral/positive affective state have demonstrated the expected reward learning bias towards the higher reward (Jen Davies and Katie Kamenish, unpublished data). Equally, mice exposed to chronic corticosterone or fighting in a group housing environment, and so in putative negative affective states, do not show this bias (Jen Davies, unpublished data- Appendix 1). It is significant that mice show the expected reward learning deficits using this assay, a species where judgement and affective bias protocols have been challenging. High vs low reward learning assays may be applicable even in cognitively simple species and so may be suitable for developing as cross-species welfare assays. Nonetheless, reward learning needs to be tested under a wider range of affective state manipulations to validate that impairments reliably associate with negative affective states. More experimental operators also need to undertake the assay to confirm a lack of experimenter effect, and that the procedure can be widely implemented by multiple operators. Finally, limited mouse numbers have also been trialled in the T-maze so far, so further information on baseline learning rates and behaviour in the apparatus is needed.

1.4 Summary

The capacity to experience different emotional states drives concern for animal welfare. While the conscious experience of animals remains unknown, other aspects of emotional responses can be used to assess welfare indirectly. Affect-induced cognitive bias assays may offer a method of objectively assessing animal affective states, improving on previous physiological or behavioural tests which have been vulnerable to misinterpretation. Within these, reward learning assays are an emerging area of interest. The potential for simpler protocols may offer benefits over earlier iterations of affective state-induced cognitive bias tests and increase accessibility to a wider range of species. Research in this area is in early stages, and further validation of reward learning deficits in rodents in hypothesised negative affective states is needed. We also need to investigate whether these deficits present similarly in negative states across other species. Consequently, the overall aims of this thesis are 1) to further validate whether mice in hypothesised negative affective states show reward learning deficits and 2) to develop a testing and training protocol suitable for developing into a reward learning assay in chickens.

1.5 Chapter aims

1.5.1 Chapter 2: Investigating reward learning deficits in mice in response to chronic social and physiological stress

Male mice have previously demonstrated reward-learning deficits when exposed to fighting in a group housing environment and when treated with chronic corticosterone. This chapter aimed to determine whether similar reward learning deficits occur following exposure to repeated restraint stress and under group housing conditions without obvious fighting.

1.5.2 Chapter 3: Developing a protocol to train and test chickens in a reward learning assay

This chapter aimed to develop a training and testing procedure to determine whether chickens show reward learning deficits in negative affective states. This would provide insight into whether affect influences reward learning similarly across taxa. As the most numerous farmed terrestrial species, objective chicken welfare measures are important.

Chapter 2: Investigating reward learning deficits in mice in response to chronic social and physiological stress

2.1 Introduction

Whether adult male laboratory mice prefer individual or group housing, is controversial. In the natural environment, mice *(Mus musculus)* live in groups (Latham & Mason, 2004). So, group housing is commonly recommended (e.g. in the UK Home Office Code of Practice) to enable natural, species-specific group behaviours (Kappel et al., 2017). However, single-sex groups are needed to avoid uncontrolled breeding. This is not the natural mouse social structure, with male mice typically holding a territory, shared with females and their offspring (Latham & Mason, 2004). The laboratory environment is also artificial, with limited space and resources, potentially influencing natural behaviours. Aggression is common in group-housed male mice (Lidster et al., 2019) and represents a major welfare concern due to potential injuries and social stress. A chronically stressful environment may also reduce the reliability and reproducibility of scientific study results using these animals (Poole, 1997).

Previously, preference and motivation studies have been used to examine the optimal social housing environment for male laboratory mice (Kappel et al., 2017; Sherwin, 1996; Van Loo et al., 2001). Mice tend to seek proximity to others, but as is often the case in this style of experiment, the motivation and relevance is unclear. Behaviour in the short-term experimental set-up, where mice are still physically separated, may not translate to long-term unimpeded group housing. Also, thermal and/or investigative needs may drive the behaviour rather than a preference for social interaction. Furthermore, animals may not choose what is best for their long-term welfare (Fraser & Matthews, 1997). Juvenile male mice have shown a socially conditioned place preference for cues associated with same-sex social housing (Panksepp & Lahvis, 2007), but the subjects' age may limit wider applicability. Overall, to tackle this welfare issue, we need to investigate the affective state of mice actually living in feasible group and single housing conditions.

Mice are the most common laboratory mammal (Hickman et al., 2017), but effective and reliable welfare measures remain a challenge. An affective state-induced cognitive bias assay is desirable and could be used to compare the welfare of single and group housed male mice. However, applying the traditional methods of assessing affect-induced cognitive bias to mice has been difficult. Over 10 published studies have attempted to demonstrate judgement bias in mice, but these were largely unsuccessful (see (Resasco et al., 2021) Table S1 for summary). Training all mice tested in the initial discrimination has often been challenging (Boleij et al., 2012; S. Jones et al., 2017; Kloke et al., 2014). Other studies have been unable to demonstrate the expected treatment effects (Bailoo et al., 2018; Boleij et al., 2012; Krakenberg et al., 2019, 2020; Novak et al., 2015, 2016; Verjat et al., 2021). Resasco was able to demonstrate expected judgement biases in an olfactory digging task when manipulating environmental enrichment, but a shortened protocol assessing the influence of cancer was only successful in male mice (Resasco et al., 2021). Altogether, an established judgement bias protocol for mice therefore remains elusive. A simpler method of assessing cognitive bias may be more successful in mice. The high vs low reward learning assay recently developed by the Robinson group has shown promise. Using the novel T-maze design, male mice have already demonstrated reward learning deficits when in negative affective states induced by chronic corticosterone exposure or fighting in a group housing environment (see Jen Davies unpublished data, Appendix 1 and Chapter 1, section 1.3.3). Nonetheless, further validation of this task is needed with other experimental operators and a wider range of putative negative affective states.

Another new reward related task has also been developed, the acute foraging task. This examines the extent to which mice will retrieve nesting material from a foraging box, an intrinsically rewarding activity. It is different to the T-maze task, as the behaviour is spontaneous, and does not rely on food motivation. Low levels of foraging activity in this task may also indicate reward-processing deficits and a depression-like state. However, these would derive from motivational and/or hedonic deficits as reward learning is not a key part of this task. The amount of nesting material collected is sensitive to effort and putative anxiety levels associated with completing the task (Megan Jackson, unpublished data). This suggests the task measures motivated behaviour, the outcome of a cost-benefit analysis where the potential benefits of an action (e.g. meeting physiological/psychological needs) are weighed against potential costs (e.g. physical/psychological effort) (Simpson & Balsam, 2016). Motivated behaviour deficits are seen in humans with affective disorders, including depression and anxiety (Simpson & Balsam, 2016).

This chapter aimed to further investigate whether deficits in reward learning and foraging behaviours would develop in mice in hypothesised negative affective states. First, a cohort of mice was trained in the novel T-maze. A pilot study comparing single vs group housed male mice was then conducted. If group housing is stressful for male mice even without explicit fighting, we would expect to see reward learning deficits. There was some evidence indicating reward learning deficits in group housed mice, but the effect was less clear than expected. This could have been due to a lack of housing manipulation effect, or task design problems. Therefore, a second study was conducted using a more established mouse stressor, repeated restraint stress, which is a depression model (Zhao et al., 2013). We expected mice subjected to the repeated restraint protocol to demonstrate reward learning deficits. These same mice were also subjected to the acute foraging task to assess reward motivation behaviours and the potential for overlap with reward behaviour in the T-maze task. Previously restrained mice were expected to show reduced foraging behaviour if the restraint protocol had induced a lasting negative state. We also thought foraging behaviour might correlate with reward learning behaviour as both tasks were reward related. Acute restraint was also used to assess the effect of acute stress on reward motivated behaviours in the foraging task. Acute stress was expected to reduce foraging behaviour in the task.

2.2 Methods

2.2.1 Subjects

Subjects were 2 experimental groups of male C57bl/6J mice supplied by Envigo, which were 6 weeks on arrival. Mice were housed in temperature-controlled conditions (21°C) on a 12:12 hour light-dark cycle (reverse lighting, Experiment 1: lights OFF at 9.30am, lights ON at 9.30pm, Experiment 2: lights OFF at 8.15am, lights ON at 8.15pm). Standard lab chow (Purina, UK) and water were provided *ad libitum* unless otherwise stated. When feed restricted, mouse weights were monitored weekly and maintained at least 85% of their free feeding weight relative to their normal growth curve. Testing took place in the animals' active phase (dark cycle) between 9.00am and 5.00pm. Potential handling stress confounds were reduced by handling mice for 3 days before starting experiments. During this period each mouse was handled for approximately 10 minutes daily during the dark phase, under red lighting. Handling consisted of allowing mice to investigate the experimenter's hands, and repeated tube and cup handling, within the home cage environment. Experiments were performed in accordance with the Animals (Scientific Procedures) Act (UK) 1986 and in line with local institution guidelines. All work was conducted under UK Home Office Licences (P9B6A09A1 to April 2022 then PP9516065, approved May 2022) and was also reviewed and approved by the Bristol University Animal Welfare and Ethical Review Body.

Experiment 1 (n = 6): Assessing the affective state of group and singly housed male mice

On arrival, mice were housed individually (n = 3) or in a trio (n = 3) in open-top cages with 2 suspended cardboard tubes, wooden chew block and nesting material (50:50 long rat nesting material: sizzle nesting material). Mice were 8 weeks at experiment start and 18 weeks at experiment end (**Figure 2.1**). Mice were first trained in the novel complex cue T-maze (CCTM). Pilot studies examining reversal learning in the CCTM and comparing reward learning in single vs group housed mice, were then conducted. Mice were fed a restricted diet of ~2.5g/mouse/day during the experiment period.

Experiment 2 (n = 8): Assessing the affective state of mice subjected to repeated restraint stress

On arrival, mice were housed individually in open-top cages with a suspended cardboard tube, wooden chew block, cardboard house, and long rat nesting material. This prevented potential confounds from in-cage fighting stress. Mice were 8 weeks at experiment start and 15 weeks at experiment end (**Figure 2.1**). Pilot studies examining the effect of repeated restraint stress on reward learning in the CCTM and foraging behaviour in an acute foraging task were conducted. Four mice were subjected to repeated restraint stress and four acted as controls. Another pilot study was then conducted examining the effect of acute restraint stress on behaviour in the foraging task. Mice were fed a restricted diet of ~3.5g/mouse/day during the CCTM study. This was more than for experiment 1 as some mice in that group were close to the acceptable weight threshold.

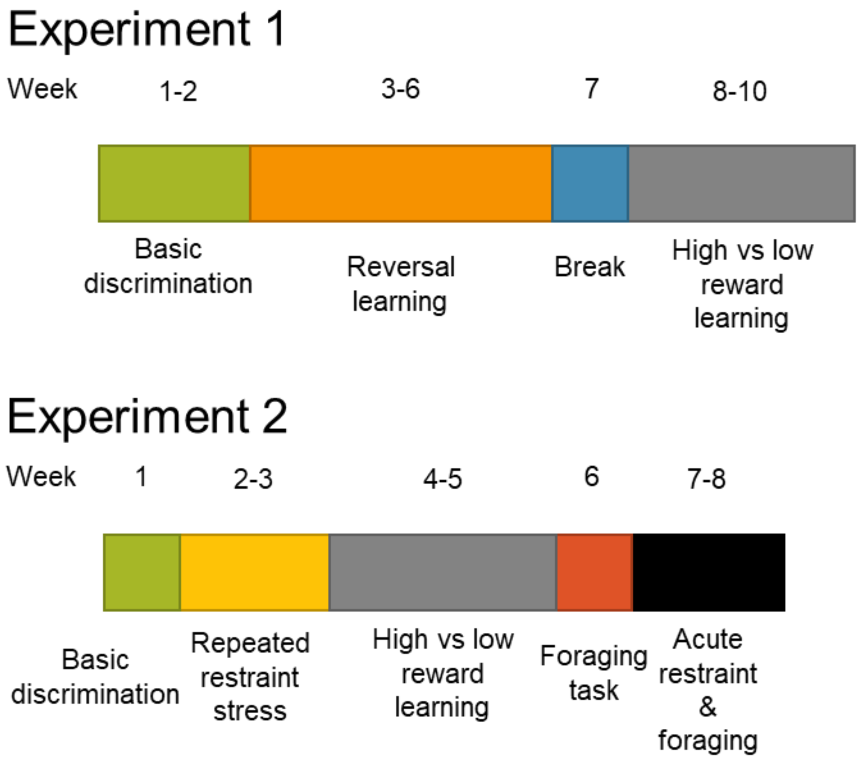


Fig 2. 1 Experiment timelines.

Mice started basic discrimination (w1) at ~8 weeks of age.

2.2.2 Apparatus

Complex Cue T-Maze (CCTM) (Figure 2.2)

A novel T-maze was used for basic discrimination, reversal, and examining reward learning bias. It was an enclosed Perspex device with a start box connected to T-maze arms via a central tube. The T-maze arms also connected back to the start box sides via tubes and corner pieces. The T-maze arm floors were removable and different textured floors were inserted (**Figure 2.2b**). The T-maze arm end walls had a small dish with a hole above where condensed milk rewards were delivered by syringe/pipette. Manual guillotine doors were operable at junctions in the maze. The apparatus was washed with disinfectant at the end of each day but not cleaned between individual animals. Mice were not tested in the same order each day so that any large running order effect would be noticeable (none was detected). The CCTM was operated under red-light conditions.

Foraging apparatus (Figure 2.3)

This was a Perspex setup with an enclosed “nest-building area” (17.5cm x 30cm x 13cm) connected by a 21cm transparent tube to a smaller transparent “foraging area” (10cm x 10cm x 13cm) with a barred floor and lid. The foraging area contained a black plastic container (8cm x 3.5cm x 10.5cm) able to hold 18g of sizzle (Datesand) nesting material, which a mouse can remove via 12, 1cm x 1.5cm rectangular holes (**Figure 2.3c**). Wood shavings covered the floors, and the nest-building area also contained a water bottle and glass food bowl. The wood shavings and sizzle nesting material were replaced between sessions, and the apparatus was cleaned with 70% ethanol after every second session. Foraging sessions were conducted in the dark.

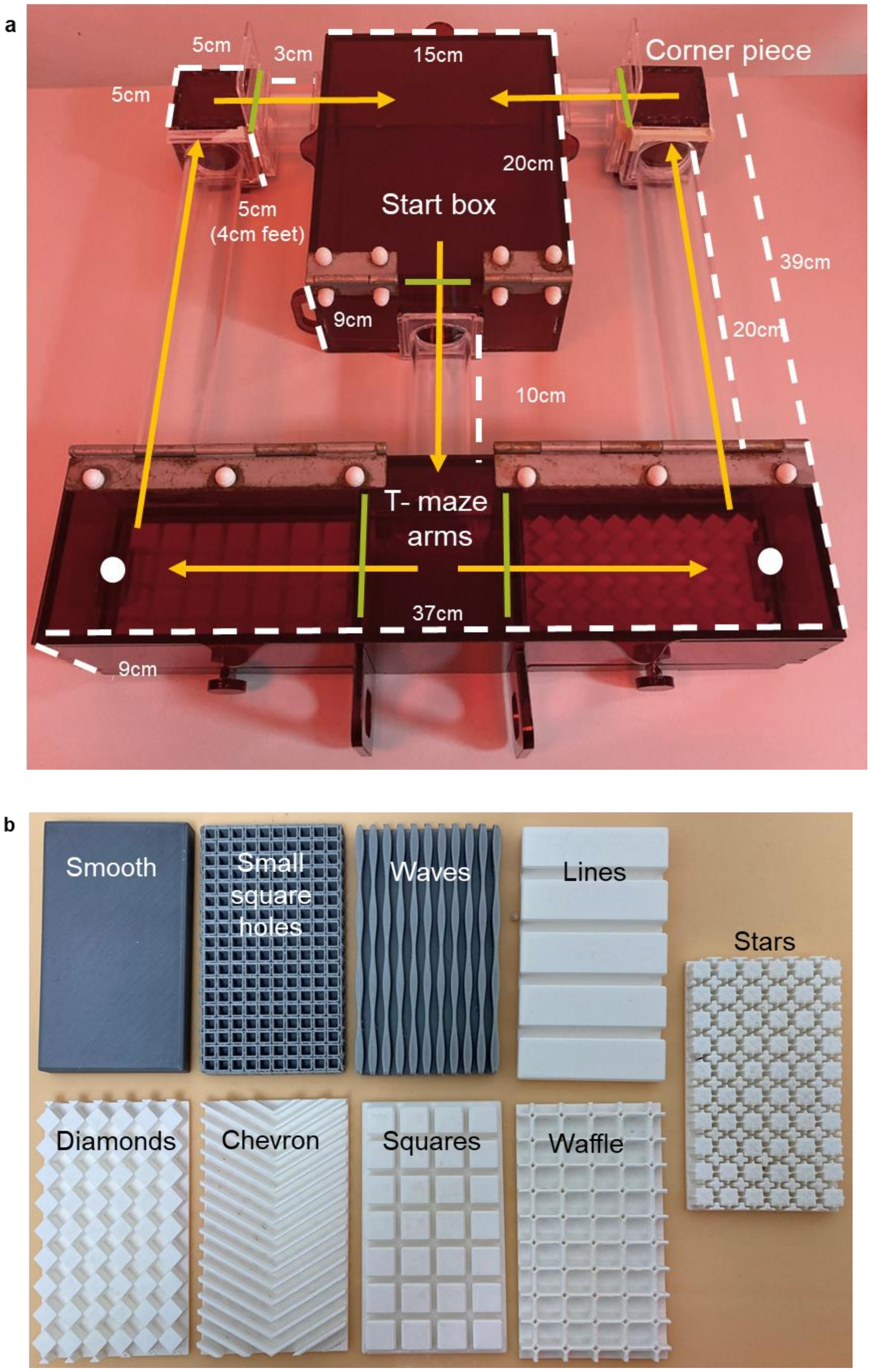


Fig 2. 2 Complex Cue T-Maze (CCTM) and textured floors.

a CCTM with dimensions. Yellow arrows indicate how mice transit the maze. Green lines show manual guillotine door positions. While circles show condensed milk reward delivery locations. **b** Textured floors for the T-maze arms. Grey floors were used for basic discrimination and white floors for other protocols. Floor dimensions: 13cm x 7.5cm x 1cm.

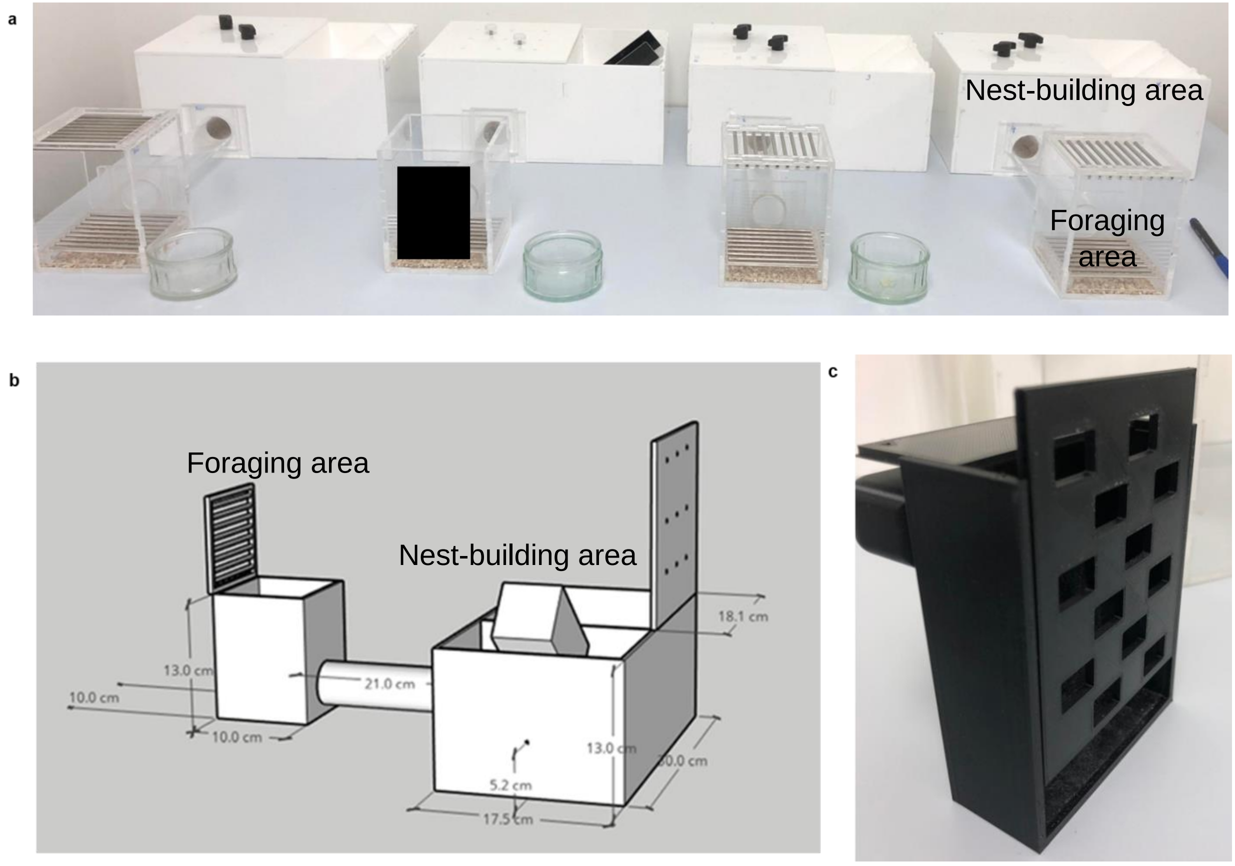


Fig 2. 3 Foraging apparatus.

(Images provided by Megan Jackson and used with permission)**. a** Four adjacent setups. Rear nest-building area contains glass food bowl visible in foreground and has water bottle on roof when in use. Black rectangle indicates position of bedding container when operational. **b** Foraging apparatus schematic with dimensions. **c** Bedding container. Front panel is pushed down, and lid encloses top when in use.

2.2.3 CCTM Reversal Learning

Training

Habituation: Individually, mice experienced two, 10-minute sessions within the CCTM without textured floors inserted, on two consecutive days. In session one, mice freely explored for 10 minutes. In session two, doors were introduced so mice could only progress cyclically through the maze. 10µl 10% condensed milk was pipetted into the maze arm dish every time the mice passed through, irrespective of chosen direction. The reward had to be consumed before mice could continue transiting the maze.

Basic Discrimination: The T-maze arms contained different textured floors (smooth vs small square holes). Choosing one floor led to a condensed milk reward delivery into the dish in the corresponding maze arm. Choosing the other floor resulted in no reward. Mice passed cyclically through the maze until 20 trials were completed. Accuracy of selecting the rewarded floor was recorded for each session. There were 10 basic discrimination sessions over two weeks.

The rewarded floor was counterbalanced across subjects. Once the mouse had 4 feet on the textured floor, the guillotine door closed, preventing exploration of the other floor. The rewarded floor position was pseudorandomised to prevent reward association with spatial cues, which we thought may encourage procedural rather than cognitive behaviour (e.g. (Hernandez et al., 2015). These conditions apply to all protocols using the CCTM.

For the first seven sessions, a 10µl 10% condensed milk reward was pipetted, and incorrect trials were not re-presented. For the final three sessions, to facilitate learning, a ~40µl 10% condensed milk reward was syringed and incorrect trials were re-presented. Incorrect trials still counted towards the 20-trial total. For the first five sessions, if six consecutive correct trials were achieved the following trials were terminated and 100% accuracy was recorded. For the following five sessions, 20 trials were completed regardless of consecutive correct trials. All mice achieved at least 70% accuracy at least once and were deemed suitable to progress to reversal.

Testing

Simple discrimination and Reversal learning: Different textured floors (lines vs diamonds) were used. Following a correct floor choice, a ~40µl 10% condensed milk reward was syringed into the dish in the corresponding maze arm. Mice undertook 20 trials per session, with incorrect trials re-presented and counting towards the 20-trial total. Accuracy of selecting the rewarded floor was recorded for each session. Mice were deemed to have learnt the rule upon achieving 70% accuracy for two consecutive sessions. Once the initial rule was learnt the contingencies were reversed in the subsequent session, so the rewarded floor became unrewarded and *vice versa.* There were 20 sessions over four weeks, with a reversal every time the criterion was met.

Mice that failed to learn one reversal were excluded from analysis (n = 1). Number of sessions required to learn the initial rule and first reversal were examined. Insufficient mice completed multiple reversals to meaningfully examine second reversal data onward.

2.2.4 CCTM High vs Low Reward Learning

For experiment 1 (comparing single vs group housed mice), this protocol followed CCTM reversal learning, so no further habituation/training was required. Experiment 2 (comparing repeated restraint stress and control mice) mice habituation progressed the same as previously described.

Experiment 2 mice underwent basic discrimination training before undertaking high vs low reward learning. Basic discrimination was largely as previously described. Smooth vs waves textured floors were used. 10µl 10% condensed milk was pipetted as the reward throughout and incorrect trials were re-presented. Incorrect trials counted towards the 20-trial total. Five sessions were completed over one week. All mice achieved at least 70% accuracy at least once and were deemed suitable to progress to high vs low reward learning. Training was completed before restraint took place.

High vs low reward learning: Two pairs of four different textured floors (chevron (experiment 1) / diamonds (experiment 2) vs squares and stars vs waffle) were presented interchangeably. One floor in each pair was rewarded. The rewards were different for each pair of floors: a “high” reward of 10µl 5% condensed milk, compared with a “low” reward of 10µl 2% condensed milk. Mice undertook 20 trials (10 trials of each floor pair) per session and incorrect trials were not re-presented. There were 10 sessions; experiment 1 ran over three weeks due to a four-day weekend, experiment 2 ran over two weeks. Accuracy for the high and low reward floor was recorded separately for each session.

Floor pairs and the rewarded floors (chevron/diamonds and stars) were consistent within each experiment, to reduce the risk of human error. The floor pair associated with the “high” or “low” reward was counterbalanced across subjects (full counterbalancing was not possible in experiment 1 due to an n of 3). The floor pairs and rewarded floor position were presented pseudo-randomly.

Visual accuracy trends for the high and low rewarded floor were examined across the 10 sessions. A reward bias score was generated across sessions 8-10 by subtracting the mean low reward accuracy from the mean high reward accuracy for these sessions.

2.2.5 Repeated Restraint Stress

Mice were restrained daily for 10 minutes in individual modified 50ml Falcon tubes, with a small breathing hole. Mice were caught and inserted into the tube, after which the lid was screwed on. The tube was too narrow for the mice to turn around but a small amount of forward and backward movement was possible. A longer period of restraint (e.g. several hours) was avoided to prevent potential confounds due to hunger and thirst and because a very severe stressor was not ethically justifiable for this study. Short periods of restraint demonstrate physiological effects in other studies (Li et al., 2020; Paré & Glavin, 1986). There were 10 restraint sessions over two weeks at ~12pm. Five restraint sessions were conducted by Jen Davies. Restraint took place in a separate room away from control animals, which remained in the holding room. The restraint tube was fully cleaned after each session.

2.2.6 Acute Foraging Task

For each session, the foraging apparatus bedding container was loaded with 18g of sizzle nesting material (Datesand). Mice were placed individually into the nest-building area with access to a water bottle and four food pellets (Purina, UK) and left for two hours. Mice spontaneously remove nesting material from the container and typically bring this back to the nest-building area. The weight of sizzle nesting material removed from the bedding container during each session was calculated (bedding container start weight – bedding container end weight). The weight of nesting material removed from the container but left in the foraging area was also recorded and expressed as a proportion of total amount foraged. Each mouse completed five sessions, which ran daily over one week.

Four mice ran simultaneously in the morning (~10am-12pm) with the other four in the afternoon (~1pm-3pm). Running time remained consistent for each mouse. Condition (repeated restraint stress vs control) was counterbalanced across running time.

The mean amount foraged by each mouse across the five sessions was calculated, alongside the mean proportion of removed material left in the foraging area.

Acute restraint foraging task

Mice performed two further foraging sessions: one following acute restraint and one control. For the acute restraint session, mice were individually restrained for 10 mins immediately beforehand.

In each session, two mice underwent restraint and two did not. The first session was conducted five days after the previous protocol ended. There was a six-day recovery period before the second session, allowing mice restrained in the first session to recover before their control session. Running time remained consistent for each mouse from the previous protocol. Acute condition (restraint vs no restraint) was counterbalanced across both chronic condition (previous repeated restraint vs control) and running time.

The weight of nesting material removed from the bedding container following acute restraint and during the control session was compared within-subject. A foraging weight ratio for the acute restraint session was calculated using *weight foraged following acute restraint/weight foraged in control session.* The overall effect of acute restraint on the amount foraged was examined as well as differences between repeated restraint and control mice. A foraging weight ratio was also calculated for the control session using *weight foraged in control session/mean weight foraged in previous five-day protocol.* Three areas were examined: 1) any overall difference between this control session and previous foraging 2) a comparison of repeated restraint and control mice 3) any effect of the order of the acute restraint and control session. Finally, the difference between the proportion of foraged nesting material left in the foraging area in restraint and control sessions was calculated.

For this acute study, an infrared activity monitoring system, developed in-house by Megan Jackson (M. Jackson, 2021), also recorded foraging area activity levels. A sensor with built-in amplifier (AMN 2,3,4 series Motion Sensor, Panasonic) was positioned ~2cm above each foraging area roof (4 sensors overall). The sensor detected movement levels every 100 milliseconds. A percentage of movement was calculated across every 10 seconds and outputted. An Arduino Mega 2560 read the output with an Arduino Mega daughter board (SchmartBoard, Mouser). Data was written as a CSV file onto an SD card using an SD card adaptor (TFT LCD w/microSD Breakout, Adafruit). Time was also outputted alongside activity data using a RealTime clock (Clock & Timer Development Tools PCF8523 RTC for RPi, Adafruit).

Each mouse's movement levels in the foraging area were recorded at 10 second intervals throughout the 2-hour foraging sessions and plotted against time. Area Under the Curve (AUC) was calculated as a proxy for activity levels in the foraging area during the task. Foraging activity levels following acute restraint and in the control session were compared within-subject. Activity levels in the first and second half of the sessions were also compared. A foraging activity ratio was calculated using *activity levels following acute restraint/activity levels in control session.* Foraging activity levels were also compared to the amount of nesting material collected.

2.2.7 Data analysis

Charts were constructed using GraphPad Prism 8.0.1. Statistical analysis, where undertaken, was also performed in this software. Significance was defined as $p < 0.05$ and the exact p values were reported. Statistical analysis was not performed where n was 4 or less per group, due to insufficient subjects to produce robust results. Unless otherwise stated, data was presented graphically showing the mean and standard error of the mean (SEM).

All statistically analysed data were subjected to normality testing using a Shapiro-Wilk test. For the simple discrimination and reversal learning, a paired two-tailed t-test was used to examine the difference between the number of sessions required to learn the initial rule and the first reversal. For the acute restraint foraging task, one sample two-tailed t-tests against a hypothetical value of 1.0 were used to determine: 1) whether the amount foraged was significantly different between the acute restraint and control sessions, 2) whether the amount foraged in the control session was significantly different than the previous five-day average and 3) whether foraging activity levels were significantly different between the acute restraint and control sessions. A one sample two-tailed t-test against a hypothetical value of 0 was used to determine whether the proportion of foraged material left exposed was significantly different following acute restraint stress. Foraging activity for each session half was examined using a two-factor repeated measures (RM) ANOVA to determine whether foraging activity was significantly affected by time. Significant main effects or interactions were reported.

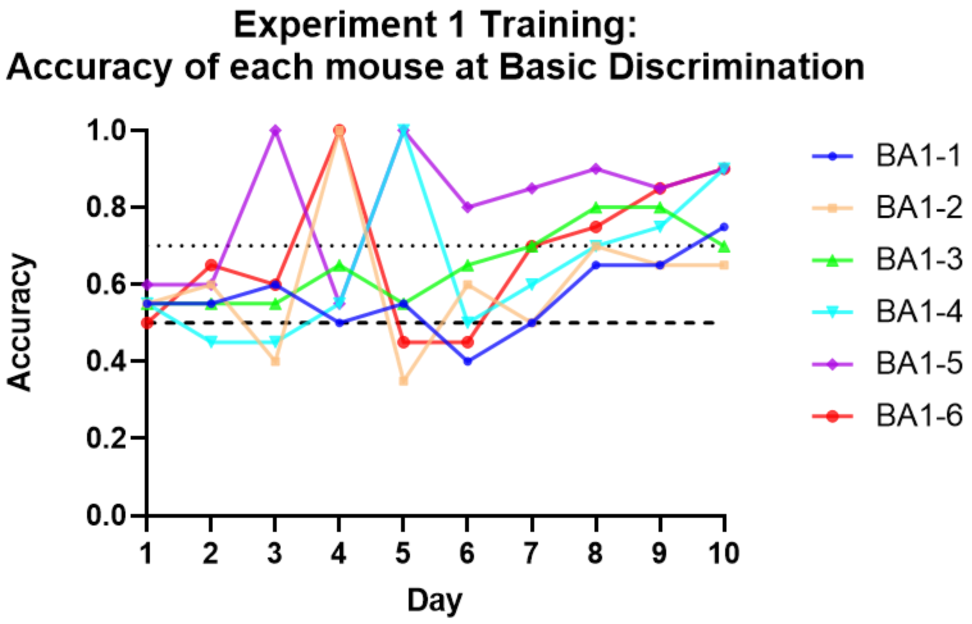
Scatter charts were used on two occasions: 1) to plot the overall mean accuracy of each mouse for the high reward floor across the 10 sessions of high vs low reward learning, against the mean weight that mouse foraged across the five foraging task sessions, 2) to plot foraging activity levels across both the control and restraint sessions against the weight of nesting material foraged. Pearson's correlation coefficient was calculated to test for an association between the two variables. For the acute foraging task, the association between the two variables was tested for four subsets of data: AUC < 25,000, AUC > 25,000, for the restraint session values only, and for the control session values only. Linear regression was used to produce a trendline and the linear model equation was reported. The foraging activity levels scatter chart displays the trendline and linear model equation for AUC < 25,000.

Visual comparisons were made for data where statistical analysis was inappropriate. For high vs low reward learning, mean accuracy scores across the sessions, and the mean reward bias score for session 8-10, were compared between condition groups (group vs singly housed and repeated restraint stress vs control). The range of individual scores within condition groups was also examined. For the foraging task, for the five sessions combined, the mean weight of nesting material foraged, and the proportion of this left in the foraging area, was compared between repeated restraint and control mice. The range of individual five-session means was also compared between condition groups. For the acute restraint foraging task, the mean foraging weight ratios were broken down to visually compare repeated restraint stress vs control mice, and mice that ran the control session before and after the acute restraint session.

2.3 Results

2.3.1 Mice learnt to associate a particular floor texture with a condensed milk reward.

All six mice learnt to associate a particular floor texture with a condensed milk reward, indicated by achieving at least 70% accuracy at least once and performing above chance (50%) for the last three sessions. Better scores were achieved in the final three sessions, potentially aided protocol modifications that increased reward size and delivery speed (**Figure 2.4**).



6 consecutive correct choices = 100% accuracy

Dashed line = 50% accuracy, chance
Dotted line = 70% accuracy, learning threshold

Fig 2. 4 Mice learnt to associate a particular floor texture with a condensed milk reward.

Over consecutive basic discrimination training sessions, mice selected the rewarded textured floor with increasing accuracy when passing through the CCTM.

2.3.2 Mice took significantly longer to learn which textured floor indicates a condensed milk reward after the rewarded floor was reversed.

Following reward contingency reversal, only five out of six mice learnt the new rule. Comparing sessions to criteria for the initial rule and subsequent reversal showed that mice needed significantly more sessions to learn the reversed rule versus the initial rule ($t_{(4)} = 7.207$, $p = 0.002$) (**Figure 2.5**).

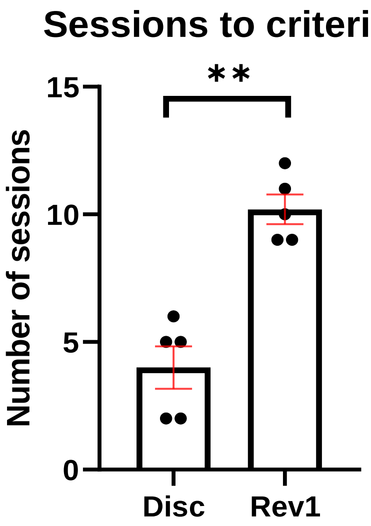
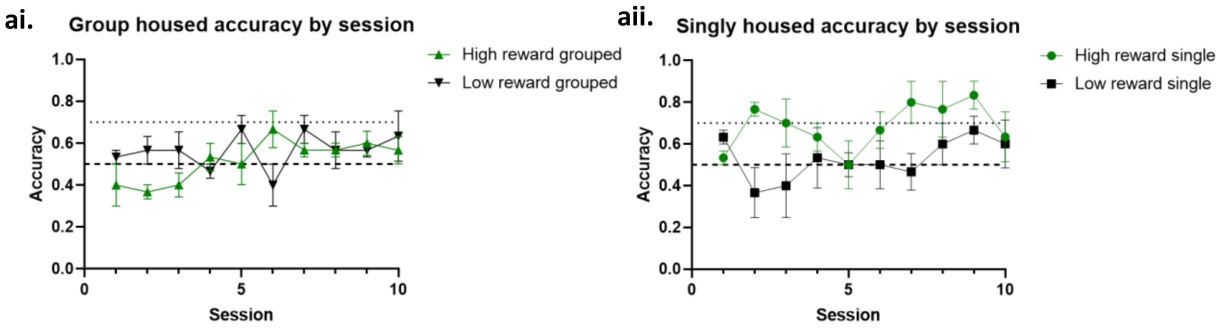


Fig 2. 5 Mice took significantly longer to learn which textured floor indicates a condensed milk reward after the rewarded floor was reversed.

Mice needed more sessions to learn the reversed rule versus the initial rule ($p < 0.01$, paired t-test). Bars are mean ± SEM with data points overlaid. n = 5. ** $p < 0.01$.

2.3.3 Singly-housed mice seemed to show better reward learning than group-housed mice in the high vs low reward learning task; however, the performance was variable.

As the sample size for this study was only three per group, no statistical analysis was performed. Visually comparing the 10 sessions of high vs low reward learning suggests that singly-housed mice showed better accuracy than group-housed mice. This was primarily driven by higher scores for the high-reward floor (mean 0.68 vs mean 0.52). Singly-housed mice selected the high reward floor with at least 70% accuracy for five sessions, suggesting these mice could successfully choose the high-reward floor. However, performance was inconsistent. (**Figure 2.6a**). Across the final three sessions, singly-housed mice showed more of a bias in accuracy for the high-reward floor the group-housed mice (mean reward bias score > 0), but the bias was very variable between singly-housed individuals (**Figure 2.6b**).



Dashed line = 50% accuracy, chance
Dotted line = 70% accuracy, learning threshold

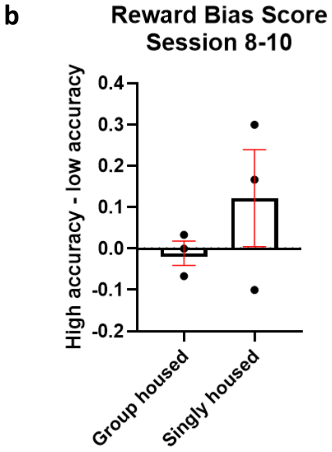
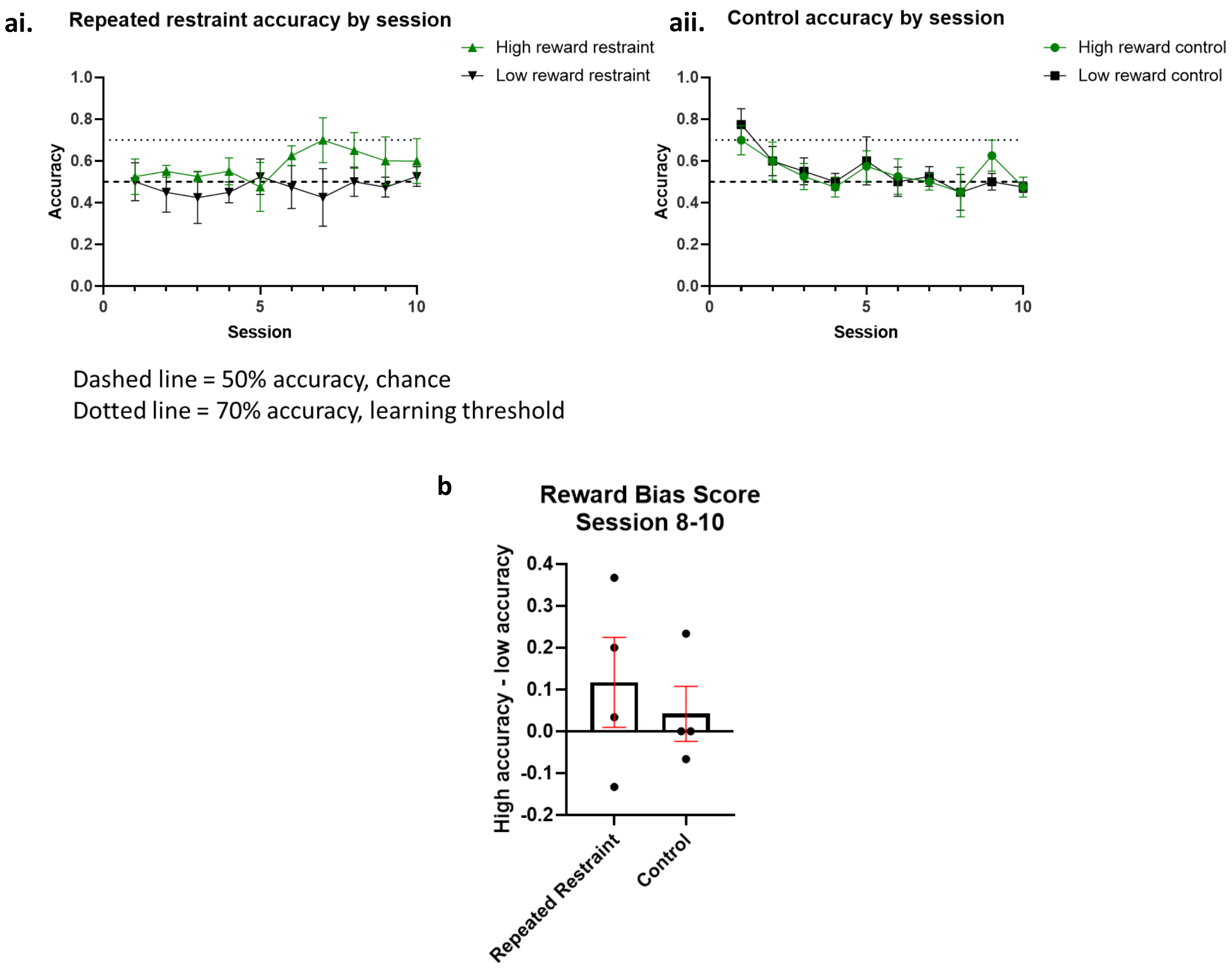


Fig 2. 6 Singly-housed mice seemed to show better reward learning than group-housed mice in the high vs low reward learning task; however, the performance was variable.

ai Group-housed mice appeared to show some improvement in accuracy for the high-reward floor but not the low-reward floor across the ten sessions. Scores remained within a range consistent with random chance. **aii** Singly-housed mice appeared to show some improvement in accuracy for the low-reward floor but not the high-reward floor across the ten sessions. Scores for the low-reward floor remained consistent with random chance. Scores for the high-reward floor for some sessions may suggest mice could successfully select the rewarded floor, however performance was variable. **b** Unlike the group-housed mice, singly-housed mice showed some bias in accuracy for the high-reward floor across the final three sessions, but this varied between inidividuals. Charts show mean ± SEM and bars have data points overlaid. n = 3 per housing type.

2.3.4 Neither control mice nor mice subjected to repeated restraint stress showed reward learning in the high vs low reward learning task.

As the sample size for this study was only 4 per group, no statistical analysis was performed. Visually comparing the 10 sessions of high vs low reward learning reveals that both repeated restraint stress and control mice failed to learn the discrimination of either rewarded vs unreward floor (**Figure 2.7a**). There was a slight upward trend in repeated restraint mice high-reward floor accuracy (mean session 1-3 = 0.53 vs mean session 8-10 = 0.62). However, mean scores remained at chance level for both cues pairs and reward values. Across the final three sessions, repeated restraint mice showed a slightly greater accuracy bias for the high-reward floor (mean reward bias score > 0), but this varied between individuals in both groups (**Figure 2.7b**). The failure of the control mice to show accuracy above chance limits the interpretaton of these results.



Dashed line = 50% accuracy, chance
Dotted line = 70% accuracy, learning threshold

Fig 2. 7 Neither control mice nor mice subjected to repeated restraint stress showed reward learning in the high vs low reward learning task.

ai Repeated restraint stress mice appeared to show some improvement in accuracy for the high-reward floor but not the low-reward floor across the ten sessions. Scores remained within a range consistent with random chance. **aii** Control mice showed no improvement in accuracy for either reward level floor across the ten sessions. Scores remained consistent with random chance. **b** Repeated restraint mice showed slightly more bias in accuracy towards the high-reward floor across the final three sessions, but this varied between individuals in both groups. Charts show mean ± SEM and bars have data points overlaid. n = 4 per treatment group.

2.3.5 Repeated restraint stress did not affect the amount of nesting material foraged in the acute foraging task. The amount foraged correlated with high-reward accuracy in the high vs low reward learning task.

Mice subjected to repeated restraint stress foraged a similar amount of nesting material to unrestrained controls across the five sessions overall (mean 9.7g vs mean 9.0g) (**Figure 2.8ai**). As the sample size was only four per group, no statistical analysis was performed. The mean proportion of foraged nesting material removed from the bedding container but left in the foraging area was higher for control mice than restrained mice (mean 4.0% vs mean 1.2%), although figures were low for both groups (**Figure 2.8aii**).

There was a positive correlation between the mean nesting material weight foraged by each individual mouse over the five sessions, and the mean accuracy achieved for the high-reward floor across the ten sessions of the previous high vs low reward learning task ($r_{(6)} = 0.766$, $p = 0.027$) (**Figure 2.8b**).

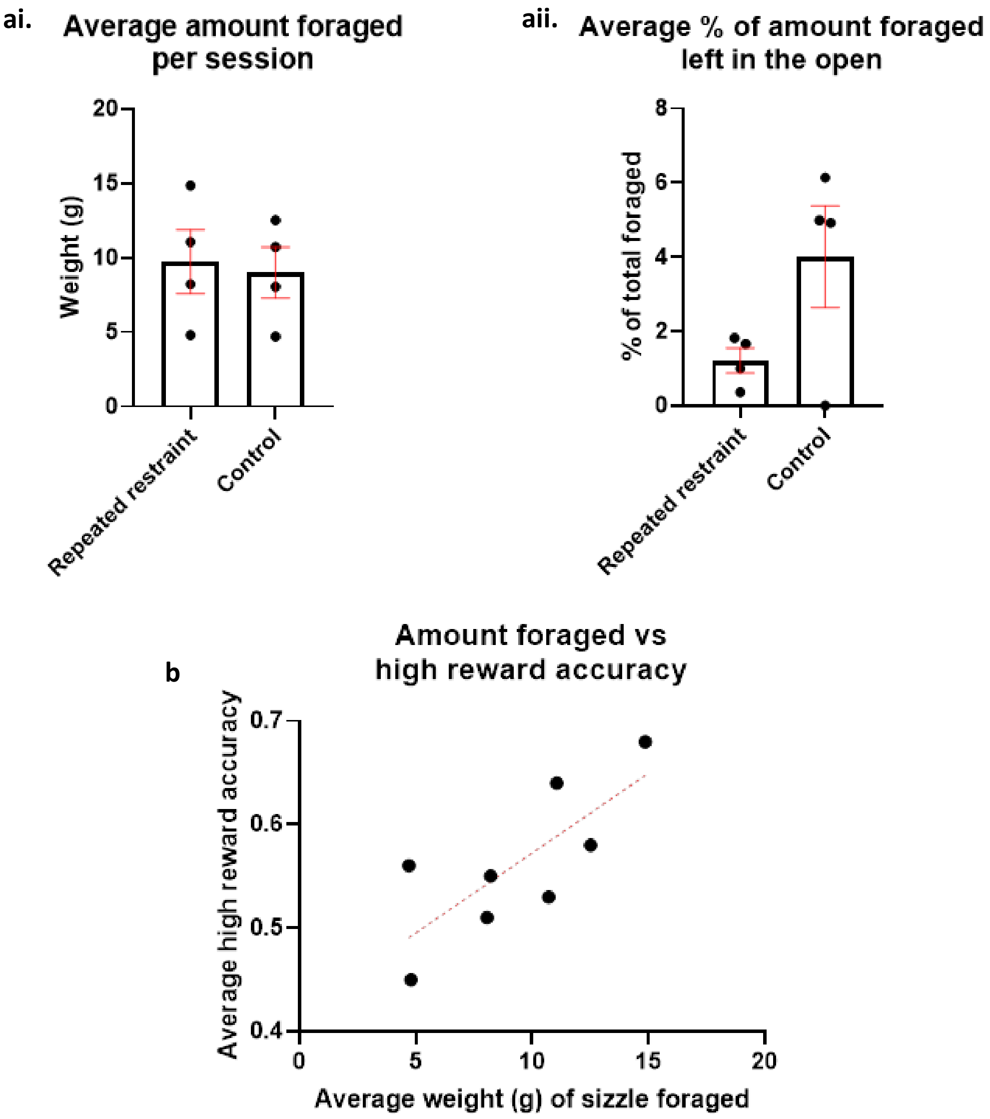


Fig 2. 8 Repeated restraint stress did not affect the amount of nesting material foraged in the acute foraging task. The amount foraged correlated with high-reward accuracy in the high vs low reward learning task.

ai There was no difference in mean nesting material weight foraged by mice subjected to repeated restraint stress and restraint-naïve controls. **aii** The proportion of foraged material left in the foraging area was higher and more variable in restraint-naïve controls, though it remained low for both condition groups. **b** There was a weak positive correlation between the mean nesting material weight foraged by each mouse over the five sessions, and their mean accuracy for the high-reward floor across the ten sessions of the previous high vs low reward learning task (linear reg. $y = 0.01543x + 0.4179$, $R^2 = 0.586$, $F_{(1,6)} = 8.505$, $p = 0.027$). Bars are mean ± SEM with individual means overlaid. Scatter plot shows individual means. n = 4 per condition group (repeated restraint vs restraint-naïve controls). Pooled n = 8.

2.3.6 Immediately following acute restraint stress, mice showed a reduction in nesting material foraged in the acute foraging task.

Following acute restraint, the amount of nesting material foraged significantly reduced ($t_{(7)} = 4.086$, $p = 0.005$) (**Figure 2.9ai**). Previously restraint-naïve mice appeared to show a larger reduction in foraging behaviour. As the sample size was only 4 per chronic condition group, statistical analysis was not performed. Mice with a history of repeated restraint stress were variable in the amount foraged following acute restraint (range 22% - 115% of control session amount), while the restraint-naïve mice were more consistent (range 6% - 48% of control session amount) (**Figure 2.9aii**).

The proportion of collected nesting material left in the foraging area was variable for both control and restraint sessions (range 0% - 8% vs range 0% - 9%). There was no difference in the proportion left exposed following acute restraint relative to the control sessions ($t_{(7)} = 0.030$, $p = 0.977$) (**Figure 2.9b**).

The amount foraged by mice in their control session was similar to the mean amount foraged in the previous 5-day protocol ("baseline") ($t_{(7)} = 0.127$, $p = 0.903$). However, for the two mice previously naïve to restraint stress that ran their control session following the acute restraint session, the amount appeared to be somewhat depressed relative to baseline (mean amount foraged in control session relative to baseline amount 58%) (**Figure 2.7c**).

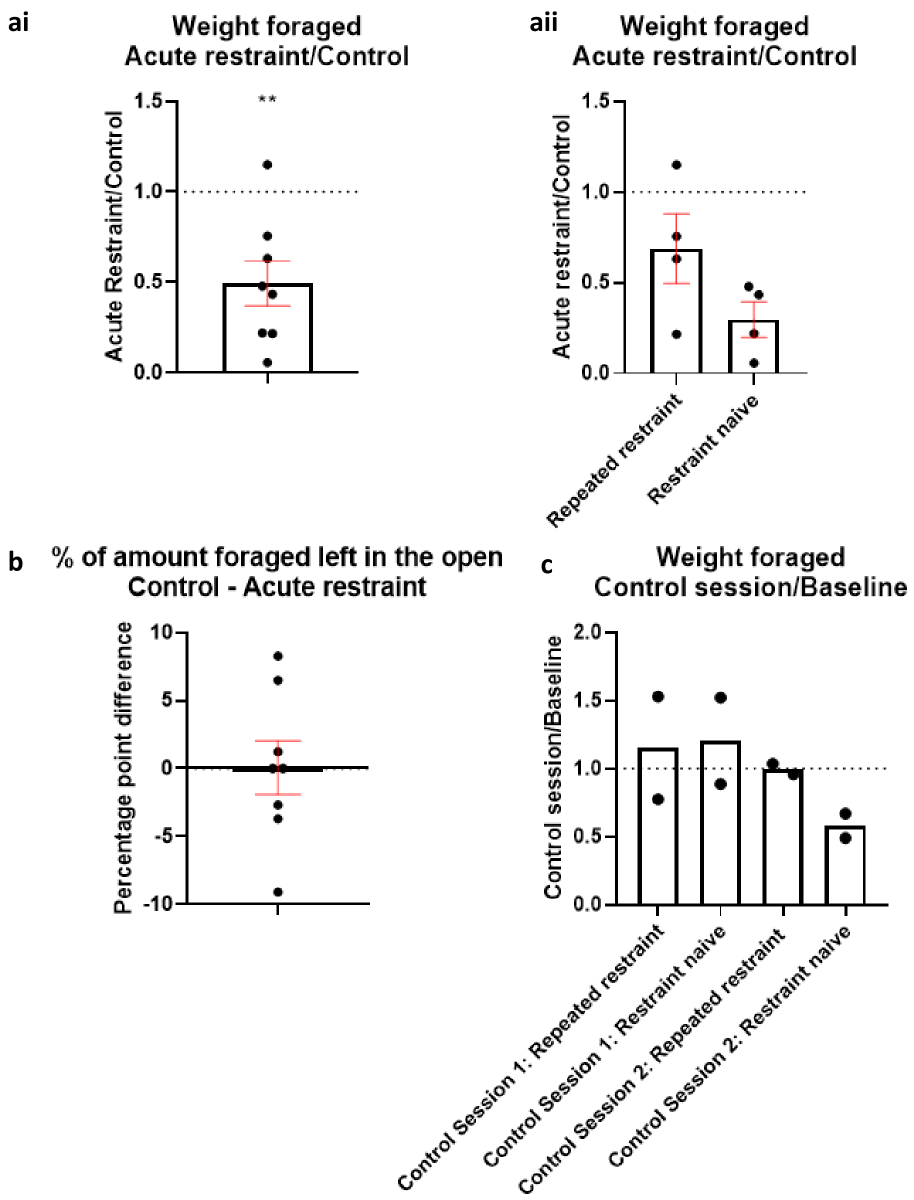


Fig 2. 9 Immediately following restraint stress, mice showed a reduction in nesting material foraged in the acute foraging task.

ai Mice showed a significant reduction in amount foraged following acute restraint, relative to their control session ($p < 0.01$, one sample t-test) **aii** with previously restraint-naïve mice seemingly more affected. **b** Acute restraint did not affect the proportion of collected nesting material left in the foraging area ($p > 0.05$, one sample t-test). **c** The amount foraged in the control session was similar to the mean amount foraged in the previous 5-day protocol (“baseline”) ($p > 0.05$, one sample t-test), though the amount foraged by previously restraint-naïve mice that ran their control session after their acute restraint session was seemingly somewhat reduced. Bars are mean ± SEM where applicable, with data points overlaid. $n = 8$. For repeated restraint vs restraint-naïve $n = 4$ for both groups. When separated by control session order $n = 2$ for all groups. **$p < 0.01$

2.3.7 Foraging area activity was reduced in mice following acute restraint stress.

Acute restraint led to significantly reduced foraging area activity levels versus the control session ($t_{(7)} = 5.256$, $p = 0.001$) (**Figure 2.10ai**). Examining activity levels in the first and second half of each session, there was a main effect of restraint stress ($F_{(1,7)} = 26.153$, $p = 0.001$) and time, where activity levels increased in the second half of the session ($F_{(1,7)} = 6.986$, $p = 0.033$). However, there was no restraint stress*time interaction ($F_{(1,7)} = 1.681$, $p = 0.236$) (**Figure 2.10aii**).

At low activity levels (AUC < 25,000), there was a positive correlation between foraging area activity and amount of nesting material foraged ($r_{(6)} = 0.850$, $p = 0.008$) (**Figure 2.10b**). At higher activity levels alone (AUC > 25000), no correlation was apparent ($r_{(6)} = 0.358$, $p = 0.354$). Similarly, activity levels correlated with amount foraged following acute restraint ($r_{(6)} = 0.874$, $p = 0.005$) but not in the control session ($r_{(6)} = 0.657$, $p = 0.077$).

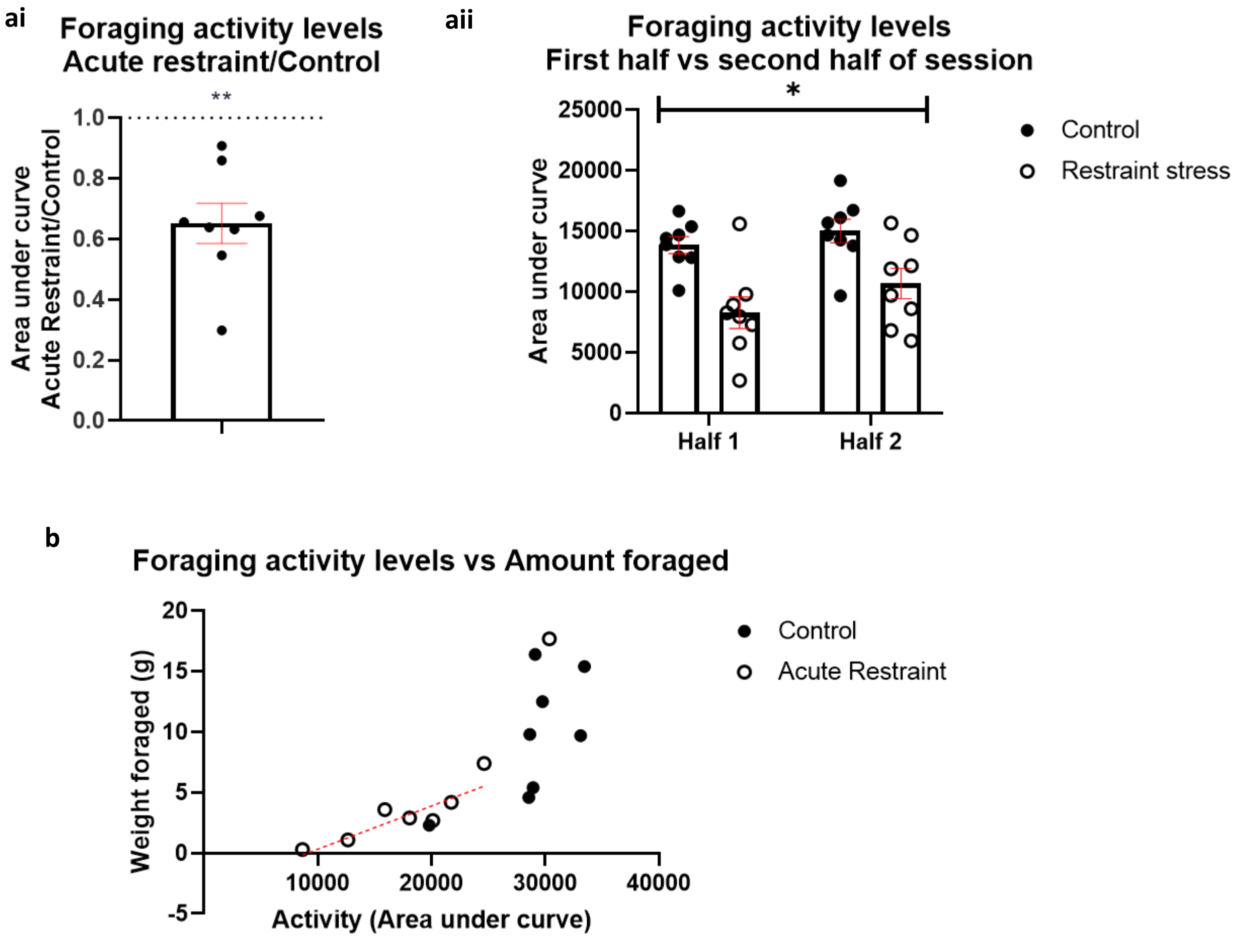


Fig 2. 10 Foraging area activity was reduced in mice following acute restraint stress.

ai Foraging area activity was significantly reduced following acute restraint, relative to the control session ($p < 0.01$, one sample t-test). **aii** When separated into the first and second half of the session, there was a main effect of restraint stress ($p < 0.01$, RM two-way ANOVA) and time, where activity levels increased in the second half of the session ($p < 0.05$). However, there was no restraint stress*time interaction ($p > 0.05$). **b** There was a positive correlation between foraging area activity and weight of nesting material foraged at low activity levels (AUC < 25,000) (linear reg. $y = 0.0003564x - 3.245$, $R^2 = 0.722$, $F_{(1,6)} = 15.561$, $p = 0.008$), primarily seen following acute restraint stress. There was no correlation between activity levels and amount of nesting material foraged at higher activity levels (AUC > 25,000) ($p > 0.05$, Pearson's correlation). Bars are mean ± SEM with individual data points overlaid. Scatter plot shows individual data points. n = 8. *$p < 0.05$, **$p<0.01$

2.4 Discussion

When comparing the group and singly housed mice in the pilot study, there appeared to be reward learning impairments in group-housed mice, indicative of negative affective states. Despite a low sample size and variable data, singly-housed mice showed a high reward accuracy bias, unlike group-housed mice.

In the subsequent study examining the effect of repeated restraint stress, both manipulated and control mice showed poor accuracy across the high vs low reward learning sessions. The mice potentially failed to learn the task as predicted. Unlike experiment 1 mice, these mice experienced fewer prior discrimination sessions and had not undertaken reversal. Their task understanding was potentially worse, and hence they were unable to learn complex high vs low reward discrimination.

The acute foraging task demonstrated evidence of reward motivation change following acute, but not chronic, restraint stress. Interestingly, mouse foraging behaviour positively correlated with accuracy from the previous task, despite low accuracy levels overall. Reward motivation may therefore be relevant to both tasks.

The relevance of these results for developing an objective measure of mouse affective state is discussed below.

2.4.1 Reward learning in the CCTM and its relationship with affective state

Simple discrimination and reversal

All experiment 1 mice reached the simple discrimination learning criteria (at least 70% accuracy for two consecutive days). The consistent choice bias for the rewarded floor shows mice can understand and respond to the floor texture - reward association. In previous work, six consecutive correct trials in two consecutive sessions was the learning benchmark (Jen Davies, unpublished data, Appendix 1). This is statistically more likely to occur by chance ($p = 0.05$ after ~five sessions) and it is encouraging that mice met a more stringent learning criterion.

Also, when the textured floor reward contingencies were reversed, the mice took longer to learn the new, opposite association between floor texture and reward. This is expected as mice must “unlearn” the original association as well as learn the new rule. An increase in sessions required to learn a rule following reversal has previously been demonstrated in a two-choice bowl digging task (Bissonette et al., 2008) (Bissonette & Powell, 2012). These findings are encouraging as it indicates that the mice are responding to the textured floor as expected, not responding to cues from the experimenter.

Mice took four sessions (80 trials), on average, to learn the initial rule before reversal. This is considerably fewer trials than for operant tasks (e.g. touchscreen visual discrimination; ~200 trials (Brigman et al., 2005)). Other non-operant associative learning tasks have reported similar trial numbers, though learning criteria are not always defined consistently, limiting comparability (e.g. spatial recognition; ~80 trials (Bannerman et al., 2003)). While 80 trials is not excessive, two-choice bowl digging has reported more rapid learning (~15 trials (Colacicco et al., 2002), ~20 trials (Kruzich & Grandy, 2004)). However, the Robinson group has found mouse bowl-digging performance inconsistent (unpublished observations).

Note that the mice underwent two weeks of basic discrimination training before the initial discrimination for the reversal task. Mice are considered capable of generalising rules between problems (Bannerman et al., 2003). This task exposure, albeit with different textured floors, potentially enhanced the learning rate (and increased the difficulty of the subsequent reversal).

High vs low reward learning

Singly-housed mice demonstrated greater accuracy than group-housed mice, with a response bias towards the high reward floor, across the high vs low reward learning sessions. However, results were variable between sessions. The low sample size of this pilot study makes drawing strong conclusions difficult. Nonetheless, only the singly-housed mice achieved scores above chance for the higher reward on multiple days, suggesting they may have learnt the higher reward cue. The lower reward cue was not learnt by mice in either housing condition.

In mice with normal reward processing, we would expect to see a reward learning bias towards the high reward cue as sessions progress. This has been demonstrated in previous mouse work (Jen Davies, unpublished data, Appendix 1) as well as an equivalent task in rats (Katie Kamenish, unpublished data). Despite variability, the results in this thesis tentatively indicate that singly-housed mice showed normal reward learning, while group-housed mice may have displayed reward learning deficits. These deficits suggest a negative affective state in the group-housed mice. This supports the hypothesis that social stress associated with group housing negatively impacts male mouse welfare, even without overt signs of aggression.

Nonetheless, a potential difficulty arises from the food restriction of the mice in this experiment. Mice were food restricted while under protocols using the T-maze to increase motivation. The singly housed mice were more affected by the food restriction and weighed less than the group-housed mice when the high vs low reward learning task began. This is probably due to singly housed mice using more energy for thermoregulation. The singly housed mice may have been more motivated by the condensed milk food reward, which might explain their generally greater accuracy in the task.

Overall, the housing study results were somewhat unclear. So, a second pilot was conducted using a method regarded as more reliable at inducing negative states in mice, repeated restraint stress. Repeated restraint aims to model chronic stress, and has been used to model depressive behaviour development in accordance with the stress-diathesis depression model (Zhao et al., 2013).

Unfortunately, in the high vs low reward learning task, neither control nor restraint-subjected mice learnt the cues associated with either reward. The mice had previously undertaken five basic discrimination training sessions to learn the principle of associating floor texture with reward. Nonetheless, the mice perhaps did not learn the task as predicted during this training phase. Compared to experiment 1 mice, which also completed a reversal protocol, these had less exposure to the CCTM before undertaking the high vs low task. Two mice only just met the minimum criterion of at least one basic discrimination session scoring at least 70% accuracy. One week of basic discrimination training has proven sufficient in previous iterations of the high vs low protocol conducted by a different experimenter (Jen Davies, unpublished data, Appendix 1). However, there may still be handler influence even in this semi-automated task. More experimenters need to try the protocol to ascertain the influence of experience and other human factors. Others may also find high vs low reward learning more successful with increased pre-training in both simple discrimination and reversal.

Ineffective training seems the likely reason for generalised poor reward learning in the CCTM. Nonetheless, another potential explanation could be a negative affective state from all the mice. This may be influenced by reduced handling exposure prior to starting the high vs low protocol compared to experiment 1 mice. Nonetheless, other mice have successfully demonstrated expected reward learning under similar circumstances (Jen Davies, unpublished data, Appendix 1), though this may again be influenced by increased handler experience. Also, the experiment 2 mice were less food restricted than those in experiment 1, potentially reducing their task motivation. Future work could examine the influence of food restriction levels on CCTM performance.

2.4.2 The acute foraging task and its relationship with reward learning and affective state

Experiment 2 mice were also tested in the acute foraging task, which is thought to examine reward motivated behaviour. There was no difference in nesting material weight foraged by mice with and without exposure to repeated restraint stress. Results should be interpreted cautiously due to the small sample size of this pilot study. Nonetheless, the motivational state of the chronically restrained animals was potentially unaffected by the treatment. Alternatively, the task may be insensitive to negative affective states/behavioural changes induced by repeated restraint stress. Also note that restraint was not continued throughout the testing period. Effects may not have persisted long enough to impact task behaviour.

Increasing effort/anxiety levels by increasing the foraging area size or tube length connecting to the nest-building area reduces the amount of nesting material foraged (Megan Jackson, unpublished data). Similarly, aged mice (which show reduced motivated behaviours (M. G. Jackson et al., 2021)) collect less nesting material than young mice (Megan Jackson, unpublished data). Altogether, this suggests the task is sensitive to motivation. The task also seems sensitive to chronic corticosterone exposure, which reduced foraging levels, but only in session one (Megan Jackson, unpublished data). Again, this result was not replicated in the repeated restraint stress group (Appendix 2). The behavioural profile of these restraint stress mice seems different from mice that had undergone a pharmacological negative affective state manipulation.

Other studies have also not seen depressive behaviours in mice following repeated restraint stress (Zhu et al., 2014). Chronic restraint's predictability may facilitate habituation (Baker & Stephenson, 2000). In rats, there is even some evidence that chronic mild stress exposure decreases depression and anxiety-like behaviours (Parihar et al., 2011). Most chronic restraint stress protocols reporting negative effects employ significantly longer restraint times than the ten minutes used here. Restraint length influences physiological effects (Paré & Glavin, 1986), and ten minutes was perhaps insufficient. It is also unclear how long any negative affective state induced by chronic restraint would persist. The foraging task was conducted two weeks after the final restraint, and negative effects may have abated. In rats, some neurophysiological changes in response to repeated restraint can reverse within 10 days (Conrad et al., 1999). Unpredictable Chronic Mild Stress (UCMS) is another protocol used to induce a depressive phenotype in mice and may more reliable (Bondi et al., 2008; Zhu et al., 2014). Further work validating the high vs low reward learning task as an affective state assay, and examining behaviour in the foraging task, should utilise UCMS.

Mouse high reward accuracy was compared to the amount of nesting material collected in the foraging task. While mouse behaviour appeared unaffected by repeated restraint in both tasks, individual mice still varied in CCTM performance and foraging levels. High-reward accuracy in the CCTM positively correlated with amount of nesting material collected. This might suggest an underlying motivational component also influenced reward learning in the high vs low task.

Personality trait-level motivated behaviour differences perhaps drove individual performance differences in this instance. Different consistent behaviour patterns are well documented in many species (Gosling, 2008). Motivational personality traits are commonly discussed in human behaviour literature (Scheffer & Heckhausen, 2008), but mouse studies are seemingly lacking. Trait anxiety is thought to underly human motivation (due to heightened risk sensitivity) (Kanfer & Heggestad, 1997), and is also a recognised mouse trait (Jakovcevski et al., 2008). Trait anxiety was perhaps driving the motivational behaviour differences seen in the mice, a suggestion supported by the observation that anxiety has been linked to motivational deficits in calmodulin signalling knockout mice (Davis et al., 2012).

While interesting, the mice in this study did not achieve high accuracy levels in the CCTM reward learning task. The correlation with foraging task performance has therefore only been evaluated for mice with poor reward learning. The relationship may not hold in groups of mice with more successful reward learning.

Acute restraint foraging task

Performance in the foraging task was also examined immediately following acute restraint. A single restraint episode induces a stress response in mice, characterised by HPA axis activation (Buynitsky & Mostofsky, 2009) (**Figure 1.2**). Acute restraint can therefore be used to model how the acute stress response affects mouse behaviour. Both depression- and anxiety- like behaviours have been reported in mice subjected to acute restraint (Casaril et al., 2019; Domingues et al., 2019).

Mice foraged less nesting material and spent less time active in the foraging area immediately following acute restraint. Foraging activity in the second half of the session remained reduced, suggesting short-term motor effects following the movement restriction were not driving the behaviour change.

Perhaps unexpectedly, foraging area activity did not display a simple linear relationship with nesting material collected. The amount of nesting material collected only correlated with foraging area activity at low activity levels. Under these circumstances, willingness to enter the foraging area may be limiting nesting material collection. Acutely stressful events, like restraint, could reduce this willingness. Once foraging area activity passes a certain threshold, other factors seemingly determine how much nesting material is collected. This suggests the task is sensitive to multiple factors affecting motivated behaviour.

Anxiety may be a key factor modulating foraging behaviour. The foraging area is less enclosed than the nest building area and may be anxiogenic to mice. Increased anxiety-like behaviour has been reported following acute restraint in mice (Casaril et al., 2019; Domingues et al., 2019). The perceived benefits of foraging may also be dulled following restraint, so mice reduce foraging area entry as foraging drive is reduced. Either hypothesis would indicate a motivational deficit. The foraging task needs testing with more affective state manipulations (e.g., UCMS, pharmacological interventions, addition or removal of enrichment) and results need to be cross-referenced with other behavioural tasks (e.g. open field, radial arm maze, sucrose preference) to build a better picture of detectable phenotypes.

As a final observation, foraging of previously restraint-naïve mice was compared with those formerly subjected to repeated restraint stress. Acute restraint seemingly had less effect on the repeated restraint mice. Furthermore, foraging seemingly remained reduced in the control session for the two restraint-naïve mice which ran this after their restraint session. This effect was not seen for the repeated restraint mice that also had sessions in this order. This suggests either a prolonged single restraint effect or some fear conditioning, but only for restraint-naïve mice. Altogether, this supports the hypothesis that there was some restraint habituation in the repeated restraint mice. This also suggests ten minutes of restraint is sufficient to produce a long-lasting behavioural response in mice. Therefore, the chronic stress protocol's short restraint time was perhaps not the primary reason for not detecting a negative phenotype in the earlier foraging task.

2.4.3 Conclusion

Given the limited sample sizes used for this study, it is difficult to draw firm conclusions from these results alone. Nonetheless, in combination with Jen Davies' previous work (Appendix 1), these data do provide further indications that mice can learn to discriminate rewarded from unrewarded non-spatial cues in the CCTM, and that this learning can take place with other experimental operators. The high vs low reward learning task also tentatively appeared to demonstrate reward-learning deficits in group housed mice. If this result is replicated in further groups of male mice, it would support the hypothesis that group-housed mice experience a more negative affective state due to the inappropriate social housing environment. Experience of fighting in a group housing environment has already been seen to negatively impact affective state (Jen Davies, Appendix 1).

The repeated restraint stress pilot was unsuccessful. Further validation of the task demonstrating impaired reward learning in mice with confirmed negative affective states, perhaps using UCMS, is still required. Further optimisation studies are also needed to ensure learning is reliably achievable for mice in neutral/positive states across operators and minimise daily variability. Increased pre-training may facilitate learning and improve results. Another potential modification is re-presenting the incorrect trails during the high vs low task, as well as in training. Full task automation could benefit results by removing any potential operator influence.

While the sample size was again small, there appeared to be no reward motivation impairments detectable by the foraging task following repeated restraint. However, acute restraint did reduce foraging behaviour. Further studies are needed to ascertain what affective states this task is sensitive to. The finding that foraging levels correlate with high-reward accuracy in the CCTM suggests an underlying motivational factor may influence both tasks under some circumstances. More work is needed to determine whether this finding stands when reward learning is more effective, and when different affective states are being compared. It is possible that CCTM reward learning is sensitive to motivational factors separable from core affective state, such as motivational personality traits (e.g., anxiety). Sensitivity to personality could be investigated further in both tasks. This may help inform cohort sizes for future studies, based on the influence and variability observed.

Chapter 3: Developing a protocol to train and test chickens in a reward learning assay

3.1 Introduction

Chickens are the most abundant domesticated species, with a global population of over 33 billion at any one time, while ~70 billion are slaughtered annually (FAO). Most chickens live in intensive production systems, which raise significant welfare concerns. In the UK, laying hens have received particular attention, with conventional battery cages banned in 2012, and over half of egg production is now free-range (Defra, 2022). Nonetheless, despite the large global chicken population, and growing interest in their welfare, understanding different production environments from the chicken's perspective remains challenging. Even with progression to free-range production, it remains unclear how much these conditions, perceived as higher welfare by humans, are experienced as such by hens.

While chickens undoubtedly have greater opportunities for behavioural expression in loose housing, significant problems remain. It is still necessary to blunt the beaks of loose-housed hens, due to abnormal injurious pecking behaviours occurring (Nicol et al., 2013). The presence of abnormal behaviour suggests the environment may still cause negative welfare in laying hens (Nicol et al., 2013). Similarly, while access to the outdoors is perceived as favourable by humans, typically less than half of hens go outside at once (Pettersson et al., 2016). How outdoor areas are often designed may be suboptimal, meaning the welfare benefits could be less than we might expect. A further issue is that a high proportion of laying hens experience keel bone fractures (Rufener & Makagon, 2020). These likely cause pain for weeks, and risk causing an overall negative welfare state regardless of available behavioural opportunities (Riber et al., 2018). Overall, we need to improve our understanding of what benefits chicken welfare from their perspective.

Many welfare assessment methods have been utilised in chickens. Preference tests (Chapter 1, section 1.2.3) have addressed questions including preferred dustbathing substrate (Shields et al., 2004) and flooring type (Hughes & Black, 1973). Research has also identified valuable resources e.g., litter substrate (Dawkins & Beardsley, 1986), and strongly motivated behaviours like dustbathing (Wichman & Keeling, 2008). Physiological stress indicators (Chapter 1, section 1.2.1) have also been used to infer welfare under different housing conditions (Paul et al., 2022). This work has enabled some positive developments in poultry housing. Nonetheless, difficult laying hen welfare problems remain unsolved, perhaps due to limitations in our current welfare assessment methods. As discussed in Chapter 1, section 1.2.3, preference studies may be confounded by factors including prior familiarity (Dawkins, 1977) and motivation study results may be difficult to explain correctly. Similarly, elevated stress measures do not correlate straightforwardly with negative affective states

(Chapter 1, section 1.2.1). To address ongoing chicken welfare issues, we need measures that can objectively assess their overall affective state under different commercial housing conditions.

Affective state-induced cognitive bias tests could be valuable for improving chicken husbandry and evaluating different farms. Fewer published studies demonstrate affective state-induced cognitive bias in avian species compared to rodents. Nonetheless, judgement bias protocols have been developed, based on visual or spatial discrimination (**Figure 3.1**). The effect of different enrichment levels on hens kept under experimental conditions has been assessed (Košťál et al., 2020). Results have been variable, with the environmental manipulations unreliable in inducing the expected judgement bias (Košťál et al., 2020; Paul et al., 2022).

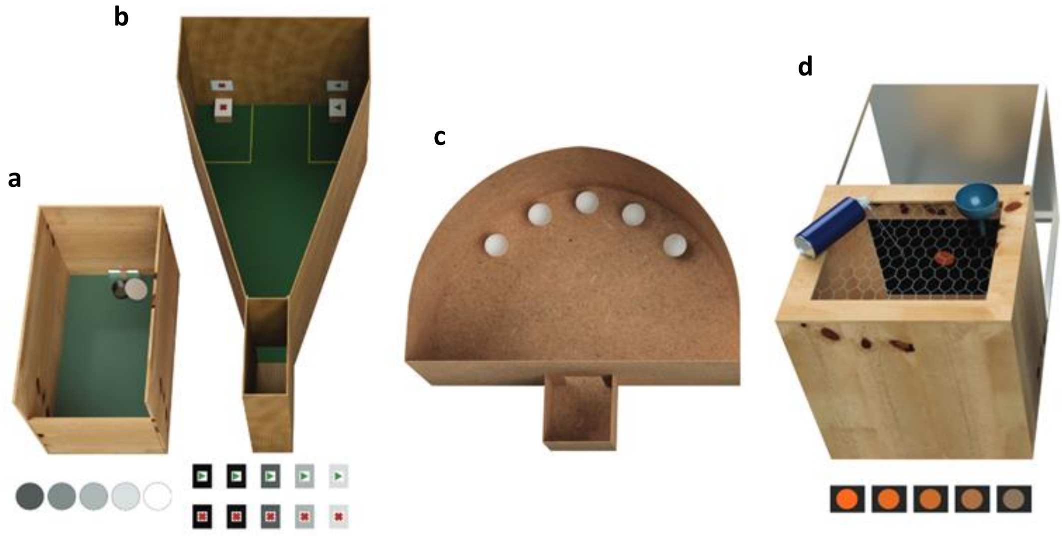


Fig 3. 1 Discrimination tasks used for Judgement Bias testing in adult chickens

a Test arena used by (Ross et al., 2019). A food dish is inserted through an opening. The lid colour indicates reward or punishment. **b** Two-choice arena used by (Hernandez et al., 2015) and (de Haas, Lee, Hernandez, et al., 2017; de Haas, Lee, & Rodenburg, 2017). Image cues indicate the presence or absence of reward, the background colour indicates reward magnitude. **c** Spatial judgement arena used by (Wichman et al., 2012). Chickens are taught to associate the far-left and far-right location with reward/punishment. Ambiguous locations are used for judgement bias testing. **d** Operant task used by (Deakin et al., 2016) and (Paul et al., 2022). A screen peck test uses colour saturation cues indicating reward or punishment, which is delivered manually. Image adapted from (Košťál et al., 2020).

Task design issues may explain the variable results. “Go/no go” protocols have been used (Deakin et al., 2016; Paul et al., 2022; Ross et al., 2019; Wichman et al., 2012), which are vulnerable to mobility and motivational confounds. Determining whether no response is an active decision or omission from the test subject is also difficult. Some tasks have been designed with punishers instead of differential reward levels (Deakin et al., 2016; Paul et al., 2022; Ross et al., 2019). These risk predisposing animals to negative judgement bias even without negative affect, as the punishment avoidance drive may be stronger than reward motivation. The tasks developed may also involve chicken handling during training and testing, and in some cases human handlers may be visible to the birds throughout (de Haas, Lee, Hernandez, et al., 2017; de Haas, Lee, & Rodenburg, 2017; Deakin et al., 2016; Hernandez et al., 2015; Paul et al., 2022; Wichman et al., 2012). This, again, risks confounding results as human behaviour may inadvertently influence bird behaviour.

Successful discrimination training is required for judgement bias, and lengthy training and testing protocols limit this technique for practically assessing chicken welfare (e.g. 42 days (Ross et al., 2019), 24 days (Hernandez et al., 2015), 29 days (de Haas, Lee, & Rodenburg, 2017), 22 days (Deakin et al., 2016)). This is pertinent if techniques were to be used on commercial farms, where time would be limited. Prolonged training may also render the behaviour less sensitive to affective state. If behaviour becomes procedural, rather than cognitive, affective state-induced cognitive biases will not be detectable. Spatial cue-based tasks seem to particularly risk becoming procedural; chickens appear prone to side-biases (de Haas, Lee, Hernandez, et al., 2017). Spatial-based discrimination tasks may be inappropriate for chickens, and care should be taken in visual tasks to control for this risk (see (Hernandez et al., 2015), (de Haas, Lee, Hernandez, et al., 2017) and (de Haas, Lee, & Rodenburg, 2017) for an illustration of not controlling for side-biases). Another problem with prolonged training is that the task may serve as an enrichment, and potentially confound the results by improving affective state over time.

There have also been difficulties with all chicken subjects achieving the learning criteria in previous studies (training success rates; 67%. (Hernandez et al., 2015), 40% (de Haas, Lee, Hernandez, et al., 2017), 75% (de Haas, Lee, & Rodenburg, 2017), 89% (Wichman et al., 2012), 63% (Deakin et al., 2016) (Košťál et al., 2020)). An effective assessment of population-level affective state cannot exclude a subset of animals.

As with rodents, developing a reward learning protocol may provide an easier and quicker way to assess chicken affective states. Chickens naturally scratch and peck in substrates for food (Thuy Diep et al., 2018). Developing an ethologically relevant task based around this naturally motivated behaviour may facilitate learning and enable differential reward learning assessment. Rats, for example, can learn a bowl-digging discrimination task, which utilises their natural foraging behaviour, in ~15 trials, whereas learning a visual discrimination task in an operant box requires ~1000 trials (Griesius et al., 2023).

If reward learning could be a cross-species affective state assay, we need to establish whether reward learning deficits associate with negative states similarly across taxa. Chickens are evolutionarily distinct from humans and rodents, where the effect has been studied so far. So, validating the phenomenon in chickens would provide useful insight into the extent to which it is conserved across species. To do this, a reliable chicken reward learning training protocol, which minimises previous challenges, is needed. Therefore, this chapter aimed to develop a semi-automated reward learning apparatus and training protocol, comparable to the mouse CCTM. Subsequently, this could be used to test whether chickens show reward learning deficits in negative affective states.

3.2 Methods

3.2.1 Subjects

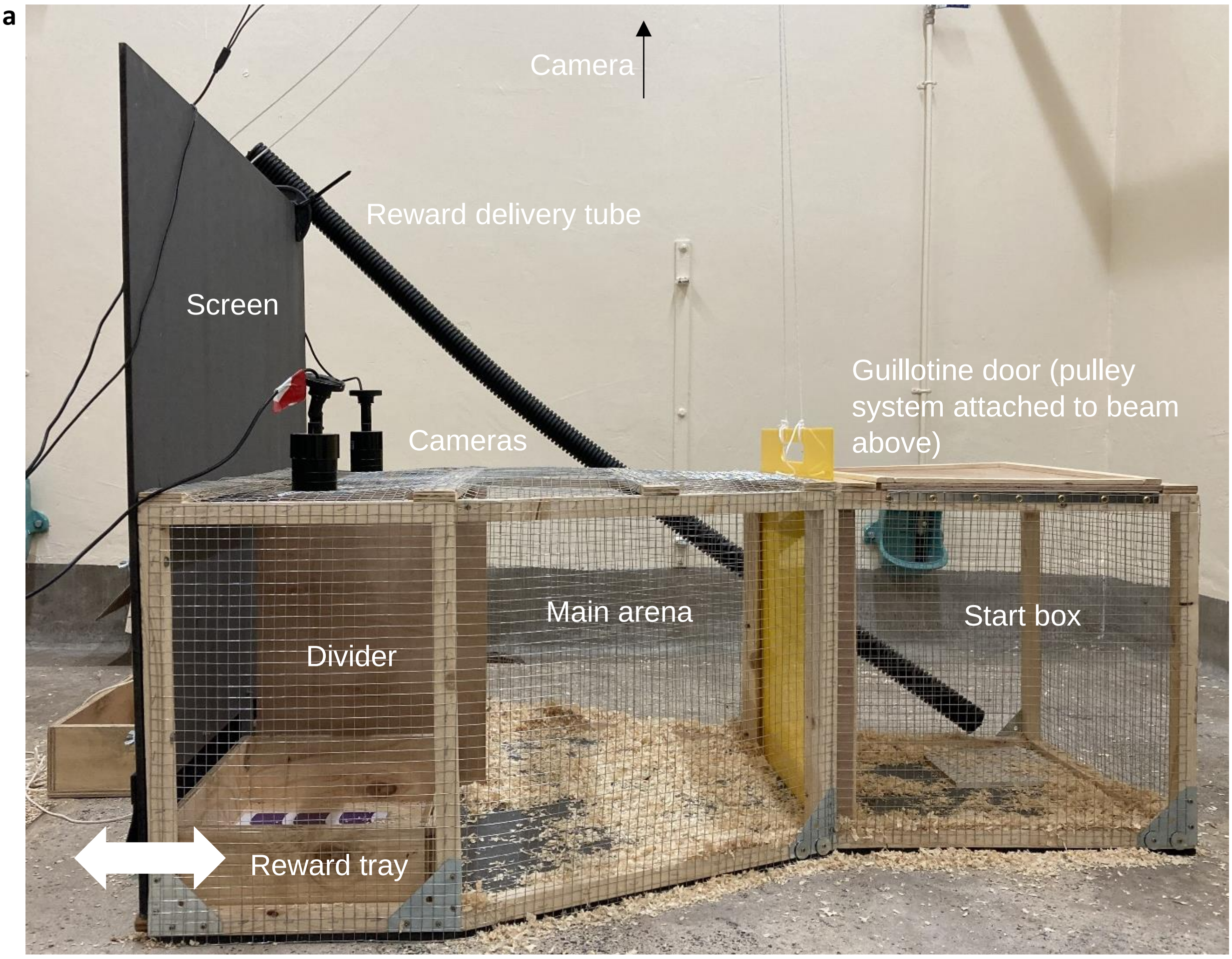
Subjects were six adult (60+ weeks) Hy-line brown laying hens, supplied by a free-range commercial farm. Hens lived together in enriched indoor housing, with wood shavings, a bale of straw, wooden perches (three structures 20cm – 1m high) and 10 nest boxes. Water and food were available *ad libitum* from suspended hoppers. Hens were kept in 12:12 artificial lighting, with lights ON at 8.00am and OFF at 8.00pm and temperature was maintained at ~19°C. Behavioural testing took place under artificial lighting between 9.00am and 5.00pm. Hens underwent a behavioural training and testing programme as outlined below. The work was reviewed and approved by the Bristol University Animal Welfare and Ethical Review Body (UIN-22-078 granted July 2022).

3.2.2 Apparatus

Hens were trained and tested in a novel enclosed wooden-framed wire arena, located in a separate room (**Figure 3.2,** Appendix 3). A Perspex guillotine door separated a start box (2500cm^2) from a main arena (7250cm^2). A seated operator, hidden by a screen (10,000cm^2), inserted wooden trays (1200cm^2) containing rewards and/or foraging substrate into the arena through slots in the screen. Visual reward cues could be attached to the tray front, inside back, or bottom. The arena floor was lightly covered with shavings from the chicken home pen.

The apparatus was designed so chickens could shuttle between the main arena and the start box without operator interference. Two ropes were attached to the guillotine door and fed through a pulley system suspended from a wooden beam above the arena. A plastic reward delivery tube was attached to the arena side, connecting the top of the screen to the start box. These enabled the experimenter to operate the door and deliver rewards to the start box from behind the screen. A ceramic tile highlighted the reward delivery location in the start box.

The operator monitored chicken behaviour via live video captured by a Swann H.264 Digital Video Recorder using an adjacent screen. One Swann SWPRO-735CAM camera was suspended above the arena from a wooden beam. Two more were positioned on top of the wire arena above the inserted wooden trays.



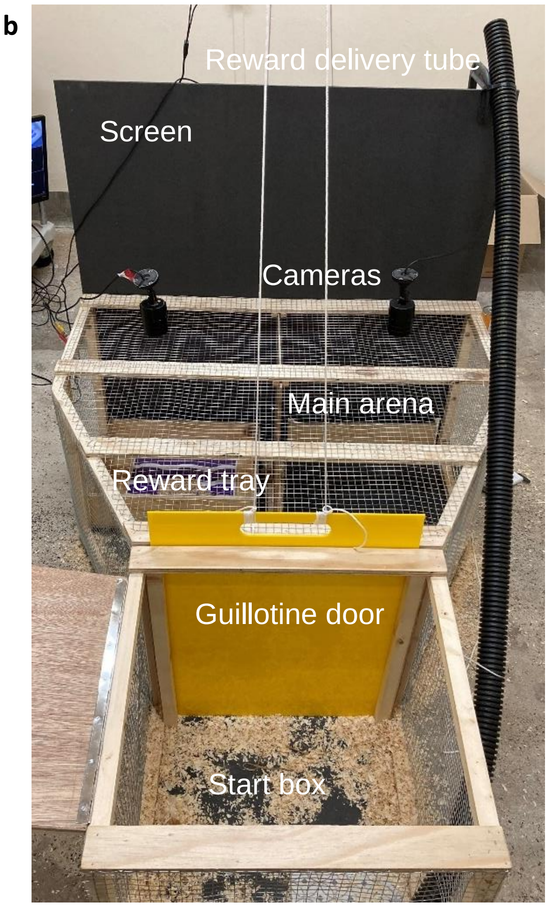


Fig 3. 2 Chicken Reward Learning Arena

(See Appendix 3 for design drawings) **a** Side view. **b** Aerial view.

3.2.3 Reward learning training protocol

Habituation

Chickens were habituated to the rewards, testing apparatus reward trays, handling, and the testing arena before training:

For one week (five days), high value rewards (tinned sweetcorn, live mealworms, corn) were put in the reward trays and placed in the chickens' home pen daily. The chickens rapidly ate the tinned sweetcorn and live mealworms by day five, though some corn was left.

Next, chickens were habituated to handling over three consecutive days. On day one, chickens were hand-fed rewards (tinned sweetcorn and live mealworms). On day two, the chickens were caught and briefly handled at least twice, and hand-fed food rewards. On day three, chickens were caught, carried outside their home pen, and given food rewards. The chickens were caught without chasing and consumed food rewards while being held.

Chickens were subsequently habituated to the testing arena over at least two days. In session one the arena was set up with the reward trays inserted but no guillotine door. Three sweetcorn kernels were placed in each tray and on the start box tile. Chickens were individually placed into the start box and given 10 minutes to consume the sweetcorn. Throughout the session, the trays and start box were rebaited when all the sweetcorn was consumed. All chickens consumed sweetcorn from all three locations in the first session and progressed to the next stage.

Stage two was as above, except the guillotine door was introduced. On setup, the guillotine door was inserted in the closed position. Chickens were individually placed into the start box and the door was opened. When the chicken consumed the start box sweetcorn and exited, the door was closed. The door remained closed until the sweetcorn in the trays was eaten and all locations were rebaited. The door then remained open until the start box sweetcorn was consumed and the chicken left the start box. This was repeated for 10 minutes. Chickens received vocal encouragement if exploring ceased for >60s. After 10 minutes the chickens were shut into the start box. Progression to the next stage was contingent on eating sweetcorn from all locations at least five times. Most chickens were fearful of the door and needed repeat sessions. The number of sessions ranged from one-five. (Appendix 4).

Developing a shuttle and forage training protocol

Chickens were taught to search a tray with foraging substrate for a hidden sweetcorn reward. **Table 3.1** describes protocol development. **Table 3.2** shows the number of sessions of each stage undertaken by the chickens. Notes regarding chicken performance and technical difficulties experienced at each stage are summarised below.

Chickens were initially trained to shuttle between the start box and the foraging trays by sequentially baiting these locations. This was straightforward for most chickens, which generally exited the start box rapidly and immediately consumed the sweetcorn. Chickens returned to the start box rapidly upon hearing sweetcorn transiting the reward delivery tube.

Training to forage for sweetcorn in a tray of gravel was attempted. Chickens found the sweetcorn easily if it was visible, including when it was partially obscured. No chickens spontaneously looked for completely buried sweetcorn and did not scratch in the gravel trays. The experimenter attempted to demonstrate looking for buried sweetcorn. Two chickens did eventually seem capable of finding buried sweetcorn, but only if gravel was piled into a mound over it. Wood chip was trialled instead of gravel to try and stimulate foraging behaviours, but there was no improvement. Another difficulty was determining if the chicken had found and consumed the sweetcorn in a tray full of substrate, due to poor video camera resolution.

For three days during this initial foraging training, the chickens were also presented with the reward trays in their home pen. Tinned sweetcorn and live mealworms were buried under wood chips in the trays, aiming to encourage spontaneous searching for hidden food in the testing apparatus. Chickens scratched in the trays under these circumstances, but this did not increase these behaviours in the testing apparatus. Reward density was much higher in the trays in the home pen, potentially influencing the foraging activity level, alongside the presence of conspecifics.

Chickens were subsequently taught to return to the start box before the sweetcorn was delivered. This was to encourage them to connect performing an action with receiving the sweetcorn, rather than just looking/listening for it. Initially, chickens would not fully re-enter the start box without hearing sweetcorn being delivered. They were first rewarded for putting their head in the start box, but subsequently all chickens were trained to enter fully before sweetcorn was received.

Foraging training was then re-attempted, hiding the sweetcorn in a wood chip pile. The chickens again initially did not look for obscured sweetcorn. The experimenter encouraged them to stand on the wood chip pile, which disturbed it and revealed the sweetcorn. After doing this once, the chickens then reliably looked for hidden sweetcorn independently and shuttled between the reward trays and start box. The smaller substrate pile also facilitated monitoring sweetcorn consumption via the camera system.

Some adaptations were made when training one chicken (“Grey”). Initially, it often stopped responding and would stand and call repeatedly. These problems ceased after stage seven. The chicken did not subsequently behave differently to the others.

Training stage	Description
1 Shuttle training (1)	The start box was baited (x3 sweetcorn) and the door closed. The centre of one reward tray was also baited (x1 sweetcorn). Chickens were individually placed into the start box and the door opened. Chickens were given as long as necessary to find and consume the reward tray sweetcorn. Then, the start box was rebaited (x3 sweetcorn) via the reward delivery tube. On entering the start box, the door was closed, and a tray was rebaited (x1 sweetcorn). 20 trials were conducted. The baited tray and sweetcorn position within the tray (centre, left, right) was pseudorandomised. Aimed to complete 20 trials in maximum 30 minutes.
2 Foraging training- gravel (1)	Chickens were trained to search for sweetcorn in gravel. (A) For trials 1-2, the trays contained no gravel and one was baited with x1 sweetcorn. (B) For trials 3-20, the baited tray contained ~2cm of gravel, with the sweetcorn on top. Sessions otherwise proceeded as for stage one.
3 Foraging training- gravel (2)	Trials 1-2 were as stage 1B. (C) For trials 3-11 the sweetcorn was partially buried. (D) For trials 12-20 the sweetcorn was fully buried. When chickens stopped/failed to search, the gravel was disturbed with a stick to reveal the

	sweetcorn, aiming to encourage chicken foraging in future attempts.
4 Foraging training- gravel (3)	Trials 1-2 were as stage 2C. Trials 3-18 were as stage 2D. Success at finding buried sweetcorn independently was recorded for this and subsequent stages where relevant.
5 Foraging training- gravel (4)	Trials were presented as stage 2D. When the chicken's head was over the baited tray, the un-baited tray was removed.
6 Foraging training- wood chips (1)	Chickens were trained to search for sweetcorn in wood chips. (A) For trials 1-2, the trays contained no wood chips, and one was baited with x1 sweetcorn. (B) For trials 3-11, the baited tray contained ~2cm of wood chips, and the sweetcorn was partially buried. (C) For trials 12-20 the sweetcorn was fully buried. Otherwise, sessions proceeded as for stage one.
7 Shuttle training (2)	Chickens were trained to return to the start box prior to re-baiting. Sessions proceeded as for stage one, except the start box was first rebaited (x1 sweetcorn) only when chickens were half-way into the start box. The door was then closed, and the start box was re-baited again (x2 sweetcorn). The guillotine door was closed after the chicken left the start box and reopened after the reward tray sweetcorn was consumed.
8 Shuttle training (3)	(A) Trials 1-10 were presented as stage 7. (B) For trials 11-20 the start box was only rebaited (x3 sweetcorn) after the chicken fully entered the start box and the door was closed.

9 Shuttle training (4)	Trials were presented as in 8B, except the start box bait was reduced (x2 sweetcorn). The guillotine door remained open while the chicken was in the main arena area. Once chickens put their heads over the baited tray, the un-baited tray was removed.
10 Foraging training- wood chips pile (1)	The chickens were trained to search for sweetcorn in a wood chip pile (~173cm^3). One tray contained a central pile of wood chips, baited with x1 sweetcorn. (A) For trials 1-10, the sweetcorn was on top of the pile. (B) For trials 11-20, the sweetcorn was partially buried. The sessions otherwise proceeded as for the previous stage. The wood chip pile location within the tray was pseudorandomised alongside the sweetcorn reward location.
11 Foraging training- wood chips pile (2)	Trials 1-2 were as in stage 10B. (C) For trials 3-20, the sweetcorn was fully buried. When chickens stopped/failed to search they were first verbally encouraged. If this did not help, they were encouraged to investigate the shavings by the handler visibly appearing next to that arena side. The start box door was also closed to prevent re-entry without finding the reward tray sweetcorn.
12 Foraging training- wood chips pile (3)	Trials were presented as in stage 11C. The tray approached first after start box exit was recorded. Approximate time to complete 20 trials was recorded.

Table 3. 1 Shuttle and forage training development stages.

Training stage	Number of sessions Chicken ID					
	Blue	Brown	Green	Grey	Red	Yellow
1	1	1	1	3*	1	2
2	1	1	1	n/a	1	1
3	1	1	1	n/a	1	n/a
4	n/a	2	1	n/a	1	n/a
5	n/a	n/a	1	n/a	1	n/a
6	1	1	1	1**	1	1
7	1	2	2	1***	1	1
8	1	1	1	1	1	1
9	1	1	1	1	1	1
10	1	1	1	1	1	1
11	1	1	1	1	1	1
12	1	1	1	1	1	1

Table 3. 2 Number of sessions by training stage and chicken ID.

Training routes already deemed unsuccessful following attempts in birds requiring fewer habituation sessions were not attempted in subsequent birds. These are marked “n/a”. *For sessions 1-2, 10 trials were completed. **Fully buried sweetcorn was not attempted, as it was this chicken’s first exposure to substrate in the trays in the testing apparatus. 16 trials were completed before responding ceased. ***The experimenter ran this session standing and was visible to the chicken. This seemed to calm them, no problems were experienced afterwards.

3.2.4 Discrimination Learning- general protocol

Chickens were tested to determine whether they could learn visual cues predicting the tray baited with a sweetcorn reward. Studies were conducted to test:

1) recognition of the wood chip pile as the reward cue

2) a reward cue image, with no image on the other tray

3) discrimination between different image cues on each tray, where one predicts reward

Chickens ran one session of 20 different trials each weekday. The spatial location of the rewarded tray was pseudorandomised for each new trial. The wood chip pile and reward location within the tray remained in the centre throughout. Incorrect trials were re-presented and did not count towards the 20-trial total. Accuracy was recorded, including for the re-presented trials. A learning criterion of at least 70% accuracy for two consecutive sessions was applied.

Chickens were placed in the closed, baited start box (x3 sweetcorn). One tray with the reward cue was baited with x1 sweetcorn buried in a central wood chip pile (~173cm^3). The other tray also contained a central wood chip pile (except for study one, which examined wood chips vs no wood chips). Where two image cues were used, the rewarded image was counterbalanced across subjects. The start box door was opened.

For trials 1-3, chickens could explore both trays to find the sweetcorn. The initial approach was recorded. An approach was defined as the chicken putting its head over the tray. If chickens approached the incorrect tray multiple times this was recorded as multiple errors. If chickens returned to the start box, the door remained open until the sweetcorn was found*.

For trials 4-20, the tray not initially approached was withdrawn. Chickens searched for sweetcorn in the wood chip, then returned to the start box where the door was closed. If the correct tray was chosen, x1 sweetcorn was delivered to the start box via the reward delivery tube. If the wrong tray was chosen the trial was repeated, otherwise the next trial was presented. The wood chip pile was reconstructed following each trial and when incorrect trials were re-presented. Wood chips were used for ~12 sessions then replaced. If five consecutive incorrect trials occurred, the wood chips were not reconstructed for subsequent presentations of that trial. If 10 consecutive incorrect trials occurred (rare), the baited tray was not withdrawn.

If chickens stopped responding and remained stationary for >60s, they were prompted to move by opening/closing the guillotine door. The operator did not interact with the chickens during trials and no time limit was imposed.

If a chicken chose the correct tray but did not find/consume the sweetcorn before returning to the start box, the door remained open. If the guillotine door was erroneously closed, the chicken was released to find the sweetcorn without resetting the trays (rare).

*If a chicken cycled between the same tray and the start box 10 times the guillotine door was closed. If incorrect approaches reached 20 times, the chicken was encouraged to the correct tray by the experimenter sitting by the tray and calling (this happened once).

Basic Discrimination- wood chip vs no wood chip

This test was a continuation of training, confirming the chickens had learnt the association between the wood chip pile and reward. One tray contained a wood chip pile with x1 buried sweetcorn. The other tray was empty. Two sessions were undertaken, on Friday and the following Monday. Accuracy was recorded. All chickens met the learning criterion in two sessions.

Basic Discrimination- image cue vs no image cue

This tested whether chickens could use an image cue to predict the rewarded wood chip pile. A laminated black and white image (~25cm x 4cm) (**Figure 3.3**) was attached to the front of the baited tray. Both trays were used as the baited tray during the sessions, to prevent chickens using other tray features (e.g., wood patterns) as the reward cue. Accuracy was recorded and compared against the learning criterion. At least five sessions were completed, with extra for chickens that needed more to meet the learning criterion in the final two sessions. This was necessary for progression. For session one, three chickens ran a session that was time-restricted to 40 minutes, due to time constraints.

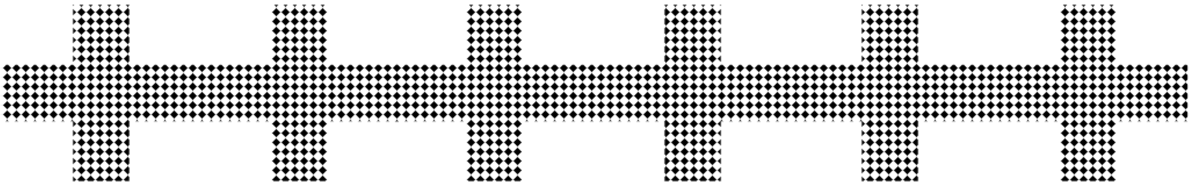


Fig 3. 3 Black and white visual cue placed on front of tray.

Image Discrimination- black and white patterns

Two different laminated black and white images were attached to the front of the reward trays, with one indicating reward. Three chickens underwent one session with the images **Figure 3.4a**. Due to suspected image bias, images **Figure 3.4b** were used subsequently. 7-8 sessions were conducted, and accuracy recorded. No chickens met the learning criterion, so the images were changed.

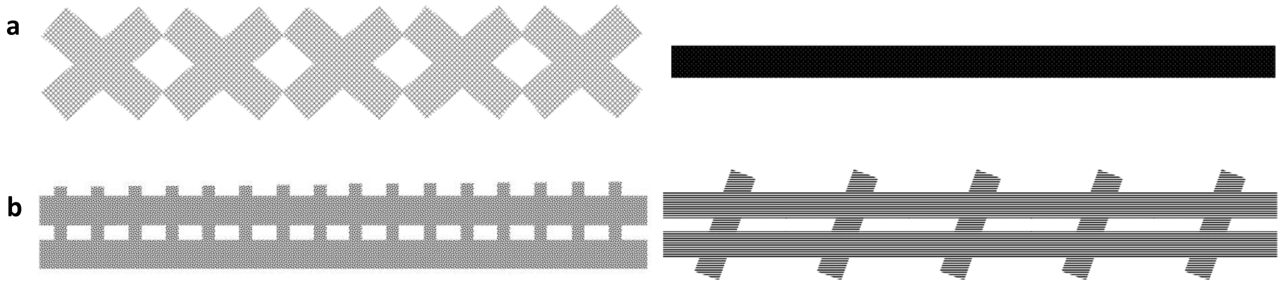


Fig 3. 4 Black and white visual cues placed on front of trays.

a The initial image pair trialled with three chickens for one session. There appeared to be a bias toward crosses, so these cues were discontinued. **b** The second black and white image pair trialled.

Image Discrimination- blue and yellow circles

Laminated images with different blue and yellow circle patterns were used as reward cues (**Figure 3.5)**. For sessions 1-5 the images were on the front of the trays.

For sessions 6-10 the images were placed on the back of the tray inside, enabling chickens to see them when searching the wood chips. None of the chickens met the learning criterion with either image placement, so the images were changed.

During session one, two chickens refused to leave the start box for over 10 minutes during trial one. Vocal encouragement facilitated their exit. These trials were discounted from accuracy recording due to interference.

Fig 3. 5 Blue and yellow visual cues placed on front or back of trays

Image Discrimination- colour and pattern in tray base

Laminated A4 sheets with different colours and patterns (**Figure 3.6**) were placed in the bottom of the trays as reward cues. The wood chip piles were placed on top, slightly towards the front of the tray, to maximise image visibility for chickens exiting the start box. Five sessions were conducted, and all chickens met the learning criterion.

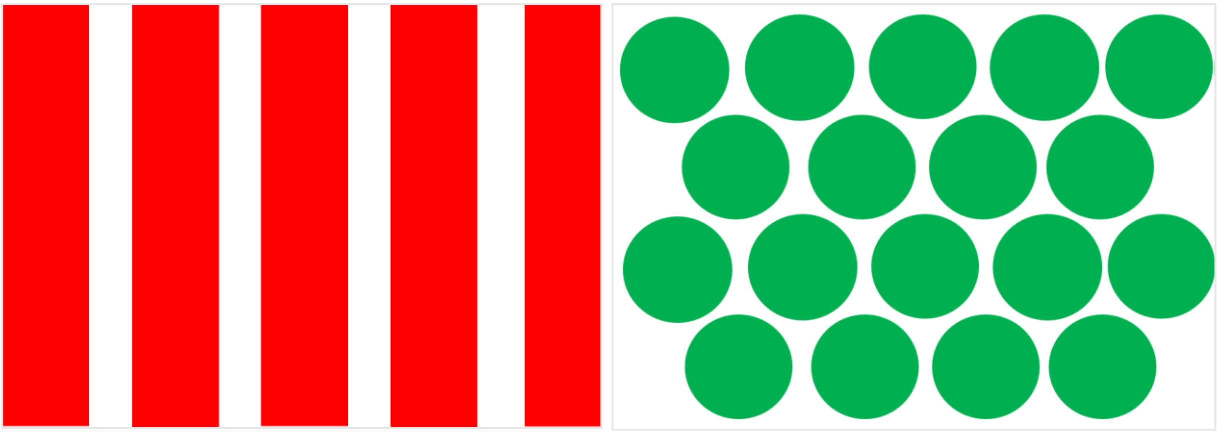


Fig 3. 6 Visual cues placed in bottom of trays.

3.2.5 Discrimination between reward levels

The successful discrimination learning protocol was adapted to assess whether chickens could discriminate between cues predicting different reward levels.

Wood chip piles were presented as previously described. However, each was baited with a higher (x3 sweetcorn) or lower (x1 sweetcorn) reward. Laminated A4 images with different colours and patterns (**Figure 3.7**) acted as reward level cues. “Incorrect” trials were not re-presented, as both outcomes were rewarded. The start box was baited after each trial (x1 sweetcorn). Accuracy for selecting the higher reward was recorded.

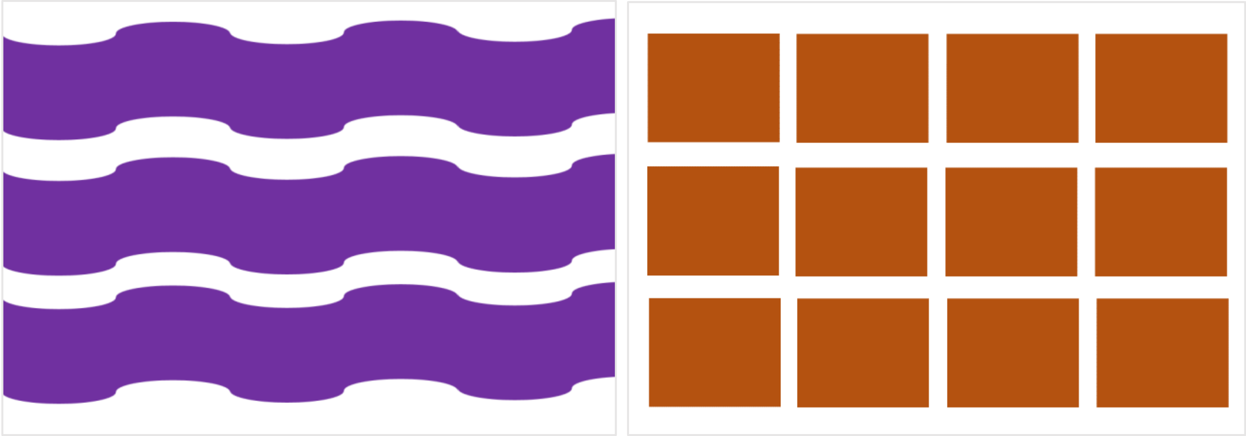


Fig 3. 7 Visual cues placed in bottom of trays for discrimination between reward levels

3.2.6 Data analysis

Charts were constructed using GraphPad Prism 8.0.1. Data were presented as line charts with individual accuracy per session. Charts were inspected to ascertain whether chickens achieved the learning criterion. For the first study (wood chip vs no wood chip) a line chart showing mean accuracy with individual data points overlaid was used for clarity. Data were also inspected for unexpected outlying accuracy scores. Polarised scores were noted for black and white patterns (1) and individual scores by rewarded image were plotted.

Statistical analysis was undertaken, assessing whether accuracy in the final session had significantly changed relative to session one. Each chicken's final session accuracy was subtracted from the first session, and a one-sample t-test against a hypothetical mean of 0.0 was conducted. For image vs no image, session 5 and session 1 were compared. For blue and yellow circles, the accuracy change was analysed separately for the different cue positions. All statistically analysed data were subjected to a Shapiro-Wilk normality test. Significance was defined as $p < 0.05$ and exact p values were reported.

For studies with unsuccessful learning, data were visually inspected for side-bias by plotting the mean proportion of left-side choices (chicken's perspective) for each study. The range of choices made in each study was visually compared. Data was presented graphically showing the mean and standard error of the mean (SEM).

3.3 Results

3.3.1 Chickens learnt to associate a wood chip pile with a sweetcorn reward.

Chickens were tested to confirm they had learnt to associate a wood chip pile with a hidden sweetcorn reward. All chickens accurately approached the tray with the wood chip pile over the empty tray (mean 98% over both sessions) and were deemed to have learnt the association (**Figure 3.8**).

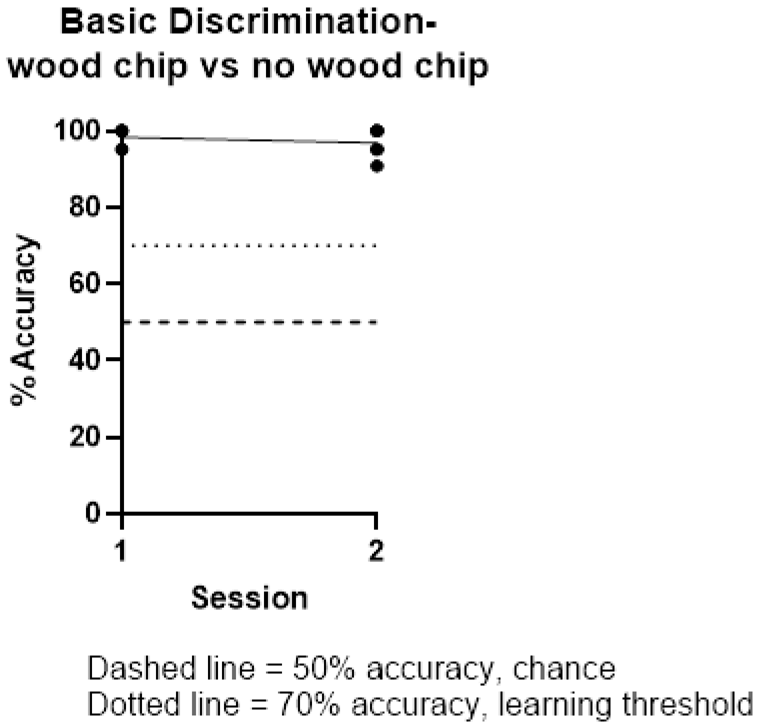


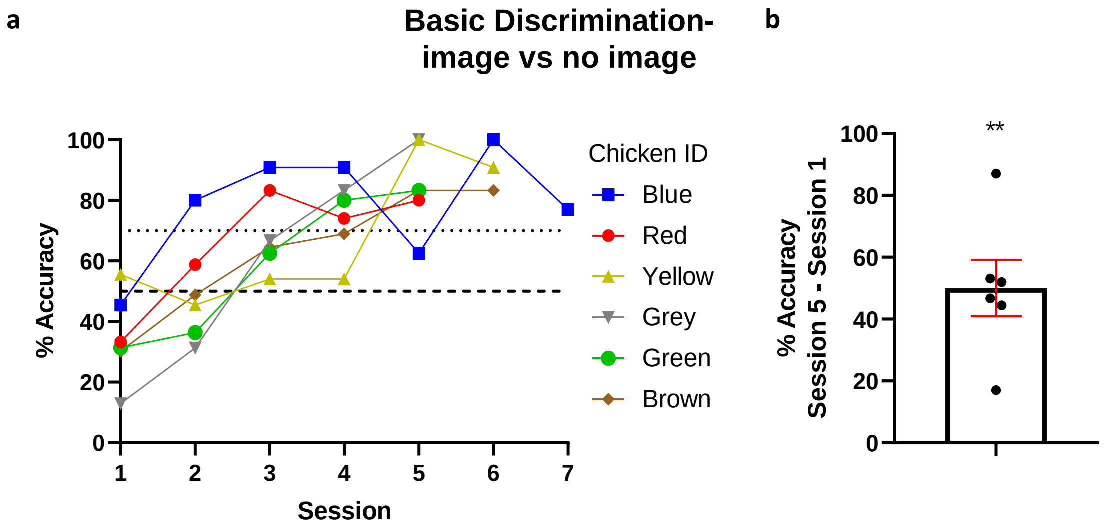
Fig 3. 8 Chickens learnt to associate a wood chip pile with a sweetcorn reward.

After training, chickens accurately approached the tray containing the wood chip pile with a hidden sweetcorn reward over the un-baited empty tray. Chart shows mean with individual data points overlaid. n = 6

3.3.2 Chickens learnt to associate an image cue with a sweetcorn reward.

All chickens met the learning criterion and could associate an image cue (**Figure 3.3**) on the reward tray front with a sweetcorn reward (**Figure 3.9a**). The mean number of sessions to meet the minimum criterion was 5 (range 3-6), with an average 167 trials (range 91-196).

Chickens completed five sessions minimum. They were significantly more accurate in session 5 than session 1 ($t_{(5)} = 5.468$, $p = 0.003$) (**Figure 3.9b**). One chicken recorded lower accuracy in session 5 despite previously meeting the learning criterion, and so completed two further sessions to confirm learning.



Dashed line = 50% accuracy, chance
Dotted line = 70% accuracy, learning threshold

Fig 3. 9 Chickens learnt to associate an image cue with a sweetcorn reward.

a All chickens learnt to associate the image cue with the sweetcorn reward and **b** and were significantly more accurate in session 5 relative to session 1 (p < 0.01, one sample t-test). Line chart shows individual data points over progressive sessions. Bar chart shows mean ± SEM with data points overlaid. n = 6, excepting session **6** where n = 3 and session 7 where n = 1. ** p < 0.01.

3.3.3 Chickens did not learn to discriminate between similar rewarded and unrewarded black and white image cues.

Three chickens initially trialled one session with two distinct black and white image cues (**Figure 3.4a**). One cue associated with a sweetcorn reward. Regardless of reward contingency, the chickens showed an apparent bias toward the “crosses” cue more like the one used previously. “Crosses” was selected in 72% of trials on average (range 68-77%) (**Figure 3.10a**). The image cues were subsequently changed to be equally like the previously used image (**Figure 3.4b**).

Following 7/8 sessions with the new images (mean 233 trials, range 215-278), no chickens had met the learning criterion (**Figure 3.10bi**). Nonetheless, final session accuracy improved slightly over the first session ($t_{(5)} = 3.599$, $p = 0.016$) (**Fig 3.10bii**)

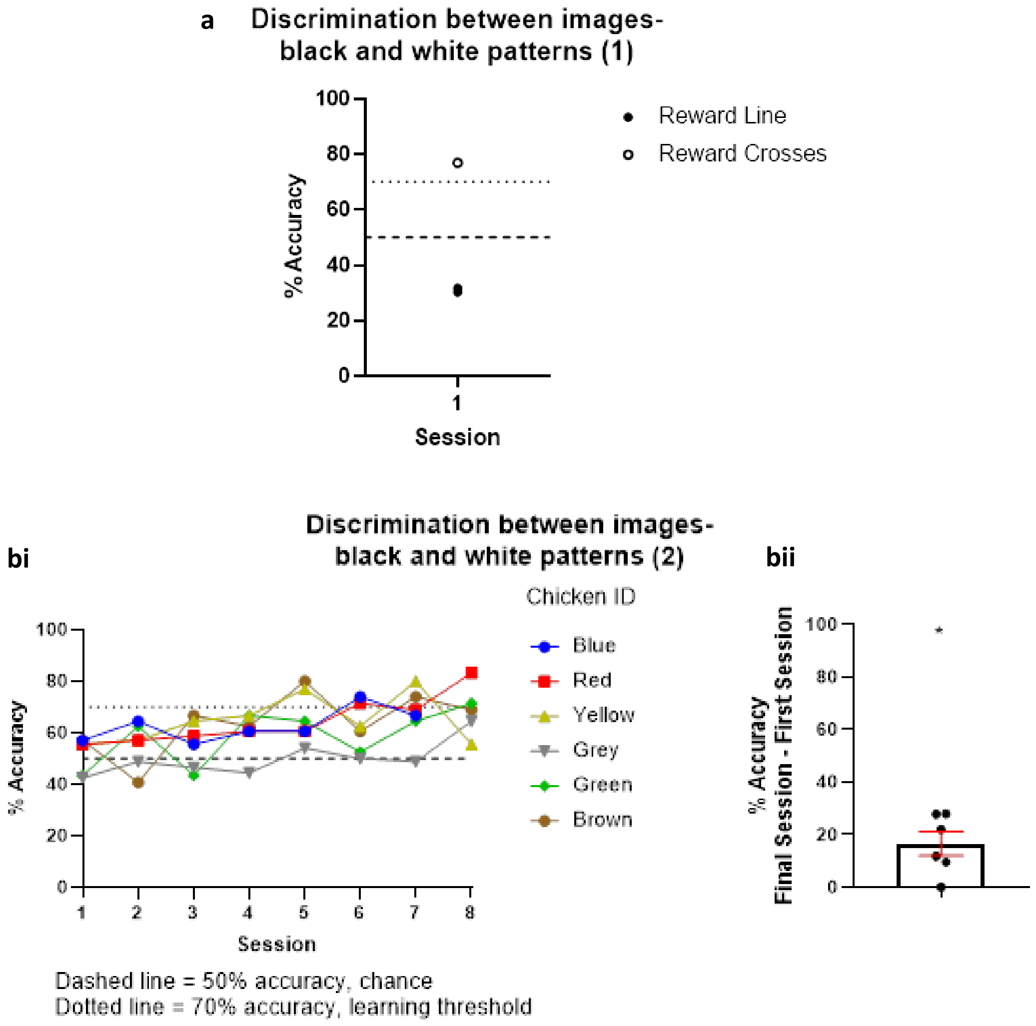


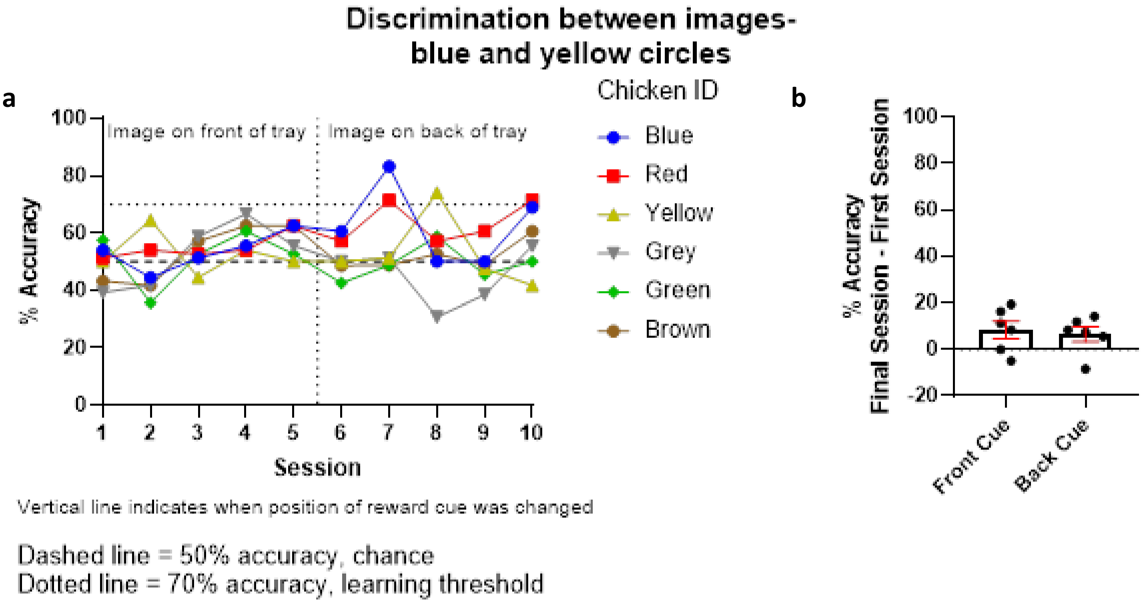
Fig 3. 10 Chickens did not learn to discriminate between similar rewarded and unrewarded black and white image cues

a Chickens seemingly showed a response bias towards the “Crosses” image more like the previous cue, regardless of reward contingency. **bi** When a pair of more similar images were used, no chickens learnt the image cue - reward association. **bii** However, final session accuracy was slightly higher than the first session ($p < 0.05$, one sample t-test). Charts show individual data points. Bar chart shows mean ± SEM with data points overlaid **a** n = 3, **b** n = 6, except for **bi** session 8 where n = 5. * p < 0.05.

3.3.4 Chickens did not learn to discriminate between rewarded and unrewarded colour pattern image cues. Cue position did not affect learning.

Chickens undertook 10 sessions (mean 384 trials, range 342-431) using blue and yellow circle patterns as image cues (**Figure 3.5**). One cue associated with a sweetcorn reward. No chickens met the learning criterion (**Figure 3.11a**).

For sessions 1-5 the reward cue was placed on the reward tray front (as previously). For sessions 6-10, the image was placed on the inside back of the tray, so it was visible while chickens consumed the reward. Accuracy did not improve in the final session relative to the first session for the front ($t_{(5)}$ = 2.195, p = 0.080) or back ($t_{(5)}$ = 2.018, p = 0.100) position. Cue position did not affect performance (**Figure 3.11b**).



Vertical line indicates when position of reward cue was changed

Dashed line = 50% accuracy, chance
Dotted line = 70% accuracy, learning threshold

Fig 3. 11 Chickens did not learn to discriminate between rewarded and unrewarded colour pattern image cues. Cue position did not affect learning.

a No chickens learnt which image cue associated with the reward. **b** Final session accuracy did not improve compared to the first session for either cue position (p > 0.05, two, one-sample t-tests). Line chart shows individual data points over progressive sessions. Bar chart shows mean ± SEM with data points overlaid. n = 6.

3.3.5 Chickens learnt to discriminate between rewarded and unrewarded image cues with differing colours and patterns in the base of the reward trays.

All chickens learnt to discriminate between images with different colours and patterns placed in the base of the trays (**Figure 3.6**) and select the rewarded cue (**Figure 3.12a**). Mean sessions required to meet the learning criterion was three (range 2-4), with an average 98 trials (range 54-120).

Chickens completed five sessions. Accuracy significantly increased in session 5 relative to session 1 ($t_{(5)}$ = 6.621, p = 0.001) (**Figure 3.12b**).

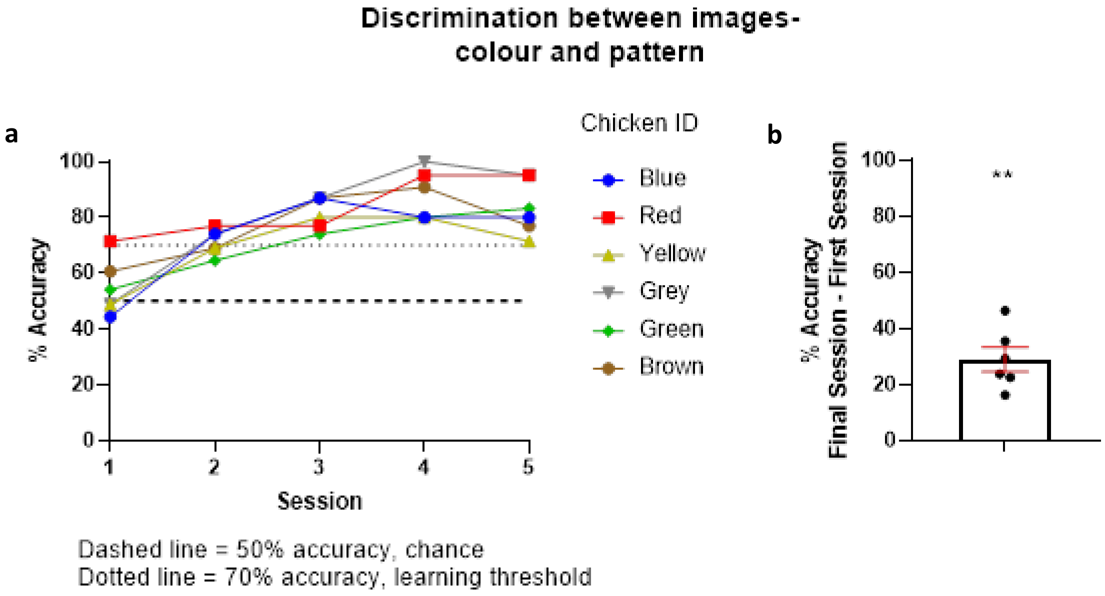


Fig 3. 12 Chickens learnt to discriminate between rewarded and unrewarded image cues with differing colours and patterns placed in the base of the reward trays.

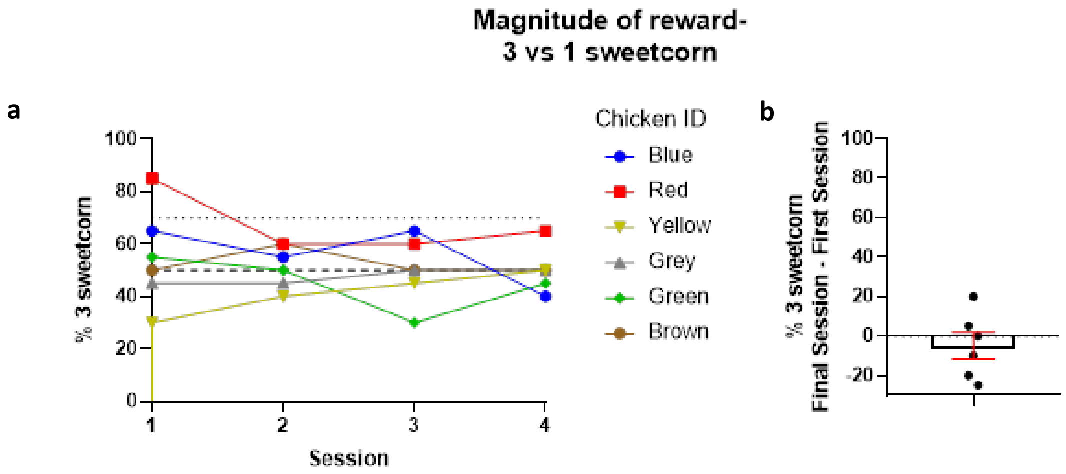
a All chickens learnt to associate an image cue with the sweetcorn reward. **b** Session 5 accuracy was significantly higher than session 1 ($p < 0.01$, one sample t-test). Line chart shows individual data points over progressive sessions. Bar chart shows mean ± SEM with data points overlaid. n = 6. ** $p < 0.01$.

3.3.6 Chickens did not develop a preference for cues predicting a higher reward magnitude.

Chickens did not develop a preference for a higher reward (x3 sweetcorn) cue versus a lower reward (x1 sweetcorn) cue (**Figure 3.13a**). There was no bias towards the higher reward cue in session 4 alone ($t_{(5)} = 0.000$, $p = 1.000$). Higher reward choices did not increase in the final session compared to the first session ($t_{(5)} = 0.732$, $p = 0.497$) (**Figure 3.13b**).

Chickens completed four sessions with 20 trials per session, giving 80 trials overall. This was fewer than previous tasks due to time constraints and perhaps insufficient for learning.

Unlike in previous tasks, some chickens demonstrated a strong side bias (**Figure 3.13c**). Incorrect trials were re-presented previously to discourage side-bias, but not in this protocol as both options were rewarded to some degree.



Dashed line = 50% accuracy, chance
Dotted line = 70% accuracy, learning threshold

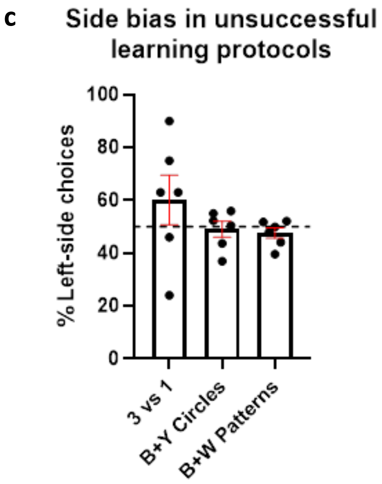


Fig 3. 13 Chickens did not develop a preference for cues predicting a higher reward magnitude.

a Chickens did not develop a preference for a cue predicting a higher reward and choices in session 4 were in line with chance ($p > 0.05$, one-sample t-test). **b** Preference for the higher reward cue was not higher in the final session than the first session ($p > 0.05$, one sample t-test). **c** Some chickens developed a side-bias, which was not seen in the other unsuccessful protocols. Line chart shows individual data points over progressive sessions. Bar charts shows mean ± SEM with data points overlaid. n = 6.

3.4 Discussion

Chickens could discriminate between rewarded and unrewarded visual cues in the novel testing arena. This suggests the testing arena and associated protocol was suitable for studying chicken reward learning behaviour. However, chickens did not develop a preference for cues predicting a higher reward magnitude when different reward levels were presented simultaneously. This could suggest chickens cannot discriminate between reward magnitudes. Alternatively, perhaps the cognitive effort to learn the higher reward was not worthwhile, considering either choice was rewarded to some degree.

This discussion considers how reward learning behaviour may be further studied using the testing arena and how observations may help understand chicken reward learning.

3.4.1 Chickens habituated to the test arena and were taught to shuttle and forage for rewards independently

All chickens were successfully pre-trained to shuttle between the start box and main arena, and search wood chip piles for buried sweetcorn. Performing this behaviour independently is important to remove potential human interference during testing. Accessibility to all chickens is also important, to avoid excluding populations from future assessment. Experimentation was initially required to determine the best way to establish the behaviour. Key findings during this phase are highlighted below:

Some chickens needed more sessions to become comfortable in the arena

Chickens responded variably to habituation and initial training. Introducing the guillotine door was particularly divisive, with large variation in willingness to explore and consume the sweetcorn (Appendix 4). Walking under the door seemed aversive for 4/6 chickens. Being individually trained in the arena was also perhaps anxiogenic, considering chickens are social. Even short isolation periods increase anxiety-like behaviour in chicks (Salmeto et al., 2011) though aged chickens are less well-studied.

One chicken was particularly difficult to habituate and train initially. Following five habituation sessions with the door, it progressed to adapted shuttling and foraging training, with only 10 trials. The chicken generally stopped exploring and would stand and call after ~five minutes. These calls were perhaps gakel-calls, associated with frustration (Zimmerman et al., 2000). The sessions were perhaps too long, with frustration increasing at the inability to access home pen resources. These difficulties were overcome following one session which the experimenter conducted standing, and so was visible to the chicken. While perhaps coincidental, inability to see the handler was potentially initially stressful for the chicken while also isolated from conspecifics.

Generally, talking or moving out from behind the screen was attractive to the chickens. These behaviours could encourage exploration when chickens were fearful or not responding. This highlights the importance of avoiding human contact during testing because results may be influenced. Arena design needs to facilitate this. However, handler interactions may be useful during habituation and pre-training, and could facilitate learning. More appropriate human interaction in early training may alleviate the nervousness of some chickens in fewer sessions. This depends on future chicken cohorts also showing positive affinity for human interaction, which remains to be seen.

Chickens did not show spontaneous foraging behaviour when food was initially hidden in the test arena

Chickens readily found visible food in the arena, even if partially buried. However, when completely buried, chickens did not spontaneously search for food under the substrate without initial human encouragement. Following previous exposure to visible food in the trays, chickens had been expected to spontaneously scratch at the substrate to find hidden food. This did not happen. Chickens pecked specifically at visible food, and initially did not attempt to disturb the substrate if no food was visible. They seemingly did not connect places that previously contained visible food with searching that area for potential hidden food. The arena was designed to exploit natural chicken foraging behaviours to facilitate reward learning, so the absence of spontaneous foraging was unexpected.

Re-initiating foraging training, so that chickens were now only rewarded *after* re-entering the start box was hoped to have a generalised effect on subsequent learning and arena behaviour (Castro & Wasserman, 2010). Reducing the amount of wood chips to a pile, increasing the searchable area's reward density and the effort required to find the single sweetcorn reward, was also implemented. This modification developed from observing that the chickens most successful at finding concealed corn in a whole tray, seemed to specifically peck at lumps of substrate.

Initially, chickens still did not disturb the wood chip pile to look for hidden sweetcorn. For the first session's first trial, the experimenter encouraged the chickens to stand on the wood chip pile, which disturbed it, revealing the sweetcorn. Contrastingly, when using a full tray of substrate, if no spontaneous searching occurred the experimenter had shown the chickens the corn's location using a stick. With the wood chip pile, after just one intervention, the chickens then spontaneously searched for hidden food independently in subsequent trials and sessions. The way the chickens revealed the corn themselves, rather than being shown, was perhaps significant.

Appendix 5 contains a recommended training protocol for future studies, based on these observations and results.

3.4.2 Chickens discriminated between rewarded vs unrewarded cues

Chickens readily learnt to associate the wood chip pile, and subsequently an image on the tray front, with the sweetcorn reward. Accuracy for wood chip vs no wood chip was consistently high across the two sessions conducted. This would be expected if chickens already learnt to associate the wood chip pile with reward in pre-training. When first using an image cue, accuracy typically started below 50%, suggesting the novel cue was initially aversive. Accuracy significantly increased as sessions progressed. Low accuracy when chickens were first presented with two wood chip piles is reassuring, as this confirms they couldn’t detect the sweetcorn by other means (e.g., scent, seeing small parts of the sweetcorn, inadvertent operator behaviour).

Chickens took an average five sessions to learn the image cue – reward association. This is comparable to sessions required by mice to learn the initial textured floor association (see Chapter 2, also Jen Davies unpublished data, Appendix 1). However, for the chickens, repeated incorrect trials did not count towards the 20-trial session total. So, chickens undertook more trials than the mice over the sessions. The chickens took nearly 170 trials on average to acquire the basic image association. Chickens often persevered on an incorrect choice for multiple trials in early sessions. High levels of spatial perseverance have previously been reported for chickens in discrimination learning tasks (Hernandez et al., 2015). This is why we re-presented incorrect trials and did not count these towards the 20-trial total, to maximise chicken learning.

Comparing with other laying hen discriminative learning procedures is difficult due to variable learning criteria. Tasks are also often designed with a visible human operator, potentially confounding reported associative learning behaviour. Nonetheless, this task seemingly requires fewer trials to develop a basic image association than an alternative (~300 trials, (Deakin et al., 2016)). Some studies also report similar trial numbers to ours (spatial recognition; ~150 trials (Wichman et al., 2012), spatial/visual; ~130 trials (lower reward) (Hernandez et al., 2015), visual; ~150 trials (lower reward) (de Haas, Lee, & Rodenburg, 2017)). Faster visual discrimination learning has also been reported (~60 trials (Kuhne et al., 2013), ~70 trials (lower reward) (de Haas, Lee, Hernandez, et al., 2017)). Significantly, in our task all chickens met the learning criterion, whereas in most other studies some were excluded for lack of learning. This would be problematic when studying reward learning, as a potentially significant population could be excluded. Numbers in this pilot study were small, but it is reassuring that all the chickens could learn the task.

Chickens need to discriminate between two visual cues presented simultaneously to examine reward learning in the testing arena. Chickens show colour preferences based on context and previous experiences (Ham & Osorio, 2007; Miklósi et al., 2002). So, discrimination was first attempted using different black and white pattern cues (**Figure 3.4a**) to avoid potential image bias.

Chickens were seemingly biased towards the image most like the basic discrimination cue (**Figure 3.3**), likely generalising the previously rewarded image. It was encouraging that the chickens apparently looked at and used the image for decision-making. Ultimately though, multiple independent reward cues will need to be presented to study high vs low reward learning. The cues used need to not significantly influence learning of other reward cue pairs.

The cues were changed to two black and white patterns equally like the original image cue (**Figure 3.4b**). No chickens met the learning criterion, though accuracy did improve. Using similar images probably made the task too difficult, disengaging the chickens. Chicken visual acuity is poorer than humans (Jarvis et al., 2009), so the images were perhaps too ambiguous to differentiate.

Blue and yellow colour pattern cues were tried (**Figure 3.5**). Chickens did not learn this discrimination with the images placed on either the front or inside back of the trays. Placing the image inside the reward tray might have aided learning, as the image would be viewed during reward consumption. This removes a working memory element that may be too difficult in a compound discrimination. Delayed reward presentation reduces chicken accuracy in matching to sample tasks studying working memory (Nakagawa et al., 2004). Nonetheless, changing the image location did not affect learning. Chickens have good colour vision and can discriminate between blue and yellow (Polt, 1969) but the pattern element was clearly not accessible. Even with their poorer visual acuity, differences between the cues ought to fall within the chicken's perceptual range. The manner the patterns were presented was perhaps too far removed from natural chicken behaviour. So, the chickens did not notice the relevant cue features.

Chickens rapidly learnt an association using different coloured (and patterned) cues in the base of the trays. Fewer trials than the original basic discrimination were needed (average 98 trials to criterion). This compares favourably with the learning rate of other chicken discrimination tasks highlighted previously. Placing cues in the base of the trays maximised visibility during reward consumption. The colour difference between cues likely also facilitated learning. Colour cues had been avoided due to potential innate or preconceived chicken colour preferences (Ham & Osorio, 2007; Huber-Eicher, 2004). However, expression of colour preferences indicates chickens notice colour signals, and other studies have successfully used colour cues (de Haas, Lee, Hernandez, et al., 2017; de Haas, Lee, & Rodenburg, 2017; Kuhne et al., 2013). It is encouraging that the chickens acquired the compound discrimination rapidly, particularly following many trials with unsuccessful learning.

One chicken did appear to show an initial image bias towards red, which was also their rewarded cue. Chicks are known to prefer pecking at red food items (Ham & Osorio, 2007) and the feed and drink hoppers in the home pen were also red. This seemingly did not overly affect chicken choices in this pilot. However, potential colour preferences should be considered in future work. Balanced study design, with enough chickens rewarded for each image, will be important. Innate biases might affect apparent cue learning rates, potentially problematic when undertaking high vs low reward learning. Chickens interpolate between colours; if red and yellow associate with food, they extrapolate this to orange (C. D. Jones et al., 2001). So, if colour-based cues are used to study chicken high vs low reward learning, we must ensure reward cue pairs remain independent to each other. A recent study presenting multiple colour-cue pairs to chickens found a purple bias in latter stages (Degrande et al., 2022). This group also speculated that prior learning may influence behaviour towards colours subsequently presented.

Despite cue design challenges, chickens could readily discriminate between rewarded and unrewarded cues in our semi-automated testing arena. This setup provides opportunities for testing reward learning.

3.4.3 Chickens did not develop a preference for cues predicting a higher reward magnitude when cues were presented simultaneously

Chickens did not develop a preference for a cue predicting a higher reward (x3 sweetcorn) versus a lower reward (x1 sweetcorn). Cues were presented simultaneously. A bias towards the larger reward was expected to develop. The larger reward should be recognised as more rewarding, enhancing learning and associated cue recall (Clayton, 1964; Madan et al., 2012; Reynolds, 1950). In an equivalent task, rats show the expected bias (Clayton, 1964), which underpins the high vs low reward learning task. The lack of bias might suggest a deficit in chicken reward processing compared to mammals. The neurobiology of reward related behaviour may differ in birds. Chickens and mammals differ in other relevant ways, e.g., they may not display successive negative contrast behaviour (Davies et al., 2015).

The experiment design may also explain the lack of reward learning bias shown. Only four sessions (80 trials) were conducted due to time constraints. This was perhaps insufficient for learning. More trials were needed to learn the previous, likely easier, discrimination tasks.

Also, half the chickens showed a consistent spatial choice bias in this task (two left-side bias, one right-side bias). This was not seen in the previous successful experimental protocols. Incorrect trials were previously re-presented until the correct choice was made. In this experiment both options were rewarded, so incorrect trials were not re-presented because this was not expected to encourage switching behaviour. Considering either choice led to reward, the cognitive effort to deduce which image indicated a greater reward was perhaps not worthwhile. The chicken tendency to develop side-preferences without study design to discourage this is well-documented (de Haas, Lee, & Rodenburg, 2017). Therefore, presenting two reward levels simultaneously may be an inappropriate method for exploring chickens' abilities to discriminate between reward magnitudes. This may be particularly relevant in this study, considering the chickens were very exposed to training where one sweetcorn was the only reward.

Nonetheless, chicken perseverative behaviour/side-bias during two-choice discrimination tasks has been linked to personality, particularly fearfulness/anxiety (de Haas, Lee, Hernandez, et al., 2017; de Haas, Lee, & Rodenburg, 2017). This might suggest that, in at least some chickens, there was a reward learning deficit associated with a negative affective state. Good housing conditions were strived for, but perhaps there was still something they found challenging. The chickens also came from a commercial farm and had potentially experienced long-term stress from which they had not recovered.

This protocol was intended to confirm chickens' ability to differentiate between high and low reward magnitudes. As no bias towards the higher reward cue developed, this ability remains unclear. However, assessing high vs low reward learning by interchangeably presenting two different reward pairs, like the mouse protocol, would still be worthwhile. In this methodology, re-presenting incorrect trials can mitigate side-bias and perseverative errors. There is already some evidence that chickens recognise different reward levels when different reward cue pairs are presented interchangeably (Hernandez et al., 2015). In the Hernandez study, chickens stopped responding to lower reward (20g food pellets) cues after ~25 trials but still responded to higher reward (eight mealworms) cues. This suggests chickens recognised the different reward levels, so a high reward learning bias should be detectable. Similarly, consummative activity (number of pecks to consume a reward) affects associated learning in young chickens (Wolfe & Kaplon, 1941).

3.4.4 Conclusion

This study demonstrates a novel, ethologically relevant, visual discrimination reward learning task in chickens. Significantly, it can be operated in a semi-automated manner, without human intervention within or between trials. In our work, chickens did not develop a preference for cues predicting a higher reward magnitude as expected based on principles described in Chapter 1. However, this may be due to study design and a limited trial number. The rodent high vs low reward learning task presents high/low reward cues interchangeably, and with this design reward learning bias may also show in chickens. The arena could be used to test this. Subsequently, if successful, the apparatus could be used in future studies ascertaining whether reward learning deficits align with negative affective states.

Some further work is also required to validate the usability of the testing arena. This pilot work only used six hens. More studies are needed to confirm that baseline discrimination learning is accessible to all chickens, and the streamlined habituation/training protocol is effective. We also need to verify problems with colour cue preference/interpolation are not seen when more cues are used in high vs low reward learning protocols. Nonetheless, our initial results are encouraging, with rapid discrimination learning achieved by the entire cohort.

Chapter 4: General Discussion

4.1 General Discussion

This thesis investigated whether reward learning deficits might make an objective cross-species negative affective state biomarker. This was with the aim of using appropriate reward learning assays for animal welfare assessment in the future.

Some evidence of reward learning deficits in group housed male mice was found, alongside reduced reward motivation following acute stress exposure. This broadly supports the link between reward deficits and negative affective states. However, reward learning difficulties were seen regardless of chronic stress exposure in the second male mouse study. This indicates further refinement to the training and experiment protocol is needed. While not fully validated, preliminary studies using the CCTM had been promising (Jen Davies, unpublished data, Appendix 1), suggesting the concept has potential. Nonetheless, to use reward learning to examine animal welfare in practice, a practical, reliable, and consistent procedure is imperative. Related to this, we also developed a semi-automated reward learning arena for chickens. Discrimination between rewarded and unrewarded visual cues was achieved, but chickens did not develop a preference for cues predicting a higher reward magnitude. However, considering other related evidence (Hernandez et al., 2015; Wolfe & Kaplon, 1941) this may reflect an unsuitable experimental method, and should be examined again with an adapted protocol.

Overall, our findings are somewhat inconclusive. Nonetheless, this discussion considers the contribution to our knowledge of the relationship between reward learning and affective state. We also consider whether our results suggest reward learning assays are developing as a practical and objective method of conducting animal welfare assessments.

4.2 Male mice: using the CCTM and reward learning deficits for welfare assessment

Interpretation of the mouse studies is limited as small sample sizes were used because the CCTM and foraging task were still under development. Pilot studies enable the feasibility of larger studies to be examined, regarding practicality, expected results, and justification of animal use. Results need to be replicated in further cohorts, building on the protocol used in these pilots. Other mouse strains should be tested going forward, as learning ability can differ by strain (Boleij et al., 2012), and could influence protocol development. Similarly, female mice have not been trialled in the CCTM so far. Reward learning deficits in the CCTM should also be validated as a biomarker of negative affect in female mice. The developed protocol could ultimately be used to address welfare questions affecting both sexes, e.g., the influence of experimental procedures on affective state.

Previous work demonstrated successful differential reward learning in mice in the CCTM, and the expected deficits when in hypothesised negative affective states (Jen Davies, unpublished data, Appendix 1). The studies in Chapter 2 were designed to support this body of evidence, however the results were variable. Further validation of the relationship between reward learning deficits in the CCTM and negative affective states is needed, perhaps using UCMS or early life stress (Wang et al., 2017). Importantly, while the concept may work in principle, optimisation of training and testing is still needed.

Some difficulties likely arose as the experimenter for this thesis was less experienced with mice and the CCTM compared to the other experimenter working in this area. The human-animal interaction is known to influence animal behaviour and welfare (Waiblinger et al., 2006). Anecdotal reports suggest individual ability to train animals in cognitive tasks varies and may be difficult to correct. The Robinson group also previously developed a mouse bowl digging affective bias task (Sørenson, 2017), but this suffered from significant inter-experimenter variability. It has similarly been difficult to successfully apply judgement bias to mice (Resasco et al., 2021). The CCTM reward learning assay was designed to be simpler than other affect-induced cognitive bias tasks, and with less direct human contact. It was hypothesised this would ensure the task was more applicable for cognitively simpler and more nervous animals like mice. However, while there has been some success, currently challenges remain. Inter-experimenter variability is undesirable in welfare assessment, limiting protocol uptake and potentially undermining objectivity.

Increasing discrimination pre-training in the CCTM may support reward learning in mice in neutral/positive affective states and reduce inter-experimenter variability. Another supportive modification could be re-presenting incorrect trials in the high vs low reward learning stage, and not counting re-presentations towards the 20-trial total (like the protocol for chicken training). This would increase reward exposure for mice with poor learning in each session and may mean fewer sessions are required. However, both these modifications risk reducing protocol practicality. More training sessions would increase the overall protocol length, while re-presenting incorrect trials would make individual sessions longer. The latter point may be particularly problematic when testing group-housed animals, potentially increasing aggression on return to the home cage. Overall, quick and simple training was hypothesised to be a strength of reward learning assays, but these modifications would reduce these advantages. Another option could be to fully automate the CCTM, which should be technically feasible given the already semi-automated design. This should correct for experimenter influence while maximising practicality.

Resolving whether male laboratory mice are in a more negative affective state when singly or group housed is a key welfare question. The CCTM reward learning assay is seemingly a viable method for investigating this. It can objectively assess the mental state of mice actually living in the housing conditions in question, something that previous studies have not done (Kappel et al., 2017). However, validation must be completed to confidently draw conclusions from this method. Also, while our pilot study found some evidence that group housing is worse for male mouse welfare, replication with more cohorts and strains is needed. Best practice housing may even vary between strains (Theil et al., 2020). If CCTM automation can be achieved, this would facilitate scaling up studies and enable wider testing in a shorter timeframe.

Increasing testing throughput would support wider investigation of other factors potentially affecting the group versus single housing question. In future cohorts, mouse husbandry/housing practices should be considered further. Transfer of nesting material during cage cleaning is recommended to reduce aggression (Barabas et al., 2021; Loo et al., 2000). This was not done in this study due to secondary behavioural observations taking place (unpublished data), but future studies should ensure best practice conditions are compared.

Non-affective state related factors that might change CCTM reward learning performance and be affected by housing condition should also be considered. Food motivation is one such area. Our group and singly-housed mice received equal food rations, but singly-housed mice weighed less, likely due to increased thermoregulatory energy use. Singly-housed mice perhaps achieved better results due to greater food motivation, rather than a more positive affective state (Lattal & Williams, 1997; Makowiecki et al., 2012). By alleviating experimenter-based time restrictions, task automation may facilitate running the CCTM reward learning protocol without food restriction. Food restriction might also influence affective state, so is undesirable in welfare assessment. The extent to which personality affects reward learning should also be considered, especially due to the correlation between foraging task behaviour and CCTM performance. Mouse personality could be profiled in other standard behavioural tests (Forkosh et al., 2019) and compared to CCTM performance +/- affective state manipulation.

4.3 Chickens: reward learning assay development and potential use for welfare assessment

Chapter 3 was a pilot study developing a chicken reward learning training and testing protocol (Appendix 5) for use in future work. We were successful in training all chickens in a visual discrimination reward learning task in a semi-automated testing arena. This offers the potential to develop a high vs low reward learning assay based on this. Judgement and affective bias tasks could also be developed based on the arena design.

Nonetheless, more chickens (including different breeds) need to be subjected to the recommended prerequisite training protocol (Appendix 5) to confirm this is achievable for all individuals, and the timeframe required. A subset of individuals cannot be excluded if a derived reward learning assay is to be used for animal welfare assessment. Equally, if training takes longer than anticipated, this will reduce protocol practicality.

We also did not confirm that chickens can distinguish between different reward magnitudes, despite some supportive evidence (Hernandez et al., 2015; Wolfe & Kaplon, 1941). The reward learning assay depends on differential reward learning rates in animals in neutral/positive affective states (Clayton, 1964; Reynolds, 1950). So, an important next step is confirming this is seen in chickens when presenting the task differently in the apparatus. Presenting the different reward levels interchangeably will likely be key as this enables side-bias correction. Presenting high and low reward cues interchangeably requires two reward cue pairs. This would also follow initial discrimination training, using another cue pair. Altogether, this means six independent reward cues will be needed. This may be challenging when using colour-based reward cues, considering chickens can interpolate between colours (C. D. Jones et al., 2001). We need to check for relationships between colour cues, learning rates, and cue history before examining potential reward learning deficits and undertaking welfare assessment. If chickens demonstrate colour preferences, this may limit the usability of the protocol. We had attempted to design pattern-based cues to avoid these challenges, but our results indicate these may be too difficult to train.

Assuming the above challenges can be overcome, we also still need to validate whether chickens show the expected reward learning deficits in negative affective states. Potential affective state manipulations include chronic ACTH administration (Puvadolpirod & Thaxton, 2000) or UCMS (Zidar et al., 2018). Commercial chickens with conditions likely to induce negative affective states; e.g., keel bone fractures (Armstrong et al., 2020), or pariah /victimised birds (Freire et al., 2003) could also be obtained. This is ethically advantageous in not needing to inflict further stress beyond what the chickens would have already experienced.

Even if the reward learning assay can be validated in chickens, it will be important to examine inter-experimenter variability. The protocol was designed to minimise experimenter influence. Nonetheless, as seemed to be the case in the mouse studies, whenever human operators are involved, there is the potential for influence. Full automation was suggested as a solution for the mouse CCTM. However, we found that the human handler may be important for initially training and calming chickens. Further cohorts will confirm whether this effect is widespread. If so, it may prevent full automation of training at least. Using wood chip, which needs to be adjusted after each trial, may also make it more difficult to fully automate the chicken arena. Other avian cognitive tasks have used a protocol based on flipping petri-dish lids (Košťál et al., 2020), which might be more practical to automate. However, this is less ethologically relevant so may be harder to train.

If validated, the novel arena setup is most applicable for studying the affective state of chickens housed under experimental conditions. Studies could be designed comparing factors relevant to commercial environments, e.g., lighting. Questions such as the level of keel bone damage that impacts affective state could also be addressed. Findings from such studies could then inform commercial housing recommendations. However, using the current setup in actual commercial environments would probably be challenging. It is unclear exactly how long testing and training may take, but the results so far suggest this is likely to be weeks. While there could be some improvement on previous chicken affect-induced cognitive bias tasks (de Haas, Lee, Hernandez, et al., 2017; de Haas, Lee, & Rodenburg, 2017; Deakin et al., 2016; Hernandez et al., 2015; Ross et al., 2019) our task is probably still too time-consuming for practical on-farm use. Even with human input reduced to a minimum, someone would at least need to transport the chickens to and from the arena. In addition, a relatively large area, with access to power, is required to set up and run the arena. Not all farms would have a suitable area available. This is a limitation to the protocol design, as ideally, we want to examine the affective state of animals actually living in the environments of interest. For farm animals, a direct translation of the high vs low reward learning task developed for laboratory rodents may be somewhat impractical.

For chickens, the reward learning protocol length also probably limits testing to laying hens, rather than broiler chickens. Broilers are typically slaughtered at six weeks of age. Testing and training would therefore need to take place in young animals over much of their life. So, the chickens would change in size, and potentially cognitive capacity (Panigrahy et al., 2017), considerably over the testing period. Broilers are also likely to experience declining walking ability, which may lead to confounds as the task requires walking (Rasmussen et al., 2022). Altogether, this may mean results are difficult to interpret in these chickens. Most chickens globally are broiler chickens (FAO), so a welfare assessment protocol accessible to these birds is highly desirable.

4.4 The future for reward learning in animal welfare assessment

We have highlighted the need for methods of objectively assessing animal welfare, from animals' own perspectives. The CCTM reward learning assay developed in rodents has shown potential to help solve animal welfare questions in laboratory environments. In this thesis, we started to translate a similar assay into chickens. The novel testing apparatus developed has the potential to validate whether reward learning deficits serve as a biomarker of negative affect in this species. It could then also be used to examine specific welfare challenges under experimental conditions. However, the reward learning assays used so far still necessitate removing individual animals from the home environment and subjecting them to trials over several days/weeks. This is impractical for testing animals outside of the laboratory environment, where suitable spaces and sufficient time are less likely to be available.

Farmed species are a dominant area of interest due to the number of animals involved globally and prevalent welfare concerns. Ideally, we would like to examine their affective state in the actual commercial environment, rather than experimental attempts to capture this. Comparison between different farms could help inform best practice for welfare. Equally, it would be valuable to continually monitor livestock affective state on-farm, to alert caretakers to real-time welfare problems. This could also potentially form part of farm assurance scheme assessments (e.g., Red Tractor or RSPCA Assured in the UK). The use of animal-based measures to assess livestock welfare is considered highly relevant (Brscic et al., 2021).

Reward learning assays have potential to be used in this manner, but protocol modifications are needed. While not a reward learning task, we also investigated a mouse foraging task, examining the relationship between this reward-related behaviour and negative states. This task is interesting, and has some advantages over the reward learning assay, because the behaviour is spontaneous and so results are obtained more rapidly. If a clear relationship with affective state was established, a welfare assay developed along these lines would be more practical to apply. However, there is currently insufficient data to ascertain exactly what this task monitors. The foraging task itself might also be difficult to translate into a wide range of species, as many do not build nests.

Lower input reward learning tasks could be developed. In mice, rapid discrimination learning within a modified home cage, therefore not requiring human intervention, has been demonstrated (Remmelink et al., 2015). The change in home cage size and enrichment provision limits the application of this method for assessment of normal laboratory animal welfare because the housing environment is significantly altered. However, learning in the home environment may be applicable in farmed species. In this case, the addition of reward learning devices may be achievable without a need for increased area, if compact devices could be developed. A potentially useful design could build around automated feed dispensers that identify interaction with individual animals using radio frequency identification (RFID) tags. There is already interest in such devices to monitor farm animal feed intake (Ketprom et al., 2007; Peng et al., 2022; Trevarthen & Michael, 2008). Similar devices could be developed that display reward cues on screens and dispense food following an instrumental response (e.g., pecking, head insertion) while the cue is displayed. Multiple dispensers displaying different rewarded and unrewarded cues could be positioned within the animals' home environment. Higher and lower reward cues could be interchangeably displayed on different devices. Each animal's relative interactions with the high reward, low reward, and unrewarded cues would then be monitored, indicating the learning rate for different reward levels. We would expect to see a bias towards high reward cue interactions in neutral animals. However, the reward learning bias should not be seen when in a negative affective state.

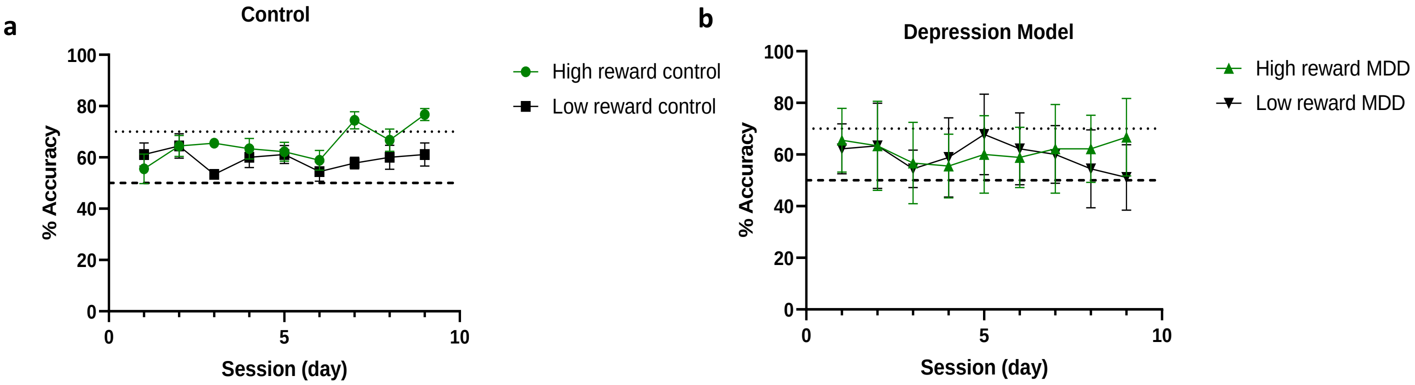
When designing a study, care would be needed to develop ethologically relevant cues and tasks to facilitate learning. Sufficient devices are also needed to mitigate resource guarding by dominant animals, to ensure testing of a representative sample. An intermittent reward schedule may also help prevent this, as well as reducing the risk of task satiety. A likely downside could be that the devices would probably act as enrichment and improve affective state relative to untested environments (Fernandez, 2022). This would need to be considered in data interpretation. Nonetheless, a welfare assessment like this would potentially be extremely useful. It could overcome challenges associated with experimenter variability and impractical training length, enabling us to objectively assess animal welfare in largely normal living environments.

4.4 Conclusion

Reward learning deficits may prove to be a suitable cross-species biomarker of negative affective states, that can practically assess welfare from the animal's perspective. Nonetheless, further validation is currently needed, and the results in this thesis indicate that optimisation of training and testing protocols is still required. The development of home environment reward learning assays may facilitate use in animal welfare assessment in environments outside of laboratory settings.

Appendices

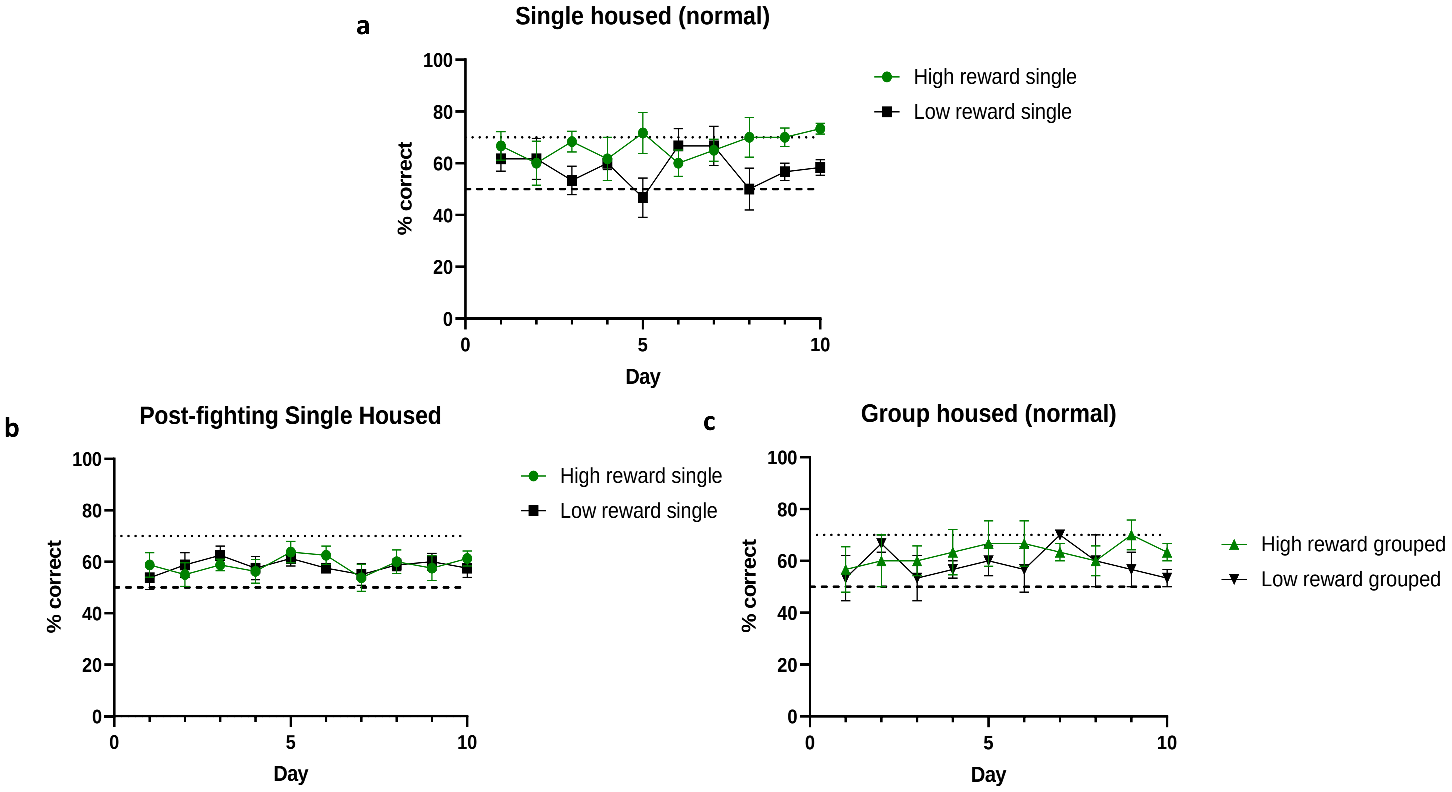
Appendix 1



Dashed line = 50% accuracy, chance
Dotted line = 70% accuracy, learning threshold

Fig A 1 Mice chronically treated with corticosterone (depression model) showed reward learning deficits compared to control mice in the high vs low reward learning task.

a Control mice showed an improvement in accuracy for the high reward floor across the nine sessions and appeared to have learnt the association. **b** Depression model mice recorded scores consistent with random chance for both the high and low reward floor. Charts show mean ± SEM. n = 9 per treatment group. These data were collected by Jen Davies.



Dashed line = 50% accuracy, chance
Dotted line = 70% accuracy, learning threshold

Fig A 2 Singly-housed mice previously exposed to fighting, and group-housed mice, showed reward learning deficits relative to singly housed mice in the high vs low reward learning task.

a Singly-housed mice showed an improvement in accuracy for the high reward floor across the ten sessions and appeared to have learnt the association. **b** Singly-housed mice that had previously been group housed and exposed to fighting (killed another mouse/received fight wounds/housed in cage where mouse was killed) recorded scores consistent with random chance for both the high and low reward floor. **c** Group-housed mice not exposed to fighting recorded some improvement in accuracy for the high reward floor across the ten sessions but scores remained consistent with random chance. Charts show mean ± SEM. n = 6 for group and single-housed normal mice, n = 8 for post-fighting single-housed. These data were collected by Jen Davies.

Appendix 2

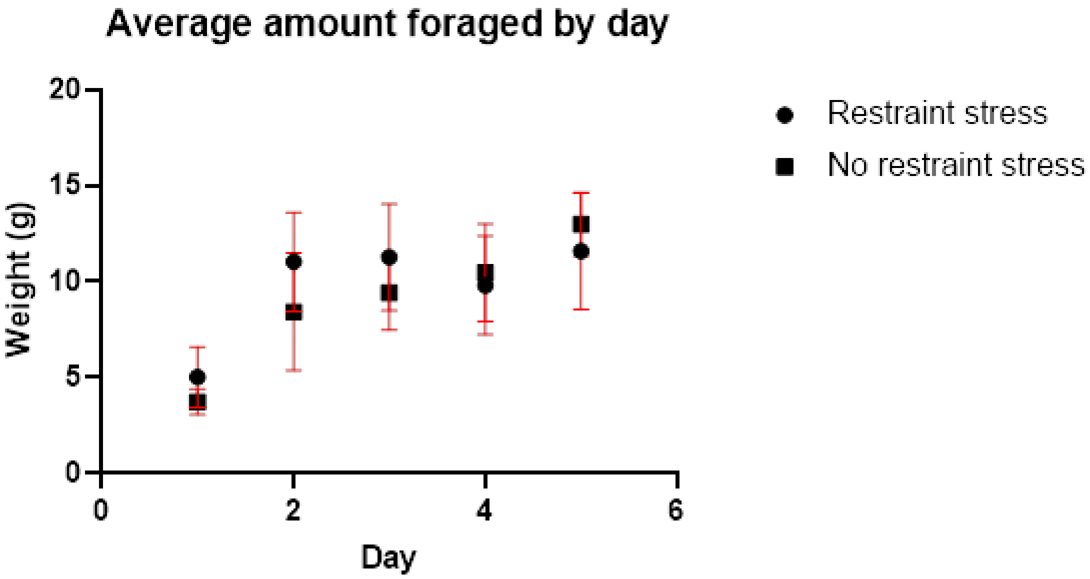


Fig A 3 Repeated restraint stress did not affect the amount of nesting material foraged in the acute foraging task in any individual session.

Bars are mean ± SEM. n = 4 per condition group (repeated restraint vs restraint-naïve controls).

Appendix 3

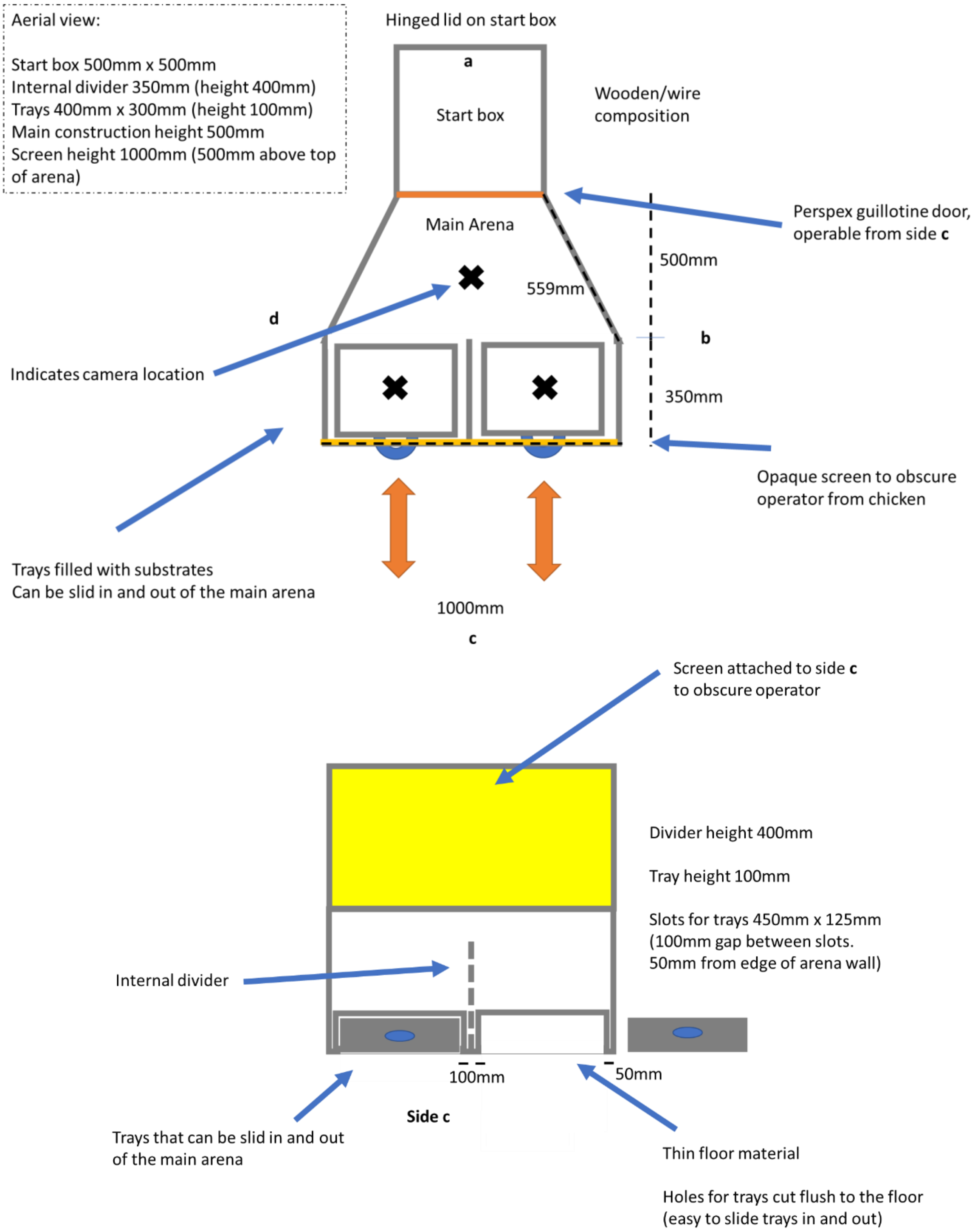


Fig A 4 Design drawings for the chicken reward learning arena.

Appendix 4

Chicken ID	Second stage habituation sessions required	Additional notes
Blue	4	Initially needed vocal encouragement to explore
Brown	2	For first session removed guillotine door to encourage return to start box as would not walk back under it
Green	1	Not fearful of the door
Grey	5	Needed vocal encouragement throughout. For first session removed guillotine door to encourage return to start box. Stopped performing and would stand and call after ~ 5mins. Chicken was moved to next training stage despite needing encouragement to meet the criteria. It was anticipated chicken would habituate further later in the training protocol, otherwise would be excluded from the study.
Red	1	Not fearful of the door
Yellow	4	Initially needed vocal encouragement to explore. For first session removed guillotine door at the end to encourage return to start box.

Table A 1 Chicken habituation process details

Appendix 5

Suggested habituation and training protocol:

Present chickens with rewards in the trays in their home pen daily while they are settling in, before training procedure starts.

1) Habituation

   i. Set up arena with guillotine door removed and both reward trays inserted. Place x3 sweetcorn in each tray and on start box tile. Individually place chickens into start box and allow 10 minutes to consume food rewards. Encourage movement and reward consumption or first five minutes, vocally and by moving around arena. Rebait trays and start box every time all food rewards are consumed. Progression to next habituation stage depends on rewards being eaten from all locations without encouragement.

   ii. As above, but with guillotine door. On setup insert guillotine door in closed position. Open door to release chicken. Then close door until chicken has eaten rewards from the trays and start box has been rebaited. Leave door open until start box rewards have been consumed and chicken leaves start box again. Shut chicken in start box after 10-minute session ends. Progression to next training stage depends on rewards being eaten from all locations without encouragement.

2) Shuttle training

   i. Set up arena with start box baited with x3 sweetcorn and door closed. Bait centre of one tray with x1 sweetcorn. Individually place each chicken into start box and open door. Conduct session while standing to be visible to chicken and offer vocal encouragement. Give chicken as long as necessary to find and consume sweetcorn in tray. Then, rebait start box with x3 sweetcorn via reward delivery tube. Close start box door when chicken enters and rebait tray. Repeat for 10 trials. Randomise baited tray position. Progression to next stage depends on completing 10 trials in maximum 15 minutes.

   ii. First trial as above. Subsequently, start training chickens to enter start box before reward delivery. Rebait start box with x1 sweetcorn only when chicken is half-way into start box. Then close door and rebait start box with x2 sweetcorn.

iii. First trial as above. Subsequently, rebait start box with x3 sweetcorn only after chicken has fully entered start box and door has been closed. Conduct session sitting down so not visible to chicken. Still offer vocal encouragement if chicken stops responding for >60s.

iv. First trial as above. Subsequently, once chicken has put head over edge of baited tray, remove unbaited tray from arena. Conduct 20 trials. Progression to next stage depends on completing trials in maximum 30 minutes.

3) Foraging training

i. Most aspects remain as previous stage. One tray contains a central wood chip pile (~173cm^3), with x1 sweetcorn on top. (A) For trials 1-10, sweetcorn is on top of pile. (B) For trials 11-20, sweetcorn is buried approx. 1cm down in wood chip pile. On first presentation of buried sweetcorn trial, encourage chicken to stand on pile to reveal sweetcorn reward. Progression to next stage contingent on independently finding at least 80% of fully buried sweetcorn (no time restrictions imposed).

ii. Present 20 trials with fully buried sweetcorn. Progression to testing contingent on chicken finding 100% of sweetcorn independently and approaching tray with wood chip pile before unbaited tray in at least 70% of trials.

Following completion of this initial training protocol, chickens should be ready to progress to basic discrimination and further testing procedures using the arena.

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