Affective state and visual decision-making in bumblebees

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ABSTRACT

Affective states, or emotions, are internal states of an organism, brought about by an appraisal of the environment, which result in specific physiological, cognitive, and behavioural responses. These states can significantly influence information processing and alter cognition and perception. Recent research has argued for similar emotion-like states in bees, using judgment bias tests, a widely accepted method for measuring emotional states in mammals. Although these findings suggest the possibility of emotion-like states in bees, alternative explanations have been suggested for these results. There is also little knowledge of how these states influence other cognitive and sensory responses. In this thesis, I develop robust tests for these states in bees and investigate their effect on multiple visual responses.

In the first experimental chapter, I develop a novel test for pessimistic cognitive biases in bees that controls for alternative interpretations and provides stronger evidence of the presence of emotion-like states in bees. Having established the presence of these states, subsequent experimental chapters delve into their impact on information processing during different stages of decision-making. In the second experimental chapter, I examine how negative emotional states affect visual acuity, revealing that such states may facilitate the ability to resolve fine spatial details, potentially aiding in threat detection. The third experimental chapter explores how negative states influence behavioural flexibility. I demonstrate that experiencing negative states facilitates the ability to estimate and update expectations of reward value, consequently enabling flexible responses to environmental change. The last experimental chapter explores methods to investigate the neural structures linked to emotion-like states and visual learning in bees for future research. Collectively, these experiments provide strong support for the existence of emotion-like states in bees and suggest that these states may serve as adaptive mechanisms to enhance survival.

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Chapter Two: Data modelling was performed by Professor Jenny Read

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TABLE OF CONTENTS

CHAPTER ONE

Introduction

1.1. Visually guided decision-making in bees

Understanding how miniature brains accumulate, process, store, retrieve and respond to sensory information has long been a popular field of study. Studying insects not only helps us understand how rather complex cognitive functions can be supported by simpler neural architectures but perhaps most importantly, it broadens our understanding of the evolutionary histories of these abilities. Among insects, social bees, particularly honeybees and bumblebees, have proved to be a valuable model. Social bees are central place foragers. These insects have therefore evolved to utilise their nests as places for storing resources, and castes whose main task is to continuously supply the colony with these resources (Goulson, 2003). Having such a high foraging specialisation might make resource gathering seem a simple task. However, both nectar and pollen availability fluctuate across space and time, not only due to species-specific flowering patterns and weather conditions but also due to intra- and interspecies competition (Núñez, 1977; Bowers, 1986; Witt *et al.*, 1999; Plos *et al.*, 2023). These pressures have predisposed bees to evolve cognitive abilities to support a high level of behavioural plasticity. The resultant flexible decision-making capacity makes bees an attractive model for studying cognition.

Foraging bees greatly rely on vision. Incoming visual information is processed by the peripheral visual system. This consists of compound eyes, located on both sides of the head capsule, and single-lens eyes, ocelli, on the top of the head capsule. The compound eye of the bee comprises multiple units, ommatidia, that hold photoreceptors. Photoreceptor spectral sensitivity, peaking at the ultraviolet (~350 nm), blue (~450 nm) and green (~550 nm) regions of the spectrum (Peitsch *et al.*, 1992), makes bee vision trichromatic. Colour processing, therefore, requires all three photoreceptors. The achromatic signal however is processed by green-sensitive photoreceptors only (Lehrer et al., 1988; Giger and Srinivasan, 1996).

Given the ecological pressures, the bee visual system has evolved the capacity to perceive and utilise different floral traits, including but not limited to colour (Gumbert, 2018), size (Essenberg *et al.*, 2015), shape (Lehrer and Campan, 2005) and even symmetry (Moller, 1994). Several factors, however, impact the extent to which visual information is used to guide bee decisions. To better illustrate how visual decision-making in bees can be modulated, I will provide examples of how innate biases, as well as both personal and social learning can contribute to this process.

Innate preferences

Bees prefer certain colours. These innate colour preferences, often referred to as sensory biases, likely represent evolutionary adaptations that existed prior to any experience with flowers. They have been suggested to have evolved to help naive bees select profitable flowers (Giurfa *et al.*, 1995; Raine and Chittka, 2007), and are even believed to drive the evolution of floral traits (Trunschke *et al.*, 2021). It has been shown that newly emerged bumblebees, which have never been exposed to flowers, display a strong preference for colours in the violet-blue range (Briscoe and Chittka, 2001; Chittka and Wells, 2004). After experiencing rewards on different flowers, however, these biases are overridden. Therefore, through experience bees can form a reward association with any colour (Gumbert, 2018). However, the learning acquisition will still be faster if the colour falls within the preferred colour range (Giurfa *et al.*, 1995).

Prior experience

Stimulus generalisation is an adaptation that is well documented in bees. However, social bees have also demonstrated the generalisation capacity that goes beyond a single stimulus feature, e.g., colour (Gumbert, 2018). For instance, visual generalisation has been demonstrated using complex visual patterns (Stach, Benard and Giurfa, 2004). In this study, bees were subjected to a discrimination learning task, where they had to learn to differentiate between patterns that only shared local orientation embedded in noise. After a series of trials, bees indeed learned to discriminate between the patterns, demonstrating the outstanding ability to extract common regularities in otherwise noisy information. Bees were also able to generalise their learning to novel stimuli with shared commonalities, and even to novel stimuli only partially related to training set. The fact that, through learning, bees were able to “fill in” missing information when test patterns contained only partial similarity with trained stimuli, shows that in addition to extracting common regularities, learning also facilitates image processing by completing missing details (Cavanagh, 1991). This ability was explored in a later study. In this set of experiments, the authors demonstrated that training bees on conspicuous shapes later facilitate discrimination between the same but highly camouflaged shapes (Zhang and Srinivasan, 1994). Importantly, without the initial familiarisation of fully visible shapes, bees failed to distinguish camouflaged shapes even after extensive training sessions. Taken

together, the few examples provided in this section demonstrate how prior experience can facilitate learning in bees and improve their visual search in noisy visual environments.

Social learning

In addition to personally acquired information, social information can also modulate foraging behaviour in bees. For example, bees can use social cues (e.g., the visual presence of conspecifics) to make their floral choices (Worden and Papaj, 2005; Leadbeater and Chittka, 2007; Dawson *et al.*, 2013). The use of social cues itself, however, can also be modified by prior experience. For instance, if bees experience the presence of visual social cues (such as conspecific-occupied flowers) always coupled with a high reward, they are more likely to rely on social information in their subsequent choices. In contrast, if the social cues do not reliably predict reward, the use of social information decreases (Leadbeater and Chittka, 2009). The use of social over personal information also depends on resource availability. When the availability of rewards is highly variable and flowers do not consistently offer high-quality rewards, bees tend to prefer flowers with visual social cues. In this situation, bees employ a "copy-when-uncertain" strategy (Smolla *et al.*, 2016). Conversely, another study has shown that when previously rewarding flowers become empty, prior exposure to the odour of new rewarding flowers within the colony does not directly facilitate switching to these new alternatives (Leadbeater and Florent, 2014). However, once the new alternative flower option is discovered, prior exposure to the olfactory information acquired within the colony, resulted in greater commitment to it. As a result, bees visit these new flowers significantly more often than bees that have no socially acquired information about this new option.

All the above behaviours are examples of appetitive learning, demonstrating the ability of bees to assign a positive value to an otherwise neutral stimulus. Bees also can learn stimuli associated with negative experiences – such as a punishment or an aversive solution - and thus avoid such stimuli (Ings and Chittka, 2008). Therefore, both positive and negative values directly impact bee decision-making. It is, however, very important to highlight that such value-driven decision-making in bees goes beyond

simple stimulus-response mapping. Instead, it involves the capacity to flexibly modulate their subsequent choices by shaping expectations and outcome evaluation. For example, compared to bees that experienced increasing reward availability, bees that experienced declining reward availability were found to be less persistent in searching for food at a patch without rewards (Gil, Marco and Menzel, 2007). Similar behaviour was observed in bees that experienced a change in reward levels of a different magnitude. If a change was large bees were more persistent in searching for rewards when flowers offered none, compared to bees that had experienced smaller changes (Gil and De Marco, 2009). These examples demonstrate that based on prior experience with the reward, bees develop certain expectations. Bees that experience declining rewards or a change in reward levels of a smaller magnitude ceased searching, arguably due to the anticipated absence of a reward. Therefore, having an internal representation of reward expectation allows bees to respond to stimuli in a more flexible way, thus adjusting their investment of time and energy while foraging.

The ability to flexibly respond to aversive stimuli has also been demonstrated in bees. This was done by employing a motivational trade-off behavioural paradigm where bees had to choose between two competing motivations: obtaining a reward or avoiding punishment (Gibbons *et al.*, 2022). First, bees were trained to forage from two types of feeders: one being a high-quality feeder offering a reward with a 40% sucrose concentration, and the alternative feeder containing either the same high reward or a lower reward (30%, 20%, or 10% sucrose concentration). Next, bees' choices were tested under two conditions. The first condition comprised of a heated high-quality feeder and an unheated alternative feeder. In the second condition both high and alternative feeders were unheated. The results showed that when an alternative feeder offered a lower reward, bees were more likely to "tolerate" noxious heat and choose to land on a heated high-rewarding feeder. This was not true, however, when both feeders offered high rewards. In this case, bees were more likely to land on unheated alternative feeders. Bees, therefore, may choose to accept aversive experience only if the payoff associated with such a decision is higher. The cost-benefit estimation associated with the outcomes demonstrated here once again showcases the flexibility behind decision-making in bees that goes beyond stimuli-response mapping.

Flexible value-based decision-making implies the existence of an internal representation of positive and negative valence (Adolphs and Anderson, 2018). This internal representation allows bees to assess potential future outcomes and make

weighted choices, whereas in the absence of such inner representation, making decisions based on expectations and cost-benefit estimation would not be possible. Systems must therefore exist within the bee brain to sustain this flexible value-based decision-making, enabling them to go beyond simple stimulus-response reactions.

To adaptively navigate their environments, animals must have the capability to ascribe value to various stimuli. Positive-valenced stimuli are generally believed to trigger approach behaviour, whereas negative-valenced stimuli tend to evoke avoidance responses (Søvik, Perry and Barron, 2015). So, how exactly do bees "assign" value to neutral stimuli? This function is usually associated with two major biogenic amines: dopamine with negative valence and aversive learning, and octopamine with positive valence and appetitive learning (Søvik, Perry and Barron, 2015). As dopamine is a key component in the mammalian mesolimbic system, i.e., the reward system (Arias-Carrián *et al.*, 2010), it may seem that dopamine has an opposite function in the brains of insects and mammals. However, recent studies show that the dopaminergic system is far more complex. Noxious stimuli can excite certain dopaminergic neuron populations while inhibiting others (Bromberg-Martin, Matsumoto and Hikosaka, 2010), suggesting a vast diversity of mammalian dopaminergic neurons. Similar valance-driven diversification has also been reported in *Drosophila melanogaster,* with anatomically and genetically distinct subpopulations of dopaminergic neurons involved in both aversive and appetitive learning (Owald and Waddell, 2015). The flexibility within dopaminergic signalling suggests that dopamine neurons do not encode motivational signals in a fixed manner. Instead, these neurons consist of multiple subpopulations that encode events in distinct value-specific ways.

Although there are currently no tools for conducting similar investigations in bees some valuable insights have been obtained relating positive value and dopamine. In a recent study, Huang et al. (2022) investigated the role of dopamine in "wanting" in bees (Huang *et al.*, 2022). "Wanting" here refers to the motivation to acquire a reward and is thought to be one of the key aspects associated with reward (Berridge, 2007). In this study, authors assessed dopamine levels in bees engaging in several behaviours, specifically reward communication (waggle dance), reward responsiveness, and reward learning. A transient increase in dopamine levels was observed with all these behaviours. Additionally, the inhibition of dopamine receptors reduces food-seeking behaviours, whereas activating these receptors increases seeking behaviour. These results provide the first evidence for the existence of a

dopamine-dependent “wanting” system in bees. Given the discovery of this system, and the numerous shared aspects of brain morphology and functionality between bees and Drosophila (Søvik, Perry and Barron, 2015), it is plausible that, as in flies, distinct dopaminergic populations in the bee brain may also drive both reward and aversive responsiveness.

In this section, I have tried to highlight certain points. First, social bees exist in a complex environment that poses multiple challenges, such as the spatiotemporal fluctuation of much-needed resources. Because of this, bees evolved cognitive abilities to sustain a high level of behavioural flexibility that can be guided not only by different innate sensory biases but also by personal or social learning. I also point to the fact that while bees are skilled at assigning value to stimuli, their value-based decision-making remains flexible. It is influenced by future expectations as well as the evaluation of possible outcomes. This suggests the presence of an inner representation of valence. While detecting and accurately responding to the value of stimuli is a complex process involving a number of neurotransmitters and neuromodulators (Even, Devaud and Barron, 2012; Søvik, Perry and Barron, 2015), the dopaminergic system seems to be flexible enough to support it. Behavioural and neuropharmacological evidence provided in this section, therefore, implies that bee brains possess the ability to respond to rewards and punishments in adaptable ways beyond simple stimulus-response mapping. As I will discuss later, in mammals, such a function is attributed to emotions. Can it be that bees also have evolved the capacity for emotions at least in its primitive form? And if so, can emotions in bees modulate bee behaviour in the same adaptive manner as it does in mammals? To answer these questions, I will first discuss how emotions have been conceptualised and linked to decision-making, as well as studies across animals.

1.2. Conceptualising Emotion

1.2.1. What is Emotion?

Since ancient times thinkers such as Socrates have tried to unpack and understand the complexity of emotional phenomena (Russell, 2003). What started as a philosophical discourse, developed into a separate discipline – affective science,

following the advancement in psychology and neuroscience of the 20th century, (Gendron and Feldman Barrett, 2009).

Affective science has gained increasing popularity with scholars of psychology, neuroscience, philosophy, economics, literature, history, sociology and computer science, all working to understand the nature of emotion. However, do we all agree on what we study? Ongoing debate over the precise definition of emotion perhaps argues otherwise (Gendron, 2010; Russell, 2012). While the field of affective science has grown exponentially, only very little progress has been made in defining the cornerstone definition - emotion. For instance, in 1981, more than 93 definitions of emotion were proposed (Kleinginna and Kleinginna, 1981). To stress this paradox, Fehr and Russell (1984) highlight that while people generally have a good understanding of what an emotion is, when asked for a precise definition, it becomes apparent that no one truly knows (Fehr and Russell, 1984). This presents a problem. The absence of a clear definition not only complicates the integration of discoveries made in different disciplines but also confuses the understanding of findings, even within the same field. Therefore, without a clear understanding of what is being studied it becomes challenging to make meaningful discoveries and draw conclusions.

By the end of the 20th century however, substantial attempts have been made to reconcile this paradox, and finally describe emotion (Izard, 2010), starting by acknowledging the complexity of phenomena. Each discipline that studies emotion only focuses on specific aspects. For example, neuroscientists might be only interested in what brain structures generate a certain emotion, while economists, would be most interested in how emotions shape human decision-making. Therefore, definitions are discipline-specific and thus are centred on a subset of aspects specific to a given field – for example, neural processes, facial expressions, appraisals, adaptive functions, action tendencies, or motivation (Nabi, 1999). Thus, no single definition comprehensively encompasses all aspects of emotion. While this is true with most complex phenomena, the key question is whether these definitions are in conflict.

To determine if there is a shared understanding among scholars of different fields regarding what “emotion” is, Izard (2010) asked 35 distinguished scientists to participate in a survey. Participants were asked several questions regarding emotion (e.g. its function, future research, etc.), as well as provide their definition. The results confirmed that emotion cannot be encapsulated by a single simple definition. However,

each of the provided definitions complemented each other pointing to different but equally important aspects of emotion. Although differences exist, some agreement was also present. First, emotions are generally considered short-lived, multifaceted states, comprising neural circuits (some of which are specialised) and response systems (physical and behavioural reactions) and subjective experiences. Furthermore, emotions are initiated by cognitive appraisals, provide information to motivate either approach or avoidance behaviour and organise cognition and actions more generally. Taken together, the most accepted definition of emotions describes them as short-lived inner states that arise from stimulus appraisal and are accompanied by changes in physiology, behaviour, cognitive and subjective experience. Can this definition, however, accommodate emotion research in non-human animals?

When it comes to including animals in emotion studies, perhaps the biggest challenge arises around whether the conscious experience of these states is a necessary requirement. When the famous question "What is emotion?" was posed by William James in the 19th century, emotions were thought of as intrinsically human, subjective experiences. Since then, little has changed. Even in recent days, "emotion" and "feelings" are viewed as synonyms and are used interchangeably. Importantly, this is true among the general population and among scholars. Making very little distinction between these two terms causes confusion. Some researchers even argue whether terms like "emotion", "fear", "sadness", or "joy" should be applied to animals (Winkielman and Berridge, 2004; Damasio, 2014; Ledoux, 2014; LeDoux, 2017; Berridge, 2018; LeDoux and Hofmann, 2018). Others suggest using different terminology such as "survival circuits", when referring to animal emotions to avoid equating animal emotion with human-like conscious experiences (LeDoux, 2012; Ledoux, 2014). Yet, other considerations argue against dismissing the idea of animal emotion. Here there are several key assumptions to consider.

The first, and most obvious consideration is about the subjective experience of emotion. It is important to explore whether emotions in humans are *always* consciously experienced. A growing body of evidence suggests that perhaps they are not (Berridge and Winkielman, 2003; Paul *et al.*, 2020). Research shows that the human brain reacts to emotion-eliciting stimuli without conscious awareness (De Gelder, Morris and Dolan, 2005; Öhman, 2005). Therefore, the neurophysiological and behavioural responses may indeed occur within an individual that is fully oblivious to the presence of emotional stimuli. Exactly this was demonstrated earlier by Winkielman et al. (2005). In this study,

subliminal images of happy and angry faces were shown to participants. Following this presentation the participants' behaviour in relation to a pleasurable stimulus such as drinking when thirsty was observed (Winkielman, Berridge and Wilbarger, 2005). While participants reported no change in their emotional state, drinking behaviour was indeed affected. Despite all participants being in a heightened motivation state (thirst), those exposed to the happy face poured and drank more of an unfamiliar beverage, contrary to the group exposed to an angry face who poured and drank less. The authors therefore concluded that emotions triggered outside conscious awareness and persisting beyond it can influence behaviour and the evaluation of emotionally charged events. Therefore, emotion need not always be conscious to trigger a response.

While not all emotions are consciously perceived many believe it is unreasonable to disregard the possibility that animals also have subjective emotional experiences (Burghardt, 2019; Paul *et al.*, 2020; Kret, Massen and de Waal, 2022). There is a recent surge in interest to finally start recognising the uniqueness of each species. It is hard to argue against the fact that each species has evolved in response to *unique* evolutionary pressures that finely tuned species brains and bodies in a *unique* way. "Feeling" fear when in danger might therefore be a very different experience in humans than, for example, flies. Just as other functions, such as light processing vary across species, the subjective experience of emotion can also differ (Kret, Massen and de Waal, 2022). While this perspective opens the door to acknowledging the potential for subjective feelings in animals, it is important to emphasise that there are currently no available methods to directly measure these feelings. However, as I will argue later, perhaps we do not need to just yet. Exploring other emotional components, such as behaviour and cognition offers a comparative approach to an emotion that permits a much deeper understanding of the phenomena beyond the mere notion of whether emotion is consciously experienced in a given species.

A final consideration addresses the function of emotion. The idea that emotion evolved to facilitate adaptive behaviour in a way that promotes an approach towards resources while avoiding harm, is not new (Bethell, 2015). Emotion is also thought to evolve from reflexes (Adolphs and Anderson, 2018). However, contrary to the latter, emotion offers a degree of flexibility in responding to environmental challenges in a way that simply surpasses that of hardwired automatic responses (Adolphs and Anderson, 2018). For example, the startle reflex, triggered by abrupt and intense

stimulation, serves an adaptive function. It may initiate a quick escape. However, it is not flexible enough to account for all the dangerous situations animals may face. This is because surviving in the presence of a threat does not always benefit from rapid movement. In some situations, staying still (freezing), or even initiating an attack, is more advantageous. Contrary to reflexes, emotions indeed possess the flexibility to accommodate behaviour that is best in a given context (Mendl and Paul, 2020). Emotion can therefore be considered as a greater functional adaptation (Dawkins, 1990; Öhman and Mineka, 2001; Nettle and Bateson, 2012; Trimmer *et al.*, 2013; Anderson and Adolphs, 2014). If so, would such functionality also be beneficial to species other than humans? While this is an open question, my thesis argues that it might, even for insects.

1.2.2. Theoretical approaches to emotion

Emotion is a complex phenomenon. There is no single simple definition that could in detail encompass all that there is to emotion. Instead, several dominant theoretical approaches have been developed to try and conceptualise emotion. These broadly can be categorised into discrete emotion, dimensional emotion, and appraisal theories. Discrete emotion theories, as the name states, view emotion as discrete, basic "programmes" that are evolutionary predetermined and exist in a limited number. Unlike discrete theories, dimensional theories conceptualise emotion as constructed within a two-dimensional space of valence and arousal. Therefore, in this view, there could be an infinite number of emotions. Appraisal theories, however, adopt a more cognitive approach viewing emotion as a multi-component process driven by information appraisal. In the following section, I will give a concise yet thorough depiction of each theory.

Discrete emotion theories

The development of the discrete emotion framework started with explorations of human bodily expressions of and responses to different emotions. In the 1960s, Ekman and others were committed to investigating if similarity in emotional facial expressions and recognition of these expressions are inherently universal (Colombetti, 2009). The pioneering study by Ekman et al. (1969) involved presenting photos depicting facial expressions of different emotions, such as fear, anger, happiness, disgust, surprise,

and sadness to participants worldwide (Ekman, Sorenson and Friesen, 1969). The results suggested that basic emotions are recognised globally. However, the study was conducted in parts of the world that are vastly impacted by Western culture, leading to doubts about whether this recognition is hardwired or acquired. To address these concerns, Ekman and Friesen conducted a follow-up study. This study took place in a culture isolated from the Western world, specifically, the Fore people in Papua New Guinea (Ekman and Friesen, 1971). The results further confirmed the initial conclusion that basic emotions exist. While the idea that certain emotions are hardwired became generally accepted, the number of such discrete emotions is still debated, with some proposing nine (Tomkins, 1980) while others proposing eight basic emotions (Plutchik, 2001). Nevertheless, most agree on the so-called "Big Six" (Moores, 2004): fear, disgust, anger, sadness, surprise and enjoyment.

Taken together, the discrete emotional framework postulated three main ideas (Izard, 1992, 2007; Ekman, 1994; Vytal and Hamann, 2010). First, there are only a limited number of "basic" emotions. Second, all basic emotions are evolutionary predetermined "programmes", therefore in the brain where the hardwired emotion-specific activation mechanism exists. The activation of such a mechanism (through cognitive appraisal) elicited specific emotions. Third, each emotion evolved to carry a distinct function.

Dimensional emotion theories

Given the emphasis of the existence of only a limited number of emotions - the "Big Six"- discrete theories of emotions are not immune to criticism. Specifically, proponents of dimensional theories (also known as constructed emotion theories) argue that the evidence supporting such ideas is rather inconsistent (Russell and Barrett, 1999; Watson *et al.*, 1999; Carver, 2001; Russell, 2003). For example, humans, when asked about their subjective emotional experiences usually fail to place their feelings into a single discrete category (Posner, Russell and Peterson, 2005). Such observations highlight the fact that human emotions are vastly intercorrelated and lack distinct boundaries, which is contrary to what discrete emotion theories imply. Moreover, the universality of emotional expressions is also questionable. Barrett (2009) argues that fear, for instance, manifests diversely across human brains and is experienced differently among individuals.

Instead of viewing emotions as distinct entities, proponents of dimensional theories offer viewing emotions as existing along a continuous spectrum, or multidimensional space (Russell and Barrett, 1999; Watson *et al.*, 1999; Carver, 2001; Russell, 2003; Posner, Russell and Peterson, 2005). This space has two dimensions - the "valence" and "arousal"/"intensity" (Russell and Barrett, 1999; Watson *et al.*, 1999; Carver, 2001; Russell, 2003). These two descriptive dimensions are also known as core affect (Russell, 2003). Here, valence refers to a degree of pleasantness or unpleasantness of a state and arousal to a degree of physiological and psychological activation/stimulation associated with it (see Figure 1). For example, fear is a state of negative valence and high arousal whereas excitement a state of pleasant valence and high arousal. Thus, the two-dimensional core affect constructed along valence and arousal is a key feature of all dimensional emotion theories. Moreover, core affect does not restrict emotion to a specific place within this space, but rather permits swift change along the valence-arousal axes in response to stimulus appraisal (Russell, 2003). similar to discrete theories, dimensional theories also consider emotion, specifically core affect, as serving functional adaptations (Burgdorf and Panksepp, 2006; Nettle and Bateson, 2012). Thus, valence and arousal indicate an animals' tendency to initiate approach or avoidance. For example, high-arousal-positive-valence states are likely to promote resource acquisition, while high-arousal-negative-valence states could assist in danger avoidance (see Figure 1).

Cognitive appraisal theories of emotion

Unlike discrete or dimensional emotion theories, cognitive appraisal theory views emotions more as processes rather than states. The core premise is that emotions are triggered by stimulus appraisal (Smith and Lazarus, 1990; Scherer, 2009; Ellsworth, 2013; Moors *et al.*, 2013; Roseman, 2013). In other words, emotion is not viewed as a direct response to the stimuli but rather as a result of stimulus appraisal. For instance, being in a state of fear when exposed to a snake is not a result of perceiving the snake *per se*; instead, fear emerges from appraising the snake as associated with danger. Therefore, cognitive appraisal is thought to be a continuous process that runs outside of the scope of awareness (Scherer, 2009; Ellsworth, 2013).

There are several criteria that stimuli are evaluated upon, and the same criteria can be used to differentiate emotions. These are not limited to valence (pleasant or

unpleasantness) but could include stimulus novelty, predictability and significance, the ability to cope with the consequences of the event(Moors *et al.*, 2013). If a person, for example, appraises a situation as being intrinsically unpleasant/punishing (valence), and sudden (predictability), she will form a multicomponent response pattern which can be called "fear". The multicomponent response, as such, can be characterised by the intensity and quality of action tendencies, neurophysiological, behaviour and subjective changes.

The simplest form of appraisal theory postulates that emotions are first triggered by the appraisal of stimuli. Stimulus appraisal is subsequently a continuous process triggering specific emotions when the set of criteria is met (e.g., "excitement" arises when the stimulus is evaluated as pleasant, novel, and significant). Finally, the outcome of such cognitive appraisal is a multi-component response characterised by changes in neurophysiology, behaviour, cognition, and subjective experience.

1.2.3. An integrative framework to study animal emotion

The frameworks presented in this section are well-used to study human emotions. However, how can these theories be applied to the study of emotions in animals? Some proponents of discrete emotions, for example, argue that basic emotions are the result of conserved **neuro**behavioral systems. Therefore certain basic human emotions are likely shared with other mammalian species (Panksepp, 2011). While this argument is valid on the basis of the existence of brain structures homologous to those in humans, it fails to accommodate species with no such similarities. Unlike discrete emotion theories, dimensional emotion theories seem to offer more flexibility in their views. Nevertheless, the significant reliance on subjective experience within this framework again poses a challenge when it comes to animals.

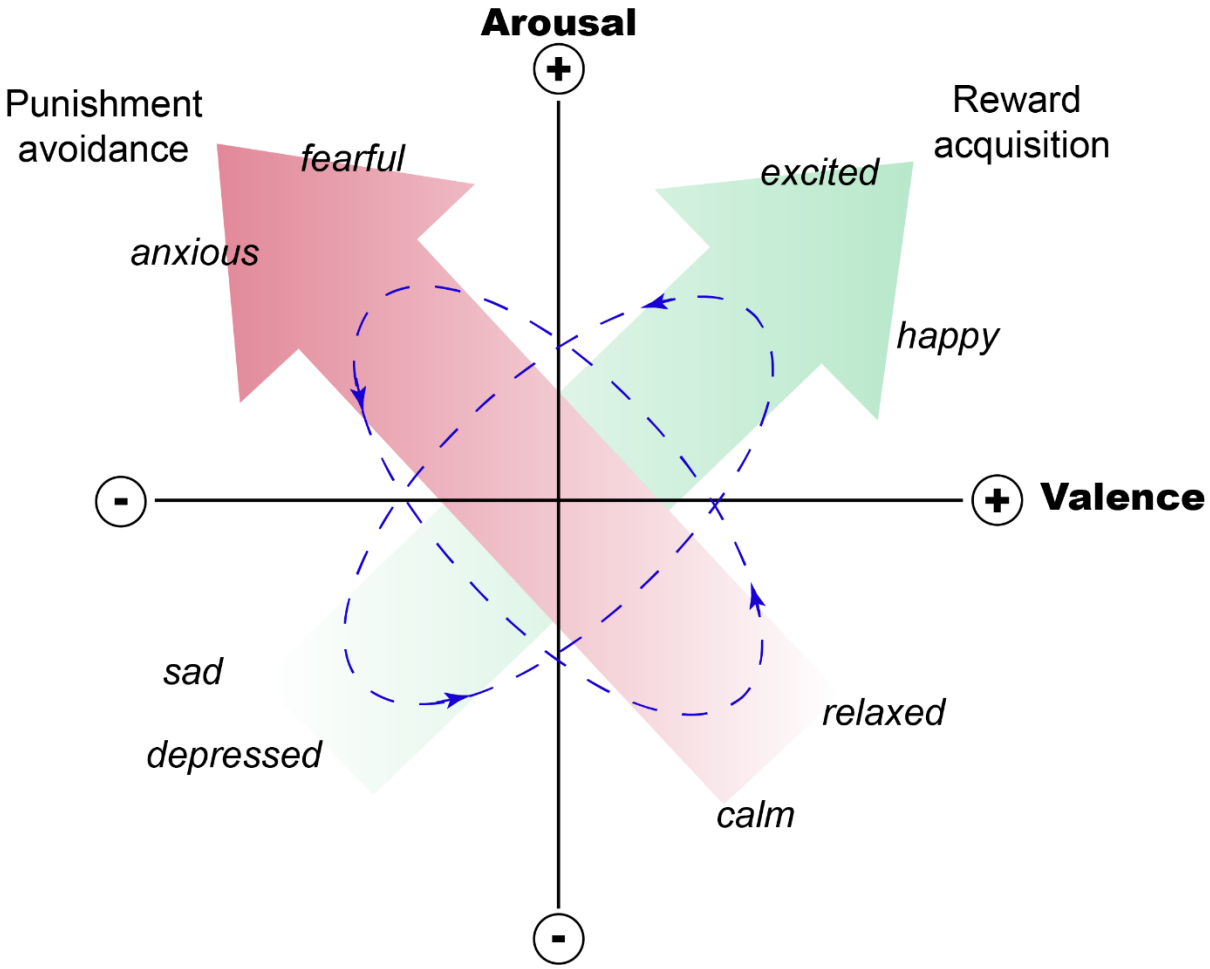

Figure 1. Two-dimensional core affective space with discrete emotions positioned within it. Arousal (ranging from low to high) and valence (ranging from pleasant to unpleasant) constitute the two axes of core affect. Words in italicised font indicate specific discrete emotions positioned within core affect, determined by their valence and arousal levels. Green arrows illustrate the tendencies for the pursuit and acquisition of rewards associated with core affect. The experience of tendencies for obtaining rewards is highest in high-arousal positive states (depicted in saturated green) and lowest in low-arousal negative states (depicted in unsaturated green). Red arrows demonstrate tendencies to avoid fitness-threatening aversive stimuli, which are most pronounced in high-arousal negative states (depicted in saturated red) and least in positive low-arousal affective states (depicted in unsaturated red). Blue dashed lines indicate how current core affect can shift in response to specific external stimuli. Adapted from Mendl et al. (2010).

Recently, there has been an effort to bridge the gap between these theories and a framework applicable to studying emotions across taxa has been proposed (Mendl, Oliver and Paul, 2010). This framework starts with acknowledging one thing that all theories agree on – emotions are valenced. The valence, however, is determined by the cognitive appraisal of stimuli/situations as being either punishing/unpleasant or

rewarding/pleasant (Mendl, Oliver and Paul, 2010). Therefore, emotions evolved as a mechanism to inform organisms about their success in maximising rewards and minimising punishments (Bethell, 2015). As a result, emotion serves an adaptive function, thus raising consideration of whether these states can be evolutionarily conserved across taxa. Mendl et al. (2010) further conceptualise discrete emotions as short-term appraisal-driven states; therefore, movement through core affect space is then determined by these discrete emotions, reflecting the animal's success or failure in obtaining rewards and avoiding punishments (Fig.1). For instance, failing to find food would shift the emotional state position in a core affective space towards a more negative-valence-**high**-arousal state, that of "frustration", which, if the struggle to acquire rewards continues, may transition into a negative-valence-**low**-arousal state of "sadness". Consequently, a given position within core affective space generally reflects how well an animal is doing in maximising fitness.

As I attempt to highlight, all theories stress the possibility that emotions carry an adaptive function. However, reconceptualising core affect as a mechanism that coordinates organisms to achieve survival goals opens the possibility of studying emotion in animals. Moreover, by focusing on the adaptive function of emotions, questioning whether animals experience these states consciously becomes secondary. This is because affective responses can be expected to those stimuli that either maximise the acquisition of fitness-enhancing rewards or minimise exposure to fitness-threatening punishers (Mendl, Oliver and Paul, 2010). Affect thus represents an animal's overall experience with reward and punishment, expressed through behaviour, neurophysiology, cognition, and (possibly) subjective experience.

1.2.4. Defining Key Terms

Through my thesis, I will adopt the componential view of emotion as proposed by Mendl et al. (2010) as discussed in earlier section. Therefore, emotions will refer to a short-lived multicomponent response tendency that are generated by stimulus appraisal and facilitate species survival (Mendl et al. 2010; LeDoux 2012; Nettle and Bateson, 2012). The response tendencies elicited by the appraisal process unfold across loosely connected component systems, encompassing subjective experience, behaviour, cognitive processes and physiological changes. On the other hand, mood will be used

to describe a long-lasting state that is not triggered by immediate appraisals (Mendl, Oliver and Paul, 2010). In simpler terms, mood represents a “background” state - it develops gradually as a combination of all previous emotional states. Finally, following the example of many researchers studying animal emotion, I will use “affect” as the overarching umbrella term that encompasses both emotions and moods (Paul, Harding and Mendl, 2005; Bethell, 2015). I also recognise the complexity of human specific emotions, especially the depth and richness of subjective experiences that accompany these states in our species. Therefore, in my research as with other scholars(Solvi, Baciadonna and Chittka, 2016; Paul *et al.*, 2020), I adopt the common practice of using terms such as “emotion-like”, “anxiety-like” and “fear-like” when referring to states in bees that display behavioural and/or physiological resemblances to human emotions. However, it is important to note that consideration of whether these emotion-like states in bees involve subjective experiences falls beyond the scope of my study.

1.3. Emotion and decision-making

In the past scholars tended to underestimate how much emotions can impact cognition especially when it comes to decision-making. Many decision-making models, like the expected utility model (Loewenstein, Rick and Cohen, 2008), assume that a decision-maker carefully evaluates information and chooses actions that maximise benefits. Yet reality contradicts this assumption, as emotions significantly influence this process (Loewenstein and Lerner, 2003; Brosch *et al.*, 2013; Lerner, 2014). Importantly, there is a two-way relationship between emotions and cognition. According to theories of emotion, emotions arise based on stimulus appraisal (Smith and Lazarus, 1990; Scherer, 2009; Ellsworth, 2013; Moors *et al.*, 2013; Roseman, 2013). Once an emotional state is triggered it also affects cognitive functions (Brosch *et al.*, 2013). For example, when an individual appraises a stimulus as dangerous a state of fear is triggered, subsequently fine-tuning cognition to facilitate the rapid allocation of attention to such stimuli (Brosch *et al.*, 2008). In another case, when experiencing heightened arousal triggered by emotion, the prioritisation of storing emotionally salient information in memory occurs (McGaugh, 2013). Hence it is important to acknowledge that cognitive processes trigger emotions, while emotions also influence cognition to support emotional response. Therefore, cognition is needed to elicit emotion, at the same time emotion modulates cognition to support emotional responses. The latter are

described as cognitive biases – for example, attention biases, memory biases and judgment biases (Paul, Harding and Mendl, 2005). In a later section, I provide more examples of how emotion-induced cognitive biases are used in affective science.

Emotion thus can modulate all processes via which individuals acquire, process, store or utilise information for later decision-making (Ono, Nishijo and Uwano, 1995; Math and Mackin, 1998; Mogg and Bradley, 1998; Kindt and van den Hout, 2001; Phelps, 2004; Phelps and LeDoux, 2005; Bar-Haim *et al.*, 2007). The cognitive decision-making process unfolds in a series of distinct stages when an individual encounters a signal (Mendl *et al.*, 2009). First, the individual perceives the incoming information through their senses and directs their attention to it. Here, at the early stages of sensory information, the information is processed into a neural percept (i.e., neural representation). Subsequently, processes of percept interpretation and evaluation take place where the expected utility of a possible decisions is calculated. The expected utility takes into account the anticipated value and the probability of a specific outcome (Loewenstein, Rick and Cohen, 2008). This process is based on the prior experience of the individual as well as their current needs. The final stage of the processing of sensory information that leads to the decision is action selection. Subsequently, the individual takes action, responding to the sensory information in a way that was evaluated as best. In the brain these processes are executed through multiple interconnected parallel-processing circuits. Therefore, affective states may modulate each process individually or cumulatively (Mendl *et al.*, 2009). At its simplest, affective modulation can occur at different stages of sensory information processing: either the early stages of signal registration or the later stages of information evaluation (Fig. 2).

Early signal processing: perception and attention

In natural environments, the stimuli perceived by animals can often be ambiguous. Take for example the scenario of a movement in the bush while in a forest. This movement could either indicate the presence of a hidden predator or simply the harmless passing of a rabbit. Thus, the rustling of the bush might serve as a signal for danger or just a neutral event. To react to this signal, it must first be perceived and attended to, and emotions can impact this early stage. For instance, fear has been theorised to have evolved as a mechanism to aid individuals in detecting environmental

threats and responding quickly and effectively to them, consequently when in such a state perceptual and attention mechanisms become finely tuned to detect potential dangers (Mogg and Bradley, 1998). This is supported by physiological studies showing that individuals in a negative affective state, particularly anxiety, tend to allocate attentional resources toward stimuli related to threats or dangers (Math and Mackin, 1998; Mogg and Bradley, 1998; Kindt and van den Hout, 2001; Bar-Haim *et al.*, 2007). Conspicuous, threatening information has a particularly strong ability to capture attention.

Emotionally charged stimuli not only capture attention but also enhance spatial attention by acting as salient cues, directing attention to the area of the visual field where they initially appear. The detection of a target among distractors has been shown to be faster if the target is threat-related (Öhman, Lundqvist and Esteves, 2001). However, the detection of a non-emotional target is also faster if it appears in the same location as the emotionally relevant cue (Pourtois *et al.*, 2006), and even faster in individuals with anxiety (Fox, 2002; Mogg and Bradley, 2002).

Moreover, emotions can directly influence pre-attentive perceptual processes, altering how individuals see. Phelps, Ling, and Carrasco (2006) initially demonstrated that the detection of a single target (a tilted sinusoidal grating) among three distractors (sinusoidal gratings oriented vertically) with decreasing contrast was improved when a fearful face, as opposed to a neutral one, was presented (Phelps and LeDoux, 2005). Subsequently, the authors aimed to determine whether the enhancement in contrast sensitivity due to the presentation of a fearful face was attributable to spatial attention or if it involved modulations in the pre-attentive mechanisms. To investigate this, fearful and neutral faces appeared at four potential locations, effectively “cueing” all four positions. Once again, the presentation of a fearful face led to an increase in contrast sensitivity. As a result, the authors reached the conclusion that emotions indeed have an impact on the ability to perceive visual information. A later study also demonstrated the emotional modulation of another low-level visual feature. Bocanegra and Zeelenberg (2009) revealed that while negative emotions improve the perception of low spatial frequencies they also impair the perception of high spatial frequencies (Bocanegra and Zeelenberg, 2009). They then suggested that this difference in perception may be attributed to the inhibition between visual pathways where each pathway specialises in processing different aspects of visual information (Bocanegra and Zeelenberg, 2009; Bocanegra, 2011).

Later signal processing: outcome valuation

In the previous example about the perception of the movement in the bush, once the signal of movement in the bush has been registered, it can be interpreted as either neutral, requiring no immediate action, or potentially dangerous, initiating a threat response. To interpret sensory signals individuals must calculate the expected utility for both possible outcomes. Expected utility-based decisions take into account not only estimations of the probability that something will happen but also estimates of the value of that outcome (pay-offs) (Loewenstein, Rick and Cohen, 2008). After comparing the expected utilities, the action believed to yield the most positive or least negative result will be selected. Emotions, once again, play a critical role in this process.

Butler and Mathews (1983) demonstrated empirically the existence of pessimistic world views among those in a negative state (Butler and Mathews, 1983). The experiment employed self-reports. Participants were presented with an ambiguous scenario such as “you suddenly wake up in the middle of the night, thinking you heard a noise, but all is quiet”. They were then asked to provide three possible answers to an open-ended question: “What could have caused you to wake up?”. Anxious and depressed participants were more inclined to interpret ambiguous scenarios as threatening. Next, participants were asked to score the likelihood of some events occurring. Those in negative states were more likely to assign a high likelihood score to negative events. Importantly, a higher likelihood of negative events was only assigned if the context of situations directly affected them. These results show that those in a negative state “choose” a more negative interpretation over a more positive one. This notion was further supported by later findings suggesting that individuals in negative states have access to both positive and negative interpretations, but they tend to select the interpretation that aligns with their current emotional state (French, 1992; Huppert *et al.*, 2007). What this suggests is that when in a negative emotional state, a more negative interpretation holds a greater expected utility either by being perceived as more likely to occur or as having a higher anticipated value. For example, negative emotions, such as fear, may not only increase the estimated likelihood of a negative outcome but also amplify the perception of the consequences as more negative (Maner and Gerend, 2007).

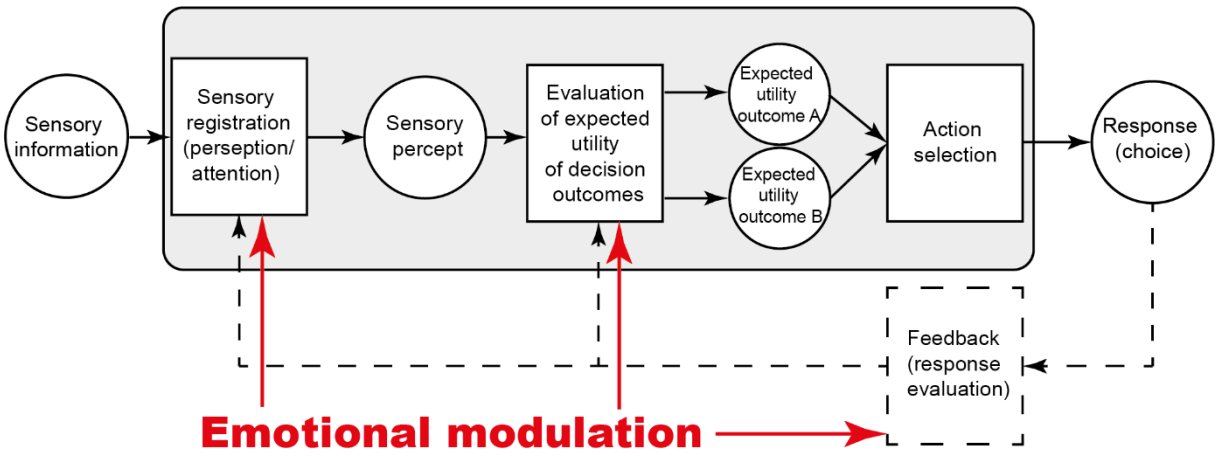

Figure 2. A simplified schematic of emotional modulation of decision-making stages. Incoming sensory information is registered through perceptual and attentional pathways. The sensory percept is then evaluated, and the expected utility of all potential outcomes is evaluated. This calculation considers the probability of each outcome and the anticipated value, or payoff, associated with each. Based on this evaluation, an action is taken, and the individual responds to the incoming signal. Emotions can impact any of these early or later processes. They can influence how the signal is perceived or attended to in the early stages, and they can also affect the estimation of the probability of an outcome occurring and the magnitude of payoffs associated with potential outcomes at later stages (figure adapted from Mendl *et al.*, 2009).

1.4. Measuring emotion across species

As stated previously, emotions are multicomponent states comprising of neurophysiological, behavioural, cognitive and subjective components. For instance, during a fear episode, several neurophysiological changes occur – the heart beats faster, breathing quickens, the eyes widen, facial expression, and posture change. Simultaneously, an overwhelming sense of terror spreads throughout the body, triggering a desire to either freeze or flee. Each of these changes serves a specific purpose. For example, the elevated heart rate and increased respiration enhance the oxygen supply to peripheral muscles improving the ability to flee. Meanwhile, pupil dilation enhances peripheral vision and, consequently, threat detection. Together, this cascade of coordinated changes in each of the emotional components enables an individual to successfully survive danger. Table 1 summarises all systems and their function that are involved in emotional response.

Table 1

The interconnection between emotional components, their functions, and the organism's subsystems (adapted from Scherer, 2005)

Emotion component	Emotion function	Subsystems within the organism and primary substrates
Cognitive (appraisal)	Evaluation of objects and events	Information processing (CNS)
Neurophysiological (bodily symptoms)	System regulation	Support (CNS, NES, ANS)
Behaviour (behaviour and motivations)	Preparation and direction of action	Executive (CNS)
	Communication of reaction and behavioural intention	Action (SNS)
Subjective (feelings)	Monitoring of internal state and organism-environment interaction	Monitoring (CNS)

CNS – central nervous system; NES – neuro-endocrine system; ANS – automatic nervous system; SMS somatic nervous system.

Each of the components, however, can and do act independently outside emotional experience. For instance, while fear causes elevated heart rate, so does digestion (Abramson and Sidney, 1941). Therefore, the key role of emotion lies in the coordination and synchronisation of all systems supporting physiological, neurological, behavioural and cognitive changes that together generate emotional response (Scherer, 2005). Studying how emotions are expressed through each of these components aids our understanding of emotion in animals (Paul, Harding and Mendl, 2005; Mendl, Oliver and Paul, 2010; Waal, 2011; Anderson and Adolphs, 2014; Bliss-moreau, 2017; Gygax, 2017; LeDoux and Hofmann, 2018; Paul and Mendl, 2018). In the following sections, I will first demonstrate the application of the multicomponent approach in studying emotions in mammals. Then, I will discuss the research conducted in invertebrates, being more dissimilar to humans. I will end by summarising what studies have been conducted in bees so far.

1.4.1. Measuring emotions in humans and mammals

Subjective approach

To date, the only methods allowing us to assess the subjective component of emotion is self-reports. These can take different forms – for instance, questionnaires, rating scales and interviews (Russell, 2003; Paul, Harding and Mendl, 2005). While these methods are perhaps most frequently used when it comes to determining emotional states in humans, they are also the most debated (Mayer, Salovey, & Caruso, 2008). This is because subjective experiences require introspection, and this capability is limited and varies greatly among individuals (Mayer, Salovey, & Caruso, 2008). The inability to recognise, describe or even process emotions in some individuals (i.e., those with alexithymia) may also skew results (Lane *et al.*, 1997). Furthermore, prior experiences, beliefs, episodic memory, culture and other aspects of rich human life also shape the self-assessment of emotional state (Robinson and Clore, 2002; Barrett, 2017). Therefore, the subjective approach may lack the desired objectivity. Moreover, this method is only accessible in humans making it unusable for study in other species.

Behavioural approach

Behaviour reflects how organisms engage with their surroundings. Therefore, observing emotion-induced changes in behaviour has proven to be a useful method of measuring emotions. For example, threat-induced freezing is a recognised negative emotional indicator in both humans and animals (Roelofs, 2017), while play behaviour is considered a sign of a positive state (Held and Špinka, 2011). More general behaviours such as those expressed in anticipation of reward/punishment can also be used (Spruijt, Bos and Pijlman, 2001).

Changes in some body parts can also be a useful measure. These could be, for example, facial expressions. Scientists developed a tool known as the Facial Action Coding System (FACS)(Ekman and Friesen, 1976) to identify basic emotions through facial expressions. FACS tracks and measures facial muscle movements based on identified pattern assigned specific emotion. Similar systems have also been developed for primates e.g. chimps (Vick *et al.*, 2007), orangutans (Caeiro *et al.*, 2013); also FACS-inspired the Grimace Scale for nonprimates including mice (Langford *et al.*,

2010) and pigs (di Giminiani *et al.*, 2016). Other body parts, such as ear position can also be used (Reefmann, Wechsler and Gygax, 2009; Reefmann *et al.*, 2012). Another popular behavioural indicator is vocalisation. Humans for instance, can identify emotion from brief vocalisation (Cowen *et al.*, 2019) whereas rats use vocalisations of different frequencies to communicate the valence of emotional experience with the environment (Brudzynski, 2009; Gloveli *et al.*, 2023). Given the social importance of vocalisation, these metrics can be a useful measure of emotion in different species.

Neurophysiological approaches

In mammals, emotional reactions are accompanied by physiological changes supported by the sympathetic and parasympathetic nervous systems. Measuring these changes such as heart rate, blood pressure, skin temperature or respiration is a common practice (Mauss and Robinson, 2009; Kreibig, 2010). Nevertheless, while physiological patterns have been identified for all basic emotions (Rainville *et al.*, 2006) they significantly overlap. The similarity in heart contractions, electrodermal activity and respiration can be observed not only in emotional states of the same valence, for example fear and anxiety, but also with emotions of opposite valence, such as happiness and excitement. Therefore, physiological measures are more likely to be related to the arousal of the state rather than valence and should be treated as non-specific indicators of emotion (Cacioppo *et al.*, 2000; Paul, Harding and Mendl, 2005).

Another impartment indicator of emotional states are biogenic amines. In mammals, dopamine, serotonin and noradrenaline are of particular interest given their role in valence-based decision-making (Lövheim, 2012; Kremer *et al.*, 2020). For example, dopamine plays a crucial part in reward processing (Bromberg-Martin, Matsumoto and Hikosaka, 2010), serotonin in how the brain appraises emotionally-valenced information (Harmer, 2008; Cowen and Browning, 2015), while noradrenaline is responsible for fight-or-flight regulation (Lövheim, 2012). Disruption in both dopaminergic and serotonergic systems is associated with emotion dysregulation not only in humans (Ruhé, Mason and Schene, 2007; Grace, 2016) but also in other animals. In rats for example, suppressed dopamine transmission to the nucleus accumbens results in increased anxiety and depression-like behaviours (Bahi and Dreyer, 2019) as does serotonin inhibition in sheep (Doyle *et al.*, 2011). While often measured separately it is important to stress that biogenic amines most likely work in

synergy to create a pathway supporting the efficient transmission of emotional information to various brain regions (Lövheim, 2012).

As emotions are brain-generated states, it is not surprising that numerous attempts have been made to try and map specific emotions to particular brain regions. In humans methods like brain imaging and electrophysiology are commonly used (Mauss and Robinson, 2009), while with less strict ethical regulation in other mammals more invasive techniques are employed, including brain lesions, electrical stimulation, single-cell recording, and others (Paul, Harding and Mendl, 2005). To date, several brain areas have been identified as involved in emotion (Paul, Harding and Mendl, 2005; Kremer *et al.*, 2020). However, attempts to detect emotion-specific neural correlates unfortunately fail to provide substantially consistent results (Barrett, 2012; Lindquist *et al.*, 2012).

Cognitive approaches

As I mentioned in earlier studies, emotions trigger various cognitive changes. Numerous tasks that require attention, memory, and judgment have been used to discover these changes.

Attention bias

Affect-congruent attention bias refers to the tendency of individuals in negative affective states such as fear or depression to exhibit heightened awareness of or increased attention towards novel or negative elements in their surroundings (Paul, Harding and Mendl, 2005). For example, in humans fear primes the processing of threatening information (Math and Mackin, 1998; Mogg and Bradley, 1998; Kindt and van den Hout, 2001; Bar-Haim *et al.*, 2007). Moreover, it also primes the specific region of the visual field previously occupied by emotional stimulus (Fox, 2002; Mogg and Bradley, 2002; Pourtois *et al.*, 2006). In mammals, attention bias has been demonstrated in sheep (Lee *et al.*, 2016; Monk *et al.*, 2018) and cattle (Lee *et al.*, 2018). When exposed to a threat-inducing stimulus, such as an image of a dog, animals that received treatment with anxiogenic drugs increase the time spent looking at the image of a dog. Other forms of attention, such as selective attention to novel

stimuli, are also utilised as emotional markers. For instance, as with humans, horses displaying signs of depression demonstrate deficits in selective attention to novel auditory stimuli (Rochais *et al.*, 2016). Although promising, these methods are not yet popular in animal studies (Jacob-Dazarola, Ortíz Nicolás and Cárdenas Bayona, 2016).

Memory bias

Another less popular method among animal researchers involves memory biases. This method is again inspired by human clinical research. Individuals experiencing sadness and depression tend to have an increased recall of memories that align with the negative valence of their emotional state (Kremer *et al.*, 2020). To date, there is only a handful of animal studies that applied these methods. For instance, whether such emotion-congruent memory recall is also present in rats has been investigated (Burman and Mendl, 2018). Rats were first trained to receive one food pellet in each arm of a radial arm maze. Subsequently, rats were subjected to forced-choice trials in which they encountered positive (12 food pellets), neutral (1 food pellet), or negative events (food pellets soaked in quinine). The hypothesis was that rats in a more positive affective state, such as those with higher social status, would demonstrate improved memory for arms associated with positive events compared to arms associated with negative events and vice versa. Memory performance was assessed based on the rats' approach and avoidance behaviours. While the experimental design was indeed promising, this study failed to demonstrate the effect of social status on memory recall. An earlier study, however, did demonstrate affect-congruent memory bias (Takatsu-Coleman *et al.*, 2013). In this study, mice that displayed depression-like behaviour induced by short-term social isolation were indeed better able to remember the arm of the plus-maze where they experienced a shock, compared to mice that were not socially isolated.

Judgment bias

Emotions can significantly affect our judgments – the process of evaluating information and making decisions based on that evaluation. When studying this phenomenon in humans, researchers often focus on how people interpret ambiguous situations and

anticipate future outcomes. What consistently emerges from these studies is that individuals in a more negative affective state such as anxiety or depression, tend to have a more pessimistic interpretation of otherwise ambiguous information (Hirsch *et al.*, 2016). The classic illustration of this state-dependent interpretation or judgment bias is captured by the question “Is the glass half empty or half full?”. Generally, people in a positive emotional state tend to make optimistic judgments about ambiguity (“glass half full”), while those in a negative emotional state tend to adopt a more pessimistic view (“glass half empty”). As a result, it is proposed that one’s emotional state can act as a valence-dependent predictor, similar to a Bayesian prior, influencing their interpretation of ambiguity and expectations about future outcomes when dealing with ambiguity (Mendl and Paul, 2020). For example, MacLeod and Byrne (1996) demonstrated how being in a negative or positive state influences people's future outlook (Macleod and Byrne, 1996). When participants were given a minute to generate as many future scenarios as possible individuals with anxiety or depression tendencies generated more negative and fewer positive future expectations compared to the control group. A similar pattern emerged in another study (Andersen, Spielman and Bargh, 1992). In this study participants with depression and a control group without depression were given prompts and asked to respond quickly with either “yes” or “no”. The prompts included statements such as “I will find a job” and “I will fall ill”. Consistent with previous research, individuals with depression were found to be more inclined to respond “yes” to negative prompts but not as much to positive prompts when compared to the non-depressed group. Furthermore, when responding, depressed individuals were much faster, indicating a more automatic response.

To investigate the possibility that animals could also display emotion-congruent judgement biases, Harding et al. (2004) developed a novel method based on tone discrimination. First, the authors conditioned rats to press a lever when they heard a tone associated with a reward (food pellet) and to withhold from pressing the lever when they heard another tone associated with punishment (white noise) (Harding, Paul and Mendl, 2004). In a subsequent test, animals were exposed to non-reinforced tones with frequencies between the two conditioned tones. The authors observed that rats exposed to “unpredictable” housing conditions (a manipulation predicted to induce a mild, depression-like state) took longer to respond to the positive tone and the tone of a frequency closer to the positive tone than rats with more regular housing conditions. They were also less likely to press the lever in response to these tones. The authors

concluded that rats in a negative affective state show diminishing anticipation of positive events resembling decreased optimism in depressed humans. These state-dependent shifts in responses to ambiguous information can be functionally defined as “optimistic” or “pessimistic” without suggesting that animals experience human-like optimism or pessimism (Bateson, 2016).

Since Harding et. al. (2004), the use of judgment bias tests to measure animal emotion has become increasingly popular. Judgment biases have been explored across modalities and involved different types of tasks. However, despite their popularity some findings have contradicted the general expectations (Lagisz *et al.*, 2020). Therefore, when designing experiments, it is important to consider non-emotional factors that can complicate later inference. It is therefore important to account for subjects’ activity or motivation levels (Mendl *et al.*, 2009, 2010), the duration of training (Roelofs *et al.*, 2016), and training process itself (Roelofs *et al.*, 2016), as all these factors may impact later result interpretation. Similarly, repeated exposure to test cues may diminish animal responses as they learn to associate such cues with no reward (Doyle *et al.*, 2010).

It is also important to note that the type of task itself can also contribute to the above issues. Most studies use go/no-go type of judgment bias tests. In this type of test, animals are trained to respond to positive stimuli (go) and withhold from responding to negative stimuli (no-go). However, as mentioned earlier, certain factors such as a lack of motivation or reduced activation levels can also lead to a lack of response. Importantly, this absence of response might then be mistakenly interpreted as a no-go response suggesting the presence of a judgment bias (Mendl *et al.*, 2009).

An alternative method, the active choice judgment bias test, can be used to address these issues. This approach ensures that animals exhibit the same behaviours (go) in response to both negative and positive stimuli. Therefore, it becomes possible to control for animals’ motivation and general activation, the reduced levels of which can manifest through choice omission. This particular type of judgment bias test has been previously utilised with birds (Matheson, Asher and Bateson, 2008; Brilot, Asher and Bateson, 2010), lab rodents (Brydges *et al.*, 2012; Novak *et al.*, 2016), monkeys (Pomerantz, Terkel and Suomi, 2012), and pigs (Murphy, Nordquist and van der Staay, 2013).

The development of an Active Choice type of Judgment Bias test for bumblebees is one of the aims of my thesis. Therefore, I delve further into the issues associated with go/no-go paradigm in Chapter Two.

1.4.2. Measuring emotion in invertebrates

Behavioural approaches

Although some behavioural measures involving the observation of specific body parts, such as facial expressions in mammals, are inaccessible in invertebrates due to obvious differences in morphology, using other measures can be an interesting avenue to explore. For instance, vocalisations imply the production of sound using a vocal cord, but insects also produce sounds with the help of different body parts. They use such acoustic communication to support different needs – for example, to attract mates, or avoid threats (Leonhardt *et al.*, 2016). Therefore, it would be interesting to investigate whether such acoustics could be used to measure emotions in invertebrates. After all, Charles Darwin was convinced that stridulation could indeed be one of the ways insects convey emotions (Darwin, 1998).

Some of the better-explored behavioural measures in invertebrates involve whole-body assessment. For example, in anticipation of punishment, invertebrates, like mammals, display adaptive behaviours to increase survival. All animals are hardwired to express these behaviours in response to naturally aversive stimuli, for example, electric shock or a shadow overhead. However, through experience, neutral stimuli can also elicit such a response. This is known as fear conditioning, a phenomenon well-documented in mammals (Ledoux, 2014). Carew and Kandel (1981) were the first to demonstrate this in invertebrates (Carew *et al.*, 1981). When exposed to noxious stimuli, like an electric shock, slugs demonstrate the defensive behaviour of head withdrawal. Following the pairing of an electric shock with a neutral stimulus, the authors were able to demonstrate the elicitation of the same defensive behaviour solely upon the presentation of the neutral stimuli. Thus, as in mammals, the conditioned fear response can be a useful measure of negative emotion in invertebrates.

The perceived threat activates an animal's fight-or-flight system which when activated, leads to initiating escape, freezing, or fighting. While all these action

tendencies are a natural and adaptive response to danger, if excessively expressed, they become traits linked to anxiety (Cisler *et al.*, 2010). Therefore, measuring, for example, the escape response can serve as a good metric to measure anxiety in animals. In mammals, this is done by using an elevated plus-maze (Walf and Frye, 2007). The maze consists of several arms: a well-lit open arm and a dark enclosed arm. When exposed to danger animals tend to seek shelter. Therefore, those with high anxiety typically avoid open arms and spend more time in the dark enclosed one. Using a similar apparatus, anxiety-like behaviour was also demonstrated in crayfish. Crayfish that experienced electric shocks (Fossat, Bacque-cazenave and Delbecque, 2014) or a decline in their social status (Bacqué-cazenave *et al.*, 2017) showed a higher preference for the dark arm. Importantly, this was not a conditioned response or response to immediate dangers. Instead, increased preference for the darker arm reflected previous negative experiences, and thus reflected the overall state of the animal.

In flies, *Drosophila melanogaster*, whole-body behaviour was also used to investigate the flexibility of their emotional responses. Most animals, including *Drosophila*, will display defence behaviours to overhead looming stimuli (Card, 2012; Pereira and Moita, 2016). Environmental factors, such as how close the predator is and the availability of escape routes, determine what this defence response will be (Schmidt *et al.*, 2008). Zacarias et al. (2018) conducted a series of studies exposing flies to an overhead looming stimulus (Zacarias *et al.*, 2018). In this study, a shift in behavioural response was observed over a period of time. Upon the initial repeated presentations of the looming threat, flies displayed the tendency to jump. However, as the stimulation continued, more flies shifted their behaviour from jumping to freezing. Gibson et al. (2015) demonstrated similar results. Here however, continuous exposure to looming stimuli not only intensified flies' defence responses but also interfered with another motivational behaviour, that of feeding. Another study showed an even more intricate mechanism underlying such switching in *Drosophila melanogaster*. Von Reyn et al. (2014) investigated what features associated with threat stimuli determine the type of escape response (Von Reyn *et al.*, 2014). When a predator approaches, flies are known to initiate either a quick but energetically costly escape or a slower energy-conserving response. The findings suggest that which of the two responses is initiated depends on the size of the threatening stimulus and its velocity. Short take-offs are initiated when the stimulus approaches fast and long ones if the stimulus approaches

slower. Although not explicitly discussed by the authors of the above studies it is intriguing to speculate that the reported change in flies' behaviour was driven by stimulus appraisal. As I discussed earlier, stimulus appraisals help determine the emotional response to stimuli (see 1.2.2. Theoretical approaches to emotion). In the given studies, two appraisals – relevance and certainty – could drive a change in flies' defence response. For example, continuous exposure to an overhead shadow (Gibson *et al.*, 2015; Zacarias *et al.*, 2018), or a larger and faster approach of the shadow (Von Reyn *et al.*, 2014) may lead to an appraisal of a threat stimulus as more relevant and perhaps more inevitable (certain). Therefore, flies switched from a more active defence strategy to a more energy-saving, passive one. Overall, these studies demonstrate a level of flexibility in the emotional response of invertebrates. Moreover, this flexibility is likely driven by stimulus appraisal.

A more long-term mood-like state has also been demonstrated in invertebrates. The inability to cope with stress, known as "learned helplessness", is associated with negative mood states, such as depression (Willner, 1986; Eisenstein and Carlson, 1997). The ability to cope with continuous stress was investigated in fruit flies, *Drosophila melanogaster* (Batsching, Wolf and Heisenberg, 2016). Two flies were placed in a dark chamber. Whenever the master fly paused to rest, both the master and yoked flies received an electric shock. Therefore, the master fly could control the delivery of a shock simply by avoiding pausing. At the same time, the yoked fly could not, and its action did not determine whether she would get shocked or not. When compared to the control group that never received an electric shock the master flies showed no difference in walking speed, number, and duration of pauses. The yoked flies however, demonstrated reduced activity in all parameters. Thus, the inability to control stress delivery resulted in reduced motivation due to the inability to cope with stress.

Neurophysiological responses

Invertebrate morphology greatly complicates the assessment of emotion-induced physiological responses e.g. heart rate. Neurochemical methods are, however, well explored and offer a promising opportunity for comparative studies. Using this approach, a degree of similarity in emotional regulation in both mammalian and invertebrate brains has been revealed. For instance, in mammals, drugs like diazepam,

reduce anxiety behaviour (Sigel and Baur, 1988). A similar effect was observed in *Drosophila* flies, where the administration of diazepam reduced anxiety-related defensive behaviours (Mohammad *et al.*, 2016). Similar anxiolytic effects were observed with another $GABA_A$-modulating drug chlordiazepoxide (Fossat, Bacque-cazenave and Delbecque, 2014), here the anxiety-like behaviour observed in crayfish exposed to electric shock or social harassment was eased with the administration of this drug. The anxiolytic effect of these drugs is linked to the modulation of $GABA_A$ receptors, which are evolutionary conserved (Robinson *et al.*, 1986).

Studies on biogenic amines in invertebrates, also point to the existence of commonalities in neurological pathways involved in emotional regulation across taxa. Both mammals and insects have the same neurotransmitters, dopamine and serotonin, unique to insect octopamine, on the other hand, is considered structural and functionally similar to mammalian noradrenaline (Roeder, 1999; Pflüger and Stevenson, 2005; Caveney *et al.*, 2006). In mammals, negative emotional states, such as anxiety are associated with lower serotonin (Ruhé, Mason and Schene, 2007; Doyle *et al.*, 2011) thus, some anxiety and depression medications have serotonin reabsorption inhibitors which ensure adequate levels of this amine in the synapses. One such drug is fluoxetine. The anxiolytic effect of this drug was shown in the crab *Pachygrapsus crassipes* (Hamilton *et al.*, 2016). In this study, fluoxetine administration led to a reduction in anxiety-like behaviours, as evidenced by the crabs' decreased time spent in the dark zone during light/dark preference assays, where increased time in the dark zone is typically associated with heightened anxiety. While this behavioural response is consistent with the drug's anxiolytic effects observed in humans, the specific neurochemical mechanisms driving these effects remain unclear.

In this overview, I only present a few instances where neuropharmacology has been utilised to address emotional "dysregulations" in invertebrates. Although this area of research is still relatively unexplored, the findings indicate a potential similarity in how the nervous systems of both invertebrates and mammals regulate emotions.

Cognitive approaches

To the best of my knowledge only the judgment bias paradigm has been used to cognitively assess emotion-like states in invertebrates. To date there are a total of five

independent studies using these tests in invertebrates, specifically insects, four of which were conducted in bees. As I dedicate a separate section for an in-depth discussion on what has been done in bees, here I will briefly cover how this paradigm was applied to another insect species, *Drosophila melanogaster*.

Judgment bias tests measure an animals' interpretation of ambiguous information. The underlying hypothesis is that such interpretation is state-dependent, pessimistic when in a negative state and optimistic when in a positive emotional state. To test this hypothesis *Drosophila* flies were first subjected to discrimination learning in a T-maze (Deakin *et al.*, 2018). Flies learned to approach the positive odour associated with a reward and avoid the negative odour associated with an electric shock. Next, flies were either shaken to induce a negative state or left unmanipulated. In a subsequent test, one arm was filled with plain air, while the alternative arm with an ambiguous odour (1:1 mixture of two conditioned odours). As compared to the control, shaken flies were less likely to approach ambiguous odours. Shaken flies thus perceived an ambiguous odour as similar to a conditioned odour that was previously associated with an electric shock and shaken flies chose not to approach it. It was therefore concluded that flies, like mammals, display a pessimistic bias when in a negative state.

1.4.3. Emotion-like states in bees

Arguably, the study that provided the first comprehensive evidence demonstrating an emotion-like state in insects was a study by Bateson et al. (2011). Moreover, it was the first time that the judgment bias test, originally introduced by Harding et al. (2004), was adopted and applied to insects. To do so, researchers used the classical olfactory proboscis extension paradigm (Bateson *et al.*, 2011). Honeybees, *Apis mellifera*, were first trained to extend their proboscis in response to a rewarding odour and withhold it for an unrewarding one. Next, half of the bees were subjected to vigorous shaking. Shaking imitated a predatory attack, thereby subjecting animals to a negative state. In the test, the extension of the proboscis to ambiguous odours (mixtures of two conditioned odours) was recorded. Remarkably, as compared to unmanipulated bees, bees that had been shaken before testing were more inclined to refrain from extending their proboscis upon the presentation of an ambiguous odour. Therefore, shaken bees

were more “pessimistic”. To further investigate the possible mechanism behind the observed judgment bias, Bateson et al. (2011) measured the levels of systemic biogenic amines in both shaken and control bees. They showed that shaking reduced the levels of dopamine, octopamine, and serotonin. Such reduced levels of biogenic amines are commonly associated with depression not only in humans (Luchins, 1976) but also in *Drosophila melanogaster* (Araujo *et al.*, 2018). These results, once again, point to the similarities in neurophysiological systems supporting emotional responses across taxa (more evidence was presented in earlier sections).

These behavioural results were replicated in another study. In their study, Schlüns et al. (2017) applied the same judgment bias test as before; the results confirmed that shaken honeybees indeed behave “pessimistically” (Schlüns *et al.*, 2017). In addition to replicating earlier studies, the authors also tested whether the same pessimistic bias would occur after exposing bees to formic acid, a standard treatment against bee pests. Unlike the shaken bees, those treated with formic acid did not show a difference in their response to ambiguous odour.

While the vast majority of judgment bias tests conducted in animals assessed the effects of negative states, only very few focused on positive states (Lagisz *et al.*, 2020). One such study was done on bumblebees, *Bombus terrestris*. Solvi et al. (2016) first trained bees to distinguish between artificial flowers of two colours, one associated with a sugar reward and the other with no reward (water)(Solvi, Baciadonna and Chittka, 2016). Bees, therefore, learned to approach rewarding and avoid unrewarding flowers. Before testing with ambiguous colours, half of the trained bees received a small droplet of high-concentration sugar reward. In humans, such unexpected snacks are known to induce positive emotions (Macht and Mueller, 2007). Interestingly, unexpected rewards also induced a positive state in bees. Unlike the control, bees that received a sugar droplet were quicker to approach flowers of ambiguous colours, indicating an “optimistic” bias. To complement these behavioural results, the authors also manipulated the levels of biogenic amines pharmacologically. To test what amines are involved in “optimistic” bias, bees were topically treated with receptor antagonists for dopamine, octopamine and serotonin. Blocking the dopamine receptor (but not the others) resulted in the elimination of “optimistic” bias, suggesting the importance of this amine in regulating positive emotion-like states in bees.

Cumulatively, the existing studies in social bees (Bateson *et al.*, 2011; Solvi, Baciadonna and Chittka, 2016; Schlüns *et al.*, 2017; Strang and Muth, 2023) indicate that their tendencies to interpret ambiguous information align with the valence of their current state. Studies also suggest that this process could be mediated through biogenic amines (Bateson *et al.*, 2011; Solvi, Baciadonna and Chittka, 2016). However, some researchers remain cautious of such interpretations. For example, when reviewing the work of Solvi et al. (2016), Baracchi et al. (2017) suggest that the observed "optimistic" bias stems from an increased motivation after receiving a high-sugar reward, and they also point out that antagonist used to block octopamine is not octopamine-specific (Baracchi, Lihoreau and Giurfa, 2017). An alternative interpretation of the results of Bateson et al. (2011) has also been put forward (Giurfa, 2013). In his review, Giurfa (2013) emphasises low discriminability between conditioned odours in the non-manipulated honeybees. Indeed, in the test, the control bees were well responsive to both conditioned odours, with a level of discriminability of approximately 35%. In the shaken group, however, discriminability increased to 65% (due to a reduced response to negative odour). Therefore, Giurfa (2013) suggests that the observed pessimistic bias could be a result of stressed bees simply becoming better at discriminating between two odours. Cumulatively, existing critiques of earlier work emphasise the necessity for an improved experimental design that could eliminate such alternative explanations. This highlights as I will argue later, the need to revisit judgment biases in bees.

1.5. Summary of aims

Affective states are internal "emotion-like" states of an organism, brought about by an appraisal of the environment that result in specific physiological, cognitive and behavioural responses. A growing number of studies show that these states can impact information processing leading to altered cognition and perception. Recently, the application of tests measuring behavioural responses that are known to be influenced by affect in humans has led to the discovery of similar affect-induced behaviours in several species, including bees. Earlier discoveries, showing that affective states can impact bees' decisions, indicate that even insects may have the capacity for emotion-like experiences. If so, emotions may be more phylogenetically

widespread than previously thought. While this idea is interesting, some remain cautious or unconvinced.

Less is known, however, about the mechanisms underlying affect-induced behavioural patterns. Affective states could, in principle, modulate several stages of decision-making, including the capture, coding and processing of a visual signal at the early stages and the later integration and interpretation of information. For example, negative emotions can facilitate the detection of an emotional signal (e.g. threat) by modulating visual acuity. Similarly, emotion could also modulate how new information is integrated and evaluated to form flexible future decisions. Affect therefore can be viewed as an adaptive mechanism promoting the allocation of cognitive and behavioural resources towards fitness-relevant stimuli.

Given this adaptive function of emotion, it is important to investigate if the same is true in insects. Therefore, the aim of my project is to provide the insights into whether and how emotion-like states in bees impact their visual decision-making. I hypothesise that affective states will modulate multiple stages of visual decision-making, including perception, cognitive flexibility and reward learning in a way that facilitates species survival. In addition, given the potential of using bees as a model for studying affect, it is essential to initiate the development of new methods for investigating neural structures in freely-moving animals. One such method could involve combining microinjections with behavioural assays within a virtual reality context. I aim to initiate the exploration of this method.

In summary, this thesis is dedicated to exploring the influence of affective states on decision-making in *Bombus terrestris*, the buff-tailed bumblebee. The specific aims of the thesis include:

1. Developing a novel judgment bias test utilising an active choice design and using it to reassess the existence of negative judgment biases in bees.

2. Assessing the impact of affective states on visual acuity in bees.

3. Investigating the influence of affective states on behavioural flexibility.

4. Evaluating the effects of microinjections on bee learning within a virtual reality setup.

CHAPTER TWO

Physically stressed bees expect less reward in an active choice judgment bias test

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2.1. Introduction

The presence of emotions in non-human animals is much debated and can have important societal implications. As pointed earlier (see 1.2. Conceptualising Emotion), most research on animal emotions has focused on vertebrates (Panksepp, 2011; Paul *et al.*, 2020), and only a handful of recent studies have explored analogous states in insects (Bateson *et al.*, 2011; Gibson *et al.*, 2015; Solvi, Baciadonna and Chittka, 2016; Schlüns *et al.*, 2017; Deakin *et al.*, 2018; Strang and Muth, 2023). In these studies, emotions are defined as valenced brain states triggered by both internal and external stimuli and comprise subjective, behavioural, physiological and cognitive components. Research on emotion-like states in insects has primarily relied on judgement bias tests, a method initially developed for assessing affective states in rats (Harding, Paul and Mendl, 2004), and discussed in earlier sections. In brief, these tests assess how animals respond to ambiguous stimuli. An animal typically is trained to associate one stimulus with a reward and another with a lack of reward or punishment. It is then tested with an ambiguous stimulus that is in-between the two learnt stimuli. Animals that respond as if this stimulus indicates a reward are considered optimistic, while those that respond as if the stimulus indicates a lack of reward or punishment are considered pessimistic.

Judgement bias tests have been used in five studies on insects, including on honeybees, bumblebees and fruit flies (Bateson *et al.*, 2011; Solvi, Baciadonna and Chittka, 2016; Schlüns *et al.*, 2017; Deakin *et al.*, 2018; Strang and Muth, 2023). Some of these studies showed that physical agitation can reduce the response of bees and flies to ambiguous odours (Bateson *et al.*, 2011; Schlüns *et al.*, 2017; Deakin *et al.*, 2018). Others showed that bees are quicker to fly towards (Solvi, Baciadonna and Chittka, 2016) and more likely to choose (Strang and Muth, 2023) ambiguous visual stimuli after encountering an unexpected reward of sucrose solution, suggesting optimistic behaviour. While these results parallel results from studies of emotions in vertebrates, other explanations have also been suggested, including changes in motivation or increased discrimination ability (Giurfa, 2013; Baracchi, Lihoreau and Giurfa, 2017).

The majority of insect studies to date have utilized go/no-go judgment bias tasks, with only one study (Deakin *et al.*, 2018) employing an alternative active choice approach, which will be discussed later. Here, the animal is trained to respond to a

positive stimulus (“go”) and suppresses the response to a negative one (“no-go”). When faced with an ambiguous stimulus, responding (“go”) or suppressing (“no-go”) a response is thought to reflect optimistic and pessimistic judgements, respectively. While very popular (Mendl *et al.*, 2009; Mendl, Oliver and Paul, 2010; Bethell, 2015), there are concerns associated with this paradigm. Firstly, the suppression of a response could result from a general reduction in activity and motivation rather than a judgment bias (Mendl *et al.*, 2009). A reduction of responses, therefore, could also indicate merely an absence of response (omission) rather than a deliberate choice (Enkel *et al.*, 2010; Baciadonna and McElligott, 2015). Moreover, the animal may fail to attend or detect a stimulus, especially when stressed. This would lead to a failure to respond, which can be mistakenly attributed to a pessimistic judgment (Bethell, 2015; Jones *et al.*, 2018). Without a test that can address these issues, we currently do not have strong evidence of emotion-like states in insects. In addition, we lack models for the mechanisms underpinning the observed behaviours – though recent work has proposed that judgement biases in bees can arise from shifts in stimulus-response curves (Strang and Muth, 2023).

One way of reducing the likelihood of confounds is to use an active choice judgment bias test (Matheson, Asher and Bateson, 2008; Enkel *et al.*, 2010; Whittaker and Barker, 2020). Unlike go/no-go tasks, the active choice paradigm requires the animal to make an active choice between two alternative responses. Such behaviour may be, for example, press the left key upon the presentation of a negative cue, and press the right key upon the presentation of a positive cue. Both actions are behaviourally comparable. In the test, to determine the state of an animal we make an inference based on which response it chooses. Therefore, employing an active choice approach can help eliminate possible confounds associated with go/no-go paradigm.

I therefore used an active choice type of judgment bias test to rigorously assess judgement biases in bumblebees *(Bombus terrestris)*. Bees had to choose between two rewarding locations depending on the stimulus displayed, clearly signalling their judgement when faced with ambiguous stimuli by moving to one of the two locations. To induce negative affective states, I used two types of manipulations simulating predatory attacks – shaking and trapping by a robotic arm. These manipulations have previously been shown to be associated with cognitive and physiological changes (Davenport and Evans, 1984; Chen, Hung and Yang, 2008; Bateson *et al.*, 2011). In addition, to further understand the mechanisms underlying our behavioural results, a

signal detection modelling framework was applied to behavioural data. Specifically, the framework was employed to test whether physical agitation affected the prior expectation of a reward in bees or their ability to distinguish between stimuli due to shifts in stimulus-response curves.

2.2. Materials and Methods

Animals and experimental set-up

All experiments were run on female worker bumblebees (*Bombus terrestris*) obtained from a commercial supplier (Koppert, UK). I transferred the bumblebees to one chamber of a bipartite plastic nest box (28.0 × 16.0 × 12.0 cm). The nest box was connected via a transparent acrylic tunnel (56.0 × 5.0 × 5.0 cm) to a flight arena (110.0 × 61.0 × 40.0 cm) with a UV-transparent Plexiglas® lid and lit by a lamp (HF-P 1 14-35 TL5 ballast, Philips, The Netherlands) fitted with daylight fluorescent tubes (Osram, Germany). When not part of an experiment, bees were fed with ~ 3 g of commercial pollen daily (Koppert B. V., The Netherlands) and provided sucrose solution (20% w/w) *ad libitum*. Although invertebrates do not fall under the Animals (Scientific Procedures) Act, 1986 (ASPA), the experimental design and protocols were developed incorporating the 3Rs principles. Housing, maintenance, and experimental procedures were non-invasive and were kept as close as possible to the natural living conditions of the animals.

Visual stimuli were solid colours covering the entire display of an LED monitor (Dell U2412M, 24", 1920 x 1200 px) and were controlled by a custom-written MATLAB script (MathWorks Inc., Natick, MA, USA) using the PsychToolbox package (Brainard, 1997). I measured the spectral reflectance of all colours used in the experiment using an Ocean Optics Flame reflectance spectrophotometer (Ocean Optics Inc., Florida, USA). The perceptual positions of the colours in the bee colour hexagon space (Fig.1B) were calculated using the spectral reflectance measurements and spectral sensitivity functions for *Bombus terrestris* photoreceptors (Chittka, 1992; Skorupski, Döring and Chittka, 2007).

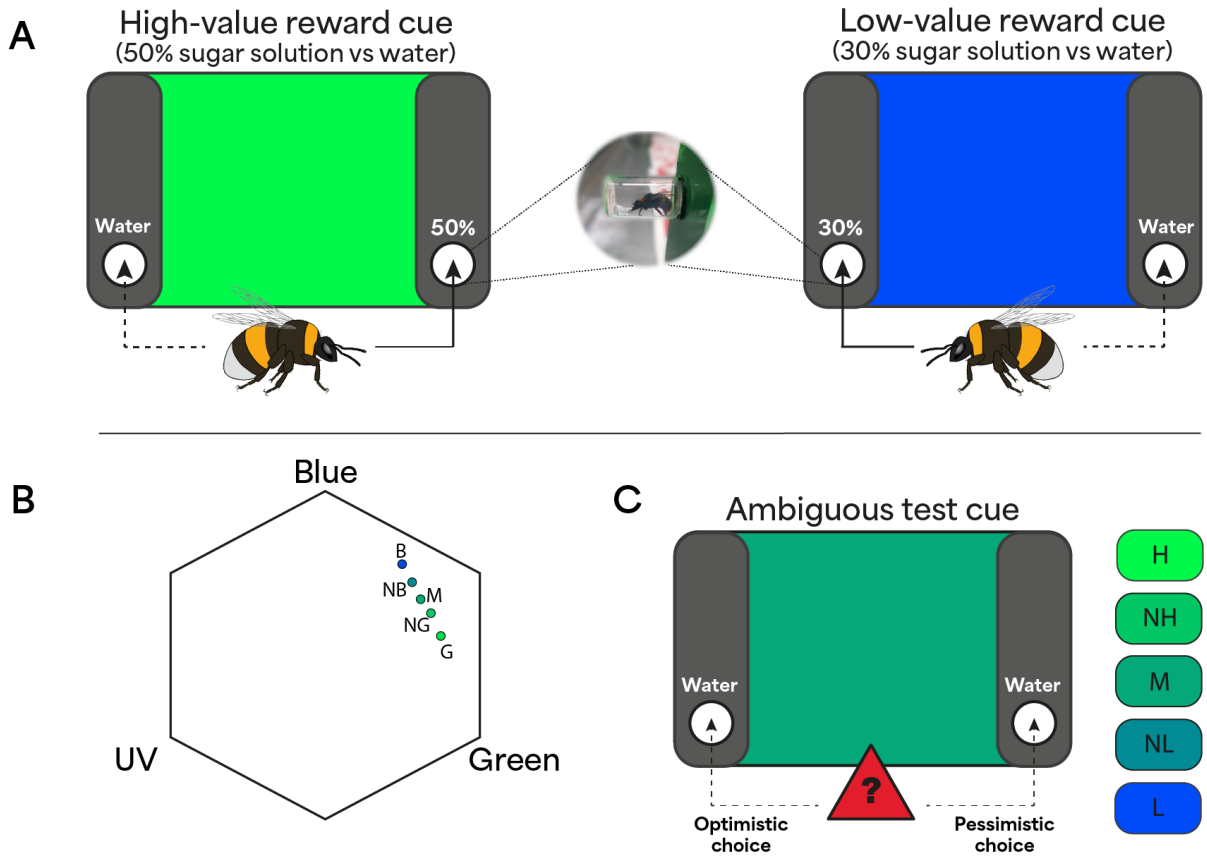

Figure 1. Experimental Protocol. A) Training phase. Bees were trained to associate two colours, green and blue, presented on an LED screen with different sugar rewards at different locations. The bees were presented one colour at a time in pseudorandomized order. The figure depicts a training scenario with green associated with a high reward (50% sucrose solution) in the right chamber and blue with a low reward (30% sucrose solution) in the left chamber. The association between colour, reward and location was counterbalanced across trials. Further details in the text. **B)** Cue colours plotted in bee colour space (colour cue: B, blue; NB, near blue; M, medium; NG, near green; G, green). The three vertices correspond to maximum excitation of photoreceptors sensitive to blue, green and ultraviolet (UV) light. The distance from the centre to any vertex is 1 and the distance between points represents hue discriminability, with 0.1 being easily distinguishable. **C)** Judgement bias testing. The test phase consisted of five trials with different colours presented on the screen in a pseudorandomized order (cue value: H, high; NH, near high; M, medium; NL, near low; L, low). The colours included the two conditioned colours and three ambiguous colours of intermediate value. In our example here, the screen shows the medium colour with blue as the low-reward colour (H) and green as the high-reward colour (L), but this was counterbalanced across bees. Entering a chamber associated with a high reward during training was considered an optimistic choice, while entering a chamber associated with a low reward during training was deemed a pessimistic choice.

I positioned two vertical panels (40.0 × 8.0 cm) 8.5 cm in front of the righthand and lefthand sides of the LED monitor, leaving the central area of the monitor open and visible. Each panel was equipped with an opening to place a reward chamber (7 ml glass vial, 10 mm inner diameter) 7 cm above the arena floor. Bees thus needed to fly from the arena entrance to the panels before entering the reward chamber. On each visit to the arena, the reward chambers were changed to ensure that pheromones and scent marks were not available during the next visit. In preparation for the next experimental day, all used chambers were washed in 70% ethanol and hot water and left to dry.

Training procedure

Before the onset of training, bees were familiarised with both reward locations. A plastic cup was used to gently capture each bee. The opening of the cup was positioned so that it aligned with the entrance of the reward chamber, inside which the bee found a droplet of sucrose solution (0.2 ml, 30% w/w). I repeated the procedure equally on each side (left and right) without displaying any colour on the LED screen. Individual bees that learnt the location of the reward and performed repeated foraging bouts were tagged for later identification using number tags (Thorne, UK). The process of tagging entailed placing every bee inside a marking cage, using a sponge to gently press it up against the mesh, then attaching the tag to the thorax with a drop of superglue (Loctite Super Glue Power Gel).

In each training trial, I presented bees (n = 48) with one of two colours on the LED screen. The two colours used were green (RGB= 0, 255, 75) and blue (RGB= 0, 75, 225). When one of the colours was displayed, the bee was provided a high-value reward of 0.2 ml 50% (w/w) sucrose solution in one of the two chambers (e.g., on the left), and an equal amount of distilled water in the other chamber (e.g., on the right). In different trials, when the other colour was displayed the bee was provided a low-value reward of 0.2 ml 30% (w/w) sucrose solution in the chamber opposite (e.g., on the right) to the one where, in the other trials, a high-reward was presented. Here again, an equal amount of distilled water would be present in the other chamber (e.g., on the left). Thus, on any given trial, the bee saw only one colour and could encounter either the high or low reward (not both), with water on the unrewarding side. In addition, the locations of the high and low rewards were on opposite sides in their respective trials.

Across bees, the combinations of each colour (green or blue), reward location (right or left) and reward type (high or low) were counterbalanced. Each bee encountered only one possible combination of each during training (e.g., green indicating a high reward on the left on half the trials, and blue indicating a low reward on the right on the other half). Trials presenting colours associated with high and low rewards were presented an equal number of times in a pseudorandom order, ensuring that no colour was repeated more than twice in a row. To ensure that the bee entered the reward chamber fully to sample its content, we placed the droplets of solutions at the very end of the reward chamber (Fig. 1A). In all cases, the reward quantity of 0.2 ml allowed bees to fill their crop within a single reward chamber visit (Pattrick *et al.*, 2020). I recorded a single choice on each trial, with a choice defined as a bee entering a chamber far enough to sample its content. Incidences of landing or partial entering (less than 1/3 of the body length) were not considered choices. Bees that reached the learning criterion (80% accuracy in the last 20 trials) continued to the test phase. 11 bees did not pass the initial conditioning test due to strong side biases. The last ten training trials were video recorded using a camera on a mobile phone (Huawei Nexus 6P phone 1440 × 2560 px, 120 fps) placed above the arena.

Predatory attack simulation

I randomly assigned individual bees (n=48 from six different colonies) that reached the learning criterion in the training phase to one of the three treatment groups. Two groups were subjected to manipulations which simulated predatory attacks and were predicted to change their affective state (Bateson *et al.*, 2011). One of these two treatments involved shaking the bee on a Vortex shaker (*Shaking*, n=16), while the other involved trapping the bee with a custom-made trapping device (*Trapping*, n=16). A third unmanipulated group served as a control (*Control*, n=16). The manipulations were applied to a bee before entering the arena for each test. Each bee in the Shaking treatment was allowed to enter a custom-made cylindrical cage (40 mm diameter, 7.5 cm length). After entering, the bee was gently nudged down with a soft foam plunger until the distance between the plunger and the bottom of the cage was reduced to ~3 cm. Once the plunger was secured, the cage with the bee was placed on a Vortex-T Genie 2 shaker (Scientific Industries, USA) and shaken at a frequency of 1200 rpm for 60 s. After shaking, the bee was released into the tunnel connecting the nest box and

experimental arena via an opening on the top of the tunnel. The bee was shaken before each test trial and released into the flight arena as soon as it was ready to initiate a foraging bout.

Each bee in the Trapping treatment was trapped using a trapping device. This consisted of a soft sponge (3.5 × 3.5 × 3.5 cm) connected to a linear actuator system (rack and pinion). A micro-servo initiated the linear motion of the trapping device (Micro Servo 9g, DF9GMS), powered, and was controlled by a microcontroller board (Arduino, Uno Rev 3). A custom-written script written in the Arduino Software (IDE) triggered an initial plunging movement of the trapping device, followed by release after three seconds. This permitted consistent trapping across all tested individuals. As in the Shaking treatment, the bee was trapped before each test trial and released into the flight arena for testing as soon as it was ready to initiate a foraging bout.

Bees in the Control treatment were allowed to fly out into the flight arena without hindrance as in the training phase.

Final sample size calculation

To determine the final sample size needed, we used a Bayes Factor approach implemented with the brms package in R (Bürkner, 2017; München *et al.*, 2017; Moerbeek, 2021). Prior beliefs about the parameters were specified using a normal distribution with mean 0 and standard deviation 1. Data collection was stopped when the Bayes Factor ≥ 3 (indicating moderate support for H_A (Moerbeek, 2021).

Judgement bias testing

The test phase consisted of five trials, each with a cue of a different colour presented on the screen. The test colours were the two conditioned colours (green and blue), and three ambiguous colours of intermediate value between the two conditioned colours (near blue (RGB=0, 140, 150); medium (RGB= 0, 170, 120); near green (RGB= 0, 200, 100) (Fig. 1B). We classified the ambiguous colours as near-high, medium, and near-low cues depending on their distance to the high or low rewarding colour for each bee. The colour presentation order was pseudorandomized between all bees, so that the

first test colour was always one of the three ambiguous colour cues. Within the test phase, all colour cues (ambiguous and learnt) were not rewarded, i.e., both chambers contained 0.2 ml of distilled water. We classified the entry of a bee into a reward chamber as a choice. After it made the first choice, we gently captured the bee with a plastic cup and returned it to the tunnel connecting the nest and the arena. Between presentations of each of the five test cues, bees were provided refresher trials consisting of two presentations of each conditioned colour with the appropriate reward at the correct location. All trials were video recorded for later video analysis using the camera of a mobile phone (Huawei Nexus 6P, 1440 × 2560 px, 120 fps). We obtained the latencies for the choices from the video analysis.

Measuring foraging motivation using ingestion rate

To assess if our manipulations changed feeding motivation in bees, I measured sugar reward ingestion rates. A separate group of bees (n=36 from six colonies) were pre-trained to forage of an elevated feeder consisting of the reward chamber used above with a 1.5 mL Eppendorf placed inside. After learning this location and completing five consecutive foraging bouts, bees were randomly allocated to one of three treatment groups as in the above experiment for the ingestion test (*Control*: n=12, *Shaking*: n=12, *Trapping*: n=12). The test consisted of a single foraging bout on a feeder with sucrose solution (~1 ml, 50% w/w). The feeder was weighed before and immediately after the test bout to determine the mass of ingested solution using a Kern Weighing Scale ADB100-4 (Resolution: mg±0.001, Kern & Sohn, Balingen, Germany). The feeding bouts were recorded using a mobile phone camera (Huawei Nexus 6P, 1440 × 2560 px, 120 fps). The recordings were used to determine the time taken for ingestion. Ingestion time was defined as the time from when the bee first touched the sucrose solution with its proboscis until the bee stopped drinking. For each bee, I calculated the absolute ingestion rate i (mg s^{-1}):

$$i = (m1 - m2)/t$$

where i is the absolute ingestion rate of a bee, *m1* is the mass of the feeder before the foraging bout, *m2* is the mass of the feeder after the foraging bout, and *t* is the ingestion time of the bee. Upon the completion of the test, the bee was sacrificed by freezing and stored in 70% ethanol at -20°C. We measured the intertegular distance

(the width between the wing bases on the dorsal side of the thorax, ITD) and the length of the glossa (nectar-gathering tongue) of each bee with a digital calliper (RS PRO Digital Caliper, 0.01 mm ± 0.03 mm) under a dissecting microscope. We then adjusted the absolute ingestion rate i to account for individual size variability using the formula:

$$I = iW^{1/3}\,G \text{ (Harder, 1983),}$$

where i is the absolute ingestion rate of a bee, G is the length of the glossa and W is the intertegular distance. This is an adaptation of the formula developed earlier (Harder, 1983) with intertegular distance instead of weight, as it has been shown to be precise at estimating bumblebee weights (Hagen and Dupont, 2013).

To control for evaporation, we located an additional Eppendorf with 50% sugar solution on the opposite side of the test chamber and recorded its weight pre-and post-test for an individual bee. This loss of mass due to evaporation was subtracted from the mass of the test feed after the foraging bout.

Video analysis

Video analysis was done using BORIS© (Behavior Observation Research Interactive Software, version 7.10.2107 (Friard and Gamba, 2016). In the judgment bias experiment, we coded two behaviours for each bee. The first behaviour, “Choice”, indicated bee entry into a reward chamber and was classified as a point event, an event which happen at a single point in time. The second coded behaviour, “Latency to choose”, was the time of making the choice and was classified as a state event, i.e., an ongoing event with a duration. For the foraging motivation experiment, we coded a single behaviour, “Drinking duration”, which was classified as a state event that indicated ingestion time.

Statistical analysis

My hypothesis and statistical analyses of the main active choice experiment were preregistered at aspredicted.com (#62198). The data were plotted and analysed using RStudio v.3.2.2 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org) and custom-written scripts. All subsequent statistical models

for the data were fit by maximum likelihood estimation and, when necessary, optimised with the iterative algorithms BOBYQA. In each analysis, several models were run and compared using the model.sel function in the MuMIn package (Barton, 2023) to select the most appropriate model based on the Akaike information criterion (AIC) scores. I considered the model with the lowest AIC score the best model, i.e., the model that provides a satisfactory explanation of the variation in the data (Johnson and Omland, 2004). Following accepted convention, models with an AIC difference of less than 2 units were considered not significantly better than the model it is being compared to (Burnham and Anderson, 2004). In such case, anova() was used to determine whether adding interaction term significantly improved model fit. I used the package DHARMa (Hartig, 2020) for residual testing of all models.

For the judgment bias analysis, I used the probability of an optimistic choice as the dependent variable, coding choices of reward chambers previously associated with high-value and low -value cues as 1 and 0 respectively. I fit a generalized linear mixed-effect model (GLMM) using the *glmer* function of the *lme4* package with binomial errors and a logit link function (Bates *et al.*, 2015). The explanatory variables included in the model were "*Treatment*" (categorical: *Control*, *Shaken*, *Trapped*) and "*Cue*" (continuous: 1-5, where 1 = high and 5 = low value cue) which refers to the colour displayed on the screen. The identity of the bee ("*ID*") was included as a random intercept variable.

For the analysis of the choice latency in the judgment bias test, I fit a linear mixed-effect model (LMEM) using the *lmer* function of the *lme4* package (Bates *et al.*, 2015). To normalise the error distribution, latency data were natural log-transformed and latencies greater than 1.5 times the Inter Quartile Range were excluded (Hubert and Van Der Veeken, 2008). The explanatory variables included in the model were "*Treatment*" (categorical: *Control*, *Shaken*, *Trapped*) and "*Cue*" (continuous: 1-5, where 1 = high and 5 = low value cue). In addition, since we expected that optimistic responses would be faster, I also included "*Response Type*" (coded as 1 for optimistic responses, and 0 for pessimistic responses) as an explanatory variable in the model selection process. Bee identity ("*ID*") was included as a random intercept variable.

In addition to the above models, I ran other statistical tests for some analyses. Data for these tests were first tested for normality and the appropriate tests were subsequently employed for analysis. I ran a one-way ANOVA on the adjusted body size

ingestion rate data (as described in the SI) to test for differences between treatment (Control, Shaking, Trapping). I also used Kruskal-Wallis tests to compare the average number of trials to the criterion in the training phase for different treatment groups, and to investigate the potential impact of the side and colour associated with a high-value cue on learning.

Signal Detection Theory model[1]

We examined whether the behaviour of the bees could be modelled with standard signal detection theory, and what could then be inferred about the underlying mechanisms. We assumed that bees learn to make their foraging decisions during training based on the value of an internal signal that is affected by noise. When this signal exceeds an internal decision boundary, the bees behave appropriately for the low reward situation and when it is less than the boundary, they behave appropriately for the high reward situation. We modelled the distribution of the noisy signal and derived the probability of an optimistic response.

We assumed that bees learn to make their foraging decision during training based on the value of an internal signal x which indicates whether they are in a high or low reward situation. We specified x as a "low reward signal" which has a high value when the cue indicates a low reward. We assumed that bees have some internal decision boundary B, such that when $x>B$, they behave appropriately for the low-reward situation, and conversely when $x<B$ for the high-reward. Although on average the value of x reflects the cue, it is affected by noise, explaining why bees do not always make the same decision in the same experimental situation.

Since we have fitted our data with a logistic link function, we modelled the distribution of the noisy signal as the first derivative of a logistic function. The standard logistic is

$$F(x) = \frac{1}{1 + \exp(-x)}$$

[1] The signal detection modelling described here was carried by Prof Jenny Read at Newcastle University

Equation 1

and its first derivative is

$$f(x) = \frac{dF}{\mathrm{dx}} = \frac{\exp(x)}{[1 + \exp(x)]^2}$$

Equation 2

which is therefore the distribution we assume for our noise. This closely resembles a Gaussian distribution with the same standard deviation but has more weight both at the centre and at the tails.

The probability density function governing the distribution of the signal *x* is $\frac{1}{\sigma} f\left(\frac{x-C}{\sigma}\right)$, where *C* represents the value of the cue and s is the noise. The probability of an optimistic response on any given trial is the probability that the value of *x* on this trial is less than the decision boundary *B*, given the value of the cue on this trial. This is

$$P_{opt} = \int_{-\infty}^{B} dx \frac{1}{\sigma} f\left(\frac{x - C}{\sigma}\right) = F\left(\frac{B - C}{\sigma}\right)$$

Equation 3

The bee's behaviour is thus influenced by the noise s and the decision boundary *B*. The noise may vary depending on factors like fatigue or attention, while the decision boundary may reflect a cognitive strategy. A common assumption is that the decision boundary is chosen so as to maximise expected reward.

During training, the expected reward is

$$< R > \; = P_{Hi} R_{Hi} F\left(\frac{B - C_{Hi}}{\sigma}\right) + P_{Hi} W\left[1 - F\left(\frac{B - C_{Hi}}{\sigma}\right)\right] + P_{Lo} R_{Lo}\left[1 - F\left(\frac{B - C_{Lo}}{\sigma}\right)\right] + P_{Lo} W F\left(\frac{B - C_{Lo}}{\sigma}\right)$$

Equation 4

where P_{H} and P_{Lo} represent the probabilities that a given trial offers high or low rewards, R_{H} and R_{Lo} represent the utility to the bee of the 50% and 30% sucrose offered

on high or low trials, and *W* represents the utility of the water obtained when the bee makes the wrong choice.

The optimal boundary B_{opt} , that maximises the expected reward then satisfies the equation

$$P_{Hi}(R_{Hi} - W)f\left(\frac{B_{opt} - C_{Hi}}{\sigma}\right) = P_{Lo}(R_{Lo} - W)f\left(\frac{B_{opt} - C_{Lo}}{\sigma}\right)$$

Equation 5

(found by taking the derivative of the expected reward, Equation 4, with respect to *B* and finding where this is equal to 0). Note that it is possible that the bee is not maximising expected reward itself, but some transform of the reward (e.g., reward squared). Since our model has only two values for reward (High and Low), we can still represent any transform as two values (R_{Hi} and R_{Lo}) and the model would not be affected by non-linear transforms.

Equation 5 has a simple graphical interpretation. First, the probability distributions for high and low reward are rescaled by their prior probability and by the additional utility of getting the trial right, compared to the water available with the wrong decision. Then, the optimal boundary is where these rescaled distributions cross over (Fig. 4). If the priors and reward utilities were equal, i.e. $P_{Hi}(R_{Hi} - W) = P_{Lo}(R_{Lo} - W)$, then the optimal decision boundary would be exactly in the middle between the two cues values: $B_{opt} = 0.5(C_{Hi} + C_{Lo})$. If the boundary was shifted to the right or left of the middle, this would indicate optimistic or pessimistic behaviour.

We fit this model to our data and obtained the decision boundary and the noise for an optimal response given the reward values we used. We compared this decision boundary to the middle value of our response variable. If the boundary was shifted to the right or left of the middle, this would indicate optimistic or pessimistic behaviour respectively.

2.3. Results

Bumblebees were trained to associate cues of one colour with a location containing a high reward of 50% sucrose solution and cues of another colour with another location

containing lower reward of 30% sucrose solution. The association of rewards with the cue colours and the locations were counterbalanced across all the bees. Bees then experienced one of three treatment conditions. Two groups of bees were physically stressed by shaking or trapping, while the third group served as a control. I then presented the bees with cues of ambiguous colours between the two learnt colours in tests and noted whether they chose the location previously associated with high or lower rewards. I also presented the bee with the cues of the learnt colours during the tests and noted their choices. All the tests were unrewarded and only offered distilled water in the previously rewarding locations.

Training

During training, a total of 48 bumblebees achieved the learning criterion (80% correct on the last 20 choices) and continued to the judgment bias test. Bees completed training within a minimum of 30 and a maximum of 60 trials. There were no significant differences in the number of trials required to reach the criterion among bees that experienced the high reward on the right or left location (Kruskal-Wallis test: $\chi^2 = 2.94$, df = 1, p = 0.09). Similarly, there was no difference in the total number of trials to criterion for bees that experienced blue or green as the high reward colour (Kruskal-Wallis test: $\chi^2 = 0.94$, df = 1, p = 0.33). The number of trials required to achieve the learning criterion also did not differ among bees used in each of the three treatment groups (Kruskal-Wallis test: $\chi^2 = 0.88$, df = 2, p = 0.64).

In the last set of trials in the training phase, bees took longer to make a choice in trials with a low-reward compared to those with a high-reward cue (Appendix Table 2, LMEM, Estimate ± standard error = 0.59±0.09, $t = 6.79$, $p < 0.001$). The median latency for choosing in low reward cue trials was 32.2 s (IQR: 35.8), and 17.3 s (IQR: 7.34) for the high reward cue trials. Thus, bees could differentiate between both the colour cues and the two rewards.

Physically stressed bees are less optimistic

The best model for my data included the main effects of cue colour and treatment (shaking, trapping and control) but not an interaction effect (see supplementary

Appendix Table 1 for model selection details). Shaking significantly reduced the probability of bees responding optimistically, i.e., choosing the location associated with a high reward (Fig. 2A, Appendix Table 2, GLMM, Estimate ± standard error = -1.49 ± 0.57, z = -2.61, $p < 0.01$). Trapping with a robotic arm also significantly reduced the likelihood of an optimistic response (Fig. 2A, Appendix Table 2, GLMM, Estimate ± standard error = -1.26 ± 0.56, z = -2.23, $p = 0.026$). Bees were also significantly less likely to respond optimistically to cues with colours further away from that of the high reward cue (Fig. 2A, Appendix Table 2, GLMM, Estimate ± standard error = -1.79 ± 0.21, z = -8.39, $p < 0.001$).

Feeding motivation and choice latencies

I examined the change in latency to make a choice in the testing phase. The model that included Cue, Response, Treatment, and their interaction had the lowest AIC (234.6447). The closest competing model, which excluded Treatment, had a slightly higher AIC (235.4639). With a delta AIC of 0.8192, indicating no strong support for one model over the other. The ANOVA comparison shows that adding Treatment to the model does not provide a statistically significant improvement ($\chi^2(2) = 4.82$, p = 0.09). The best-fitting model, therefore, included cue value and response type (optimistic or pessimistic) as fixed predictors and an interaction between them (Appendix Table 1). Including Treatment as an explanatory factor did not improve model fit, suggesting that the treatment group had limited power in explaining choice latency. All bees were significantly slower to make a choice when the cue colour was further away from that of the high reward cue (LMEM, Estimate ± standard error = -0.09 ± 0.03, t value = -2.9, p = 0.0139). Additionally, bees were faster when making optimistic choices compared to pessimistic ones (LMEM, Model Estimate ± standard error = -0.91 ± 0.16, t = -5.6, p < 0.001). Importantly, the interaction between Cue and Response was significant, indicating that the effect of the cue on latency differed depending on whether the response was optimistic or pessimistic (LMEM, Estimate ± standard error = 0.26 ± 0.05, p < 0.001).

I also tested the ingestion rate of sucrose solution as a measure of the feeding motivation of the bees. The mean (± s.d.) ingestion rate by shaken and trapped bees was 3.42 ± 0.67 mg/s, and 3.17 ± 0.61 mg/s respectively. These rates were not

significantly different from control bees (Fig. 2C, ANOVA: F(2, 33) = 0.642, p = 0.533), whose average ingestion rate was 3.17 ± 0.55 mg/s.

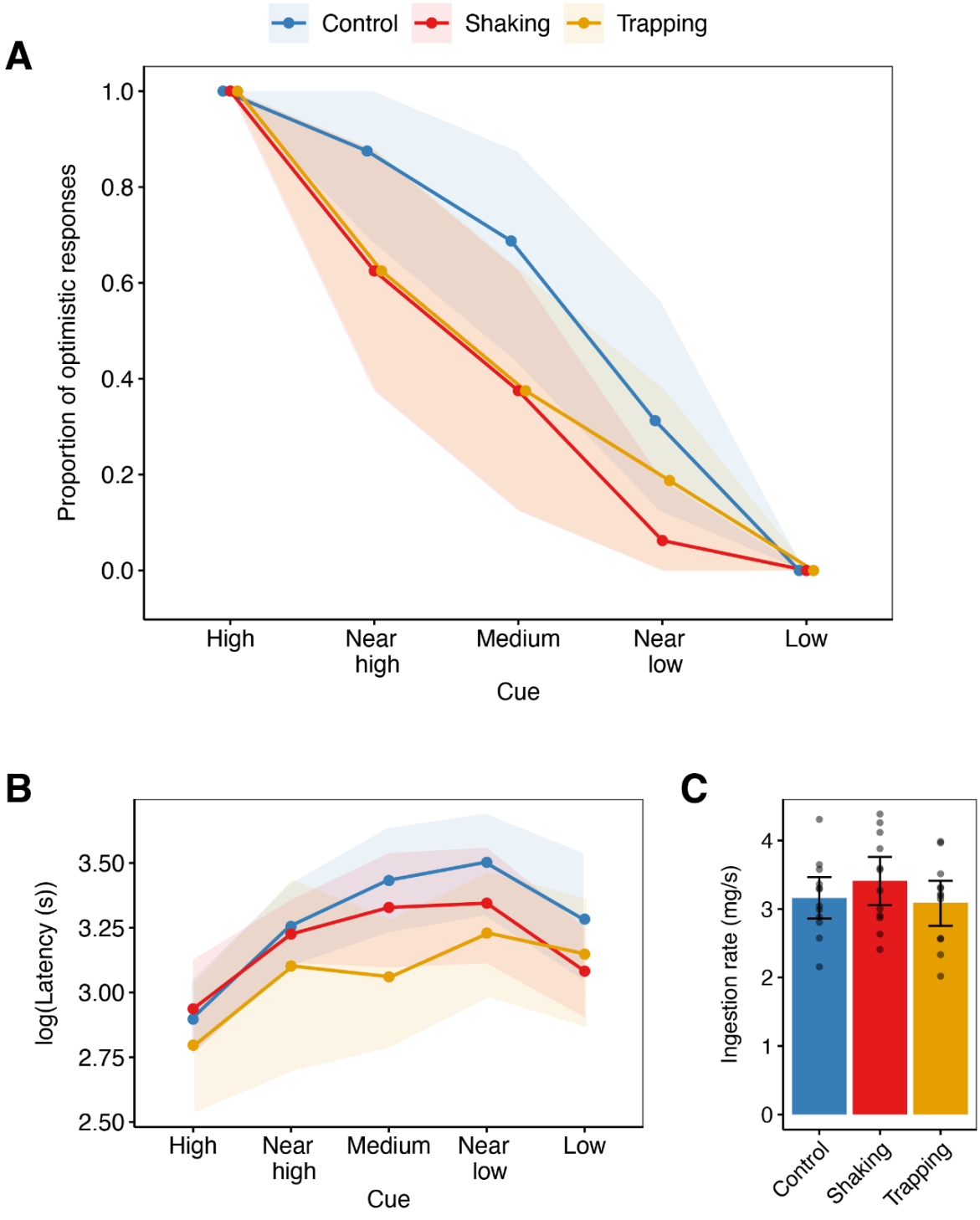

Figure 2. Bee responses to test cues. A) Proportion of bees (N = 16 per treatment) making an optimistic choice (choosing a reward chamber associated with a high reward) in response to each of

five cues. **B**) Latency of making the choice in response to each of five cue values (N = 16 bees per treatment). **C**) Average ingestion rate of high reward (50% sugar solution) for bees in each treatment group (N = 12 bees per treatment). The treatment groups were control (blue), shaking (red), and trapping (orange). The test cues were high, near high, medium, near low, and low value cues depending on their distance to the colours of high- and low-reward cues. Points and bars represent means, and the shaded areas and error bars represent 95% bootstrapped confidence intervals. Grey dots in panel C represent values form individual bees.

Signal-detection theory model

According to a standard signal-detection theoretic approach, the probability that a bee makes an optimistic choice for Cue level *C* is (Equation 3)

$$P_{opt} = F\left(\frac{B - C}{\sigma}\right),$$

where **σ** is the noise on the internal signal, *B* is the decision boundary, and F is the logistic function. This is exactly the model fitted by our generalized linear mixed model (GLMM, see above), with the fitted gradient for *Cue* corresponding to $-1/\sigma$ and the intercept corresponding to B/σ. Thus, the fact that no interaction between *Cue* and *Treatment* has been found indicates that the effective noise level is not changed by our manipulations. The estimate of -1.79 for the gradient (Appendix Table 2) allows us to infer an effective noise level of **σ** = 0.56, in our units where Cue runs from 1 (high reward) to 5 (low reward).

However, the significant main effect of *Treatment* indicates that the decision boundary was different in the two cases. The estimate of 6.05 (Appendix Table 2) for the intercept in the control condition implies that the decision boundary in this condition is 3.38. Bees in the Control treatment (Fig. 2A) are thus equally likely to make the optimistic or pessimistic response when the cue is a little closer to “near low” than medium (3). The fact that the intercept drops by -1.49 for the Shaking treatment and -1.26 for Trapping (Appendix Table 2) implies that the boundary shifts leftward to 2.55 and 2.68, respectively, in these conditions. The point at which these bees are equally likely to make optimistic and pessimistic choices is closer to “near high” than to medium (Fig. 3B).

In the fitted model, weighted probability distributions for both low and high rewards have an equal spread, reflecting the noise level inferred from the GLMM. In

the Control treatment, the shift of the decision boundary reflects the greater weight given to the high reward. Quantitatively, the extent of the shift, together with the fitted noise level, implies that the high reward is given 3.6 times the weight of the low reward. This result also cannot be explained merely by the bees not perceiving the medium colour as midway between blue and green since both the high and low reward trials combine data from trials where the cue was blue and trials where it was green. Instead, this result might, for example, indicate that the bees understand that both rewards are equally likely (P_{Hi} = 50%) and find the 50% sucrose solution 3.6 times as rewarding, relative to water, as the 30% solution.

The fact that the decision boundary is to the left of neutral in the Shaking and Trapping treatments indicates that here, greater weight is given to the low reward (Fig. 3B). Assuming we can discount the possibility that the reward value has inverted (i.e., that stressed bees find 30% sucrose more rewarding than 50%), this must represent a shift in the priors, such that stressed bees now consider high-reward trials less likely. To match the extent of the leftward shift, given the noise level inferred from our GLMM fit, the low reward must be weighted 4.6 times as much as the high reward. If the reward ratio were 3.6, this would imply that the bees behave as if the perceived probability of the high reward was 6%. However, if stressed bees find 50% and 30% sucrose equally valuable, i.e., the stress has removed the difference in reward utility, then the observed shift in decision boundary could be produced with a less dramatic shift in the priors, with perceived probability of the high reward being 18%.

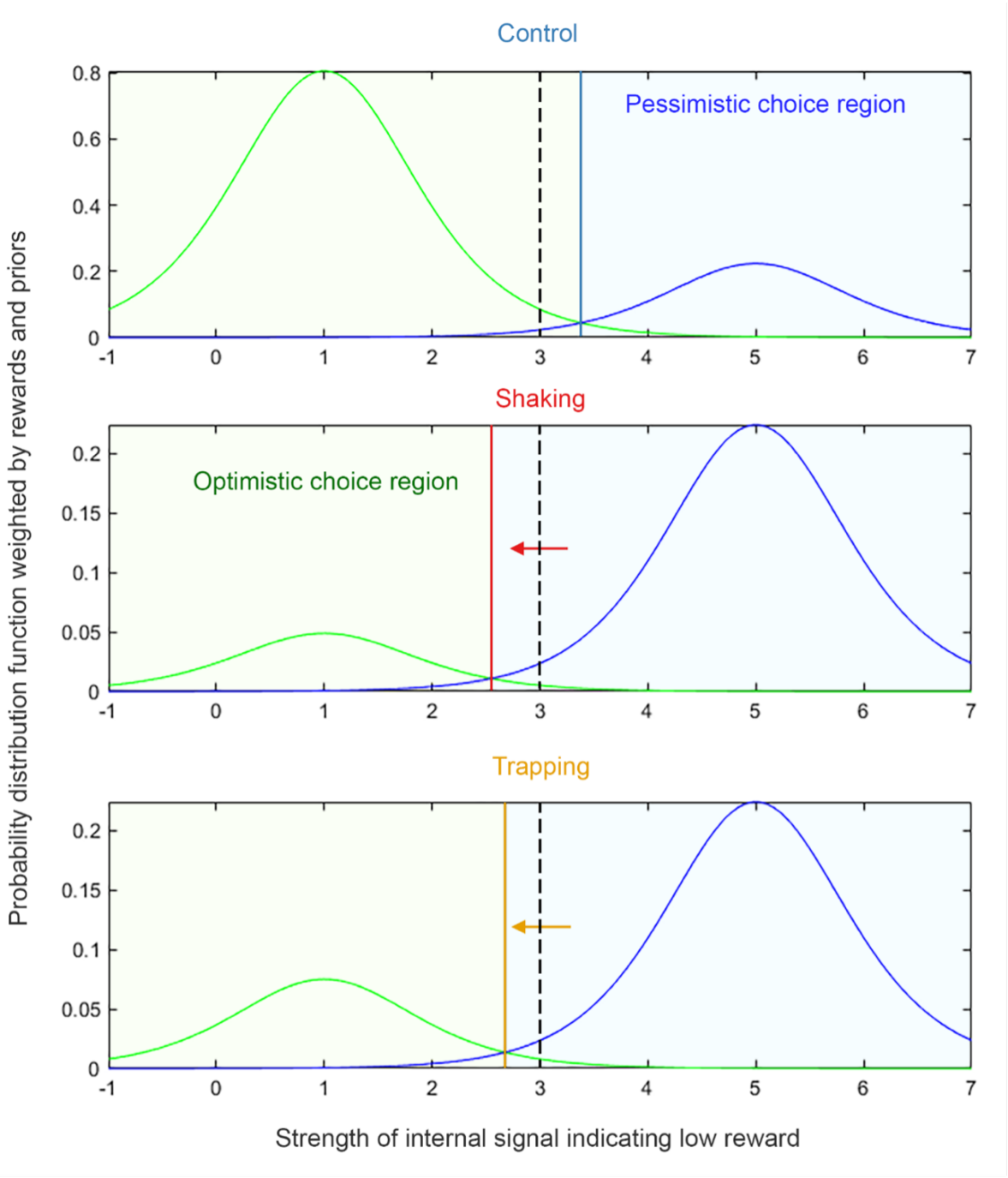

Figure 3. Bee decision-making boundaries and priors fitted by a signal-detection model. Curves depict the probability density functions for responses based on the internal signal *x* indicating a low reward. In each case, the original distribution has been weighted by the product of the value of that reward and its probability of occurring (Equation 5). The two curves in each panel depict the probabilities that the cue indicates high reward (green, centred on 1) or low reward (blue, centred on 5). Solid lines depict the decision boundary B inferred from the Generalized Linear Mixed Model fit to our data. Dotted lines indicate the medium point for comparison. Regions to the right of the solid boundary line are regions where the bee makes pessimistic choices (shaded blue). Regions to the left are regions where the bee makes optimistic choices (shaded green). Arrows depict the shift in boundaries compared to the control condition. The three panels depict the conditions for the **A)** Control, **B)** Shaking and **C)** Trapping treatments. Note the change in axes in the lower two panels.

2.4. Discussion

I developed a novel task to assess emotion-like states in bees. Using an active choice judgment bias task, we demonstrated that physically stressed bees are more likely to make pessimistic choices when faced with ambiguous stimuli. A signal detection model of our data suggests that this behaviour is explained by a reduced expectation of rewards. I thus provide strong evidence for bee judgement biases and a possible explanation for bee behaviour in judgement bias tasks.

Most studies of judgement bias tests have used a go/no-go paradigm. The results of these studies can be challenging to interpret due to confounds from other factors that do not involve stimulus judgements such as, for example, motivation. The active choice design presented here avoids these complications. Motivation alone cannot therefore explain the observed shift in responses in the manipulated bees in my experiment. This is further supported by the results of ingestion rate experiment, where I do not find differences in feeding motivation. Only one previous study has used an active choice design to study judgement biases in insects (Deakin *et al.*, 2018). In that study, flies had to choose between two odours, one associated with a reward and another with punishment. Rather than using reward and punishment, we developed a novel paradigm for insects that uses two rewards of different quality. This allowed to investigate the mechanisms underlying the judgement bias in further detail and test how negative states modulate expectations and perceptions of reward. Using previous paradigms involving reward and punishment as the expected outcome can make it easier to detect affect-dependent judgement bias (Lagisz *et al.*, 2020). I, however, find a bias in bee behaviour when using two rewards and an active choice paradigm, providing stronger evidence for affect-dependent processing in insects.

Bees learnt the stimulus-outcome associations

When performing an active choice task, it is important to ensure that the rewards used to condition the animals' responses are not perceived as equally favourable. If so, the results of tests using ambiguous stimuli would reflect the animal's colour preferences rather than its value-based interpretation. Bumblebees, however, can use colour cues to discriminate between rewards of varying value and prefer higher concentrations of sugar solution, including the colours and concentrations I used in my experiments

(Nityananda and Chittka, 2021). In my experiments, too, the bees chose high rewards significantly faster than lower rewards at the end of the training phase. In the tests, bees in all treatment groups also made slower choices as the cue value moved further away from the one indicating a high reward. The faster choice latency towards the high reward cue suggests that bees maintain their preference for higher rewards even after experiencing stress. This demonstrates that the bees distinguished between the high and low rewards, regardless of the associated colour.

Physical stress was not detrimental to bee sensory perception

Manipulations in judgement bias tasks need to change decision-making without impairing sensory perception or discrimination. In one previous test of judgement biases, shaken honeybees showed a decreased response not only to ambiguous odour mixtures but also to the conditioned negative odour (Bateson *et al.*, 2011). This decrease has been suggested to indicate an improved ability to differentiate odours rather than a negative bias in judgement (Giurfa, 2013). In my experiment, however, the bees were perfectly accurate when responding to both conditioned cues (high and low) in the tests. The manipulations thus did not impair the colour discrimination abilities and memory of the bees. The preservation of high colour discrimination abilities is not surprising, as previous studies on Drosophila have successfully used shaking in aversive learning paradigms (Bicker and Reichert, 1978). Similar trapping mechanisms to the ones we used have also been employed in aversive learning tasks in bees (Ings, Wang and Chittka, 2012).

Active choices are better indicators of judgments than latencies

Latency is often used in go/no-go judgment bias tests to evaluate the emotional states of animals (Solvi, Baciadonna and Chittka, 2016). When evaluating an emotional state, it is important to determine whether it is positive or negative (known as valence). However, relying solely on latency as a measure of valence is not always reliable, as it can be affected by other factors unrelated to emotions. An increase in approach latency may be associated with a general increase in reactivity and arousal, for example, due to the increased energetic demands after experiencing stressful events (Even, Devaud and Barron, 2012). It may also indicate a shift in the perceived value of

the reward and differences in motivation (Karagiannis, Burman and Mills, 2015). Relying solely on latency can therefore make it challenging to interpret the results of judgment bias tests. For instance, exposure to a positive event has been reported to cause both longer (Burman *et al.*, 2011) and shorter (Verbeek *et al.*, 2014) response times to ambiguous stimuli.

Only one study has used latencies to measure emotion-like states in bees (Solvi, Baciadonna and Chittka, 2016). This study used go/no-go type of judgment bias test to demonstrate an optimistic bias in bumblebees after receiving an unexpected reward of sugar solution. As predicted, unexpected rewards reduced the latency with which bees approached ambiguous stimuli. However, the treatment also caused an increase in thoracic temperature which has been linked to increased motivation for foraging in other studies (Sadler and Nieh, 2011). Further experiments did indicate that optimism was a more plausible explanation, but choice latency clearly could be influenced by motivational changes as well as judgements. The results of the present study showed that after trapping, bees had shorter latencies than the control bees. This could, in principle, have indicated a positive state, again demonstrating the difficulty of using latencies alone to interpret judgement bias data. However, since our study was an active choice design, I could more reliably use the choices made by the bees rather than their latencies. Choices can better indicate affective valence, showing that the trapped bees were in a pessimistic state. This makes a strong argument in favour of active choice judgement bias tasks such as the one we used in my study.

Pessimistic choices by bees is related to a significant change in prior expectations

To unravel the potential mechanisms underlying the choices made by the bees, a signal detection approach has been employed. This framework has previously been suggested as a valuable tool for investigating affective biases (Locke and Robinson, 2021). A recent study has suggested that judgement biases in bees may be caused by a shift in stimulus-response curves (Strang and Muth, 2023). However, this study did not investigate the underlying causal mechanisms of this shift. In our model, the estimation of future outcomes combines estimates of the probability of an outcome occurring and the magnitude of the payoff from an outcome. The signal detection analysis demonstrates that control bees exhibit a higher probability of responding

optimistically to ambiguous cues, indicating an expectation of high rewards. Such a bias would not be suboptimal as it is in fact what is predicted by a rational, fully informed strategy which optimises expected reward. Even if the bees are estimating the priors correctly as 50-50, the difference in reward utility will still shift the decision boundary towards the cue indicating low reward (Fig. 4A). Our model shows that the control bees are behaving as if 50% sucrose is 3.6 times more valuable, relative to water than 30% sucrose. Thus, the data admit the possibility that the bees' behaviour is completely rational and unbiased, and the 50% sucrose is much more rewarding.

However, the decision boundary for the stressed bees is harder to interpret. Here, the decision boundary is to the ***left*** of neutral. Previous studies have shown that acute stress can increase an animal's sensitivity to the reward (Hernandez *et al.*, 2015). However, the observed left shift of the decision boundary in stressed bees cannot plausibly reflect such a change in reward sensitivity since a leftward shift could only be produced if the value of high and low rewards were swapped, i.e., if 50% sucrose became less rewarding than 30%. However, it could reflect a pessimistic bias in expectations, i.e., that the stressed bees behave as if high-reward priors are less likely ($P_{Hi} < P_{Lo}$), perhaps because in nature high rewards are indeed scarcer when conditions are stressful. This can account for a leftward shift, but the large quantitative extent of the shift is still surprising. Since the noise remains relatively small, as indicated by the perfect performance for high and low cues, we have to postulate enormous changes in the priors to produce the observed shift. To obtain the decision boundary of 2.55 inferred for shaken bees, we would have to postulate that shaken bees estimate P_{Lo} = 94%, i.e., they expect a high reward to be available on only one trial in 20. This assumes that the reward utility remains the same, with a high reward 3.6 times as valuable as a low. If the relative utility of the high reward increased, e.g., because of an increased need for sucrose after stress (Even, Devaud and Barron, 2012), the priors would have to shift even further from 50%. However, one possibility is that, contrary to the assumptions of our model, the noise was not uniform for all cues, and there was more sensory noise on intermediate values of the cue. If this were so, the change in priors would not need to be as dramatic, although the basic result of changed priors would remain true.

By employing an active choice judgment bias task, my results support the possibility of emotion-like states in bees, further suggesting the widespread nature of these states. Moreover, I also provide robust evidence that neither motivational factors

nor colour discrimination alone can account for the observed cognitive biases. Importantly, the modelling indicates that the pessimistic-like behaviour results from a significant shift in prior expectations of rewards occurring after stress. These novel insights into the underlying causal mechanisms of state-dependent judgment biases in insects open new avenues for exploring state-dependent decision-making in insects.

2.6. Appendix

Table 1

Summary of model selection to analyse the impact of stress treatments (shaking/trapping) on performance in the judgement bias task

Explanatory variables					
Dependent variable	Fixed	Random	d.f.	AIC	ΔAIC
Optimistic Response	**Treatment+Cue**	**ID**	**5**	**179.81**	**0.00**
	Treatment*Cue	ID	7	183.24	3.43
	Cue	ID	3	184.39	4.57
	Treatment	ID	4	335.19	155.38
	(1|ID)	ID	2	336.11	156.30
log(Choice latency)	Cue*Response+Treatment	ID	8	234.64	0.00
	Cue*Response	**ID**	**6**	**235.46**	**0.82**
	Cue+Treatment+Response	ID	14	242.35	7.70
	Cue*Treatment*Response	ID	6	256.38	21.73
	Response	ID	4	256.47	21.83
	Cue+Response	ID	5	257.39	22.74
	Cue+Treatment+Response	ID	7	257.46	22.82
	Cue*Treatment+Response	ID	9	259.14	24.50
	Cue+Treatment*Response	ID	8	260.15	25.51
	Cue	ID	4	260.17	25.52
	Cue+Treatment	ID	6	260.92	26.28
	Cue+Treatment*Response	ID	9	261.28	26.64
	Cue*Treatment	ID	8	262.74	28.09
	(1|ID)	ID	3	273.35	38.71
log(Training latency)	**Cue**	**ID**	**4**	**137.36**	**0.00**
	Cue+Treatment	ID	6	144.68	7.32
	Cue*Treatment	ID	8	150.98	13.62
	(1|ID)	ID	3	162.20	24.83
	Treatment	ID	5	169.53	32.17

Three models were fit to analyse 1) the likelihood of choosing reward chamber associated with high reward (optimistic response) and 2) Choice latency in the test and 3) Choice latency during training in trials with high and low cues. Only summaries of the best fit models are shown. For each model, fixed and random explanatory variables, degrees of freedom (d.f.) and Akaike's Information criterion (AIC) are detailed. For each dependent variable, the selected model, i.e., the one with the lowest AIC, is indicated in bold.

Table 2

Summary of the best fit statistical models analysing the impact of stress treatments (shaking/trapping) on performance in the judgement bias task.

Response variable		Estimate	Std. Error	z value/ t value	Pr(>|z|)
Optimistic response	(Intercept)	6.05	0.81	7.46	0.000
	Cue	**-1.79**	**0.21**	**-8.39**	**0.000**
	Treatment(Shaking)	**-1.49**	**0.57**	**-2.61**	**0.009**
	Treatment(Trapping)	**-1.26**	**0.56**	**-2.23**	**0.026**
log(Choice latency)	(Intercept)	3.63	0.15	24.87	0.000
	Cue	**-0.09**	**0.03**	**-2.48**	**0.014**
	Response(Optimistic choice)	**-0.91**	**0.16**	**-5.59**	**0.000**
	Cue*Response(Optimistic choice)	**0.26**	**0.05**	**5.06**	**0.000**
log(Training latency)	(Intercept)	2.87	0.08	35.95	0.000
	Cue	**0.59**	**0.09**	**6.79**	**0.000**

The table provides summaries of the best fit models for the effects of the treatments, shaking and trapping, on the performance of subjects in the judgment bias task. The data is presented in terms of estimated coefficients, standard errors, z-values (Optimistic response model) or t-values (Choice latency model), and the associated p-values for response variables and predictor variables.

CHAPTER THREE

Stress increases the resolution of bee vision

3.1. Introduction

Bees inhabit an environment rich in visual cues. To support a colony's needs, social bees have evolved the capacity to use different floral traits to obtain a reward. For example, bees use colour (Gumbert, 2000) and size (Ronacher, 1992; Spaethe, Tautz and Chittka, 2001) to guide their flower choices. They can also discriminate visual patterns based on orientation (Wehner, 1971; Srinivasan, Zhang and Rolfe, 1993; Giger and Srinivasan, 1996), shape (Zhang and Srinivasan, 1994), and even symmetry (Moller, 1994). In addition to using single traits to guide their behaviour towards reward, bees can also extract regularities from more complex visual patterns to achieve their goals (Stach, Benard and Giurfa, 2004).

Not all visual cues in the bee environment, however, are reward-related. Some cues signal dangers. For example, crab spiders, sit-and-wait predators, can camouflage themselves on flowers, making flowers dangerous foraging sites, and therefore reducing pollinator visitation frequency as well as the overall time spent on flowers (Romero, Antiqueira and Koricheva, 2011). To maximise energy intake, bees therefore constantly need to balance their "attention" between visual cues needed for flower identification and cues indicating danger (Wang *et al.*, 2013). Given that past experiences shape how animals perceive the world (Snyder *et al.*, 2015), it is reasonable to assume that this attention allocation trade-off would prioritise processing threat-reated stimuli if previously encountered with a predatory attack. Consequently, negative past experiences can result in attentional bias, i.e., an increased processing of threat-related stimuli (Math and Mackin, 1998; Mogg and Bradley, 1998; Kindt and van den Hout, 2001; Bar-Haim *et al.*, 2007). The pronounced presence of attentional biases seen with negative emotional states suggests the inherent functionality of these states — for instance, swift threat detection (for examples see 1.2. Conceptualising Emotions). The mechanisms for such adaptation can, for example, involve a fine-tuning of perception towards the detection of specific features within enviroment that potentiate survival (Mogg and Bradley, 1998). Given this adaptive function of negative states, could bees also benefit from it? Could experiencing a sudden predatory event that induces a negative state in bees (as discussed in Chapter Two) potentially enhance their visual perception and improve the detection of task-relevant information?

While visual perception is predetermined by the optical quality of the eye (e.g., eye size), this only sets its upper boundary (Spaethe and Chittka, 2003). In humans, affective states can play a crucial role in directly influencing visual perception (Vuilleumier, 2005; Mathewson, Arnell and Mansfield, 2008; Bocanegra and Zeelenberg, 2009; Zadra and Clore, 2011). Recent studies demonstrate that emotion-induced modulation can occur at the level of early vision, thereby directly altering the processing of low-level visual information. For instance, in mammals, an early-warning system mediated by the amygdala has been proposed (LeDoux, 2000; Phelps and LeDoux, 2005). Here, the amygdala drives a rapid and pre-attentive evaluation of threat-related cues in a way that enables a swift shift of attention toward such cues and initiates subsequent behavioural responses. Thus, affect can prepare the visual system for detecting relevant information in the environment (Schwarz and Clore, 1983; Zadra and Clore, 2011).

Several human studies have shown that fear can modulate early visual perception, heighten stimulus-driven attention, and, consequently, facilitate visual search. For instance, Phelps, Ling, and Carrasco (2006) demonstrated that the brief presentation of a fearful face facilitates target detection by enhancing contrast sensitivity. Similarly, fear-induced states have been found to facilitate the detection of non-threatening but task-relevant objects (Becker, 2009). Researchers have also shown increased sensitivity to low-level spatial frequencies, another crucial component of visual acuity, in response to fear (Bocanegra and Zeelenberg, 2009; Bocanegra, 2011). Similarly, emotional arousal leads to a shift in peak contrast sensitivity towards lower spatial frequencies (Lee *et al.*, 2014).

Recent research has shed light on how acute exposure to negative events simulating predatory attack can influence the internal state of bees, resembling negative emotional states in other animals (see Chapter Two). Although little is known about the underlying mechanisms, the findings clearly show state-induced modulations of responses to ambiguous information. We do not however know at what stages of information processing these modulations occur.

In this study, I therefore explored whether the emotion-like states previously observed in bees have a similar influence on how bumblebees perceive their visual environment as they do in humans. To address this question, I adopted the assay developed to characterise visual acuity in *Bombus terrestris* (Chakravarthi *et al.*, 2016).

Bees were trained to discriminate rewarding horizontal sigmoidal gratings from unrewarded vertical ones in a Y-maze set-up. Subsequently, bees were tested on grating pairs with varying spatial frequencies and decreasing contrasts to determine their spatial resolution and contrast thresholds. I hypothesised that exposing bees to simulated predatory attacks, and the resultant negative emotional states would lead to a shift in contrast and spatial resolution thresholds.

3.2. Materials and methods

Animals and housing

For this experiment, I used four commercially raised *Bombus terrestris* colonies (Agralan, UK) that I transferred to bipartite plastic nest-boxes (28.0 × 16.0 × 12.0 cm). The nest-box was connected to the Y-maze via a transparent acrylic tunnel (56.0 × 5.0 × 5.0 cm). Multiple shutters allowed me to control which bees accessed the maze. Bees were kept under standardized temperature conditions (23 ± 2℃). To provide illumination, I attached double LED tubes (Philips CorePro LEDtube UN 600mm HO 8W865 T8) above the flight arena. The luminance was equally spread in the Y-maze, with an average of 1100 lux in both arms and 1020 lux at the entrance to the maze, as measured by a digital light meter (Dr. meter, LX1010BS, USA).

Through the experimental period, colonies were fed with ~ 3g commercial pollen daily (Koppert B. V., The Netherlands) and provided with sucrose solution (20% w/w) *ad libitum* outside the experimental work. Before the onset of training or testing, bees were food-deprived by removing the feeder for the arena. Although invertebrates do not fall under the Animals (Scientific Procedures) Act, 1986 (ASPA), the experimental design and protocols were developed incorporating the 3Rs principles. Housing, maintenance, and experimental procedures were non-invasive and were kept as close as possible to the natural living conditions of the animals.

Training Apparatus

Bumblebees were individually trained in a Y-maze custom-built at Newcastle University (see Fig. 1A). This Y-maze design followed the specifications of a previously published

experiment designed for measuring visual acuity in *Bombus terrestris* (Chakravarthi *et al.*, 2016). The Y-maze comprised three identical arms, each measuring 20 cm in height, width, and length, and covered with UV-transparent Plexiglas sheets. Two of these three arms served as decision arms, while the third functioned as the entrance arm. The entrance arm contained a transparent Plexiglass tunnel (3 cm x 3 cm x 3 cm), which connected to the other two arms within the maze. This tunnel in the entrance arm provided direct access to an outer tunnel leading to the nest-box. The back wall of each decision arm was equipped with a hole (1cm in diameter) used for the insertion of a reward chamber.

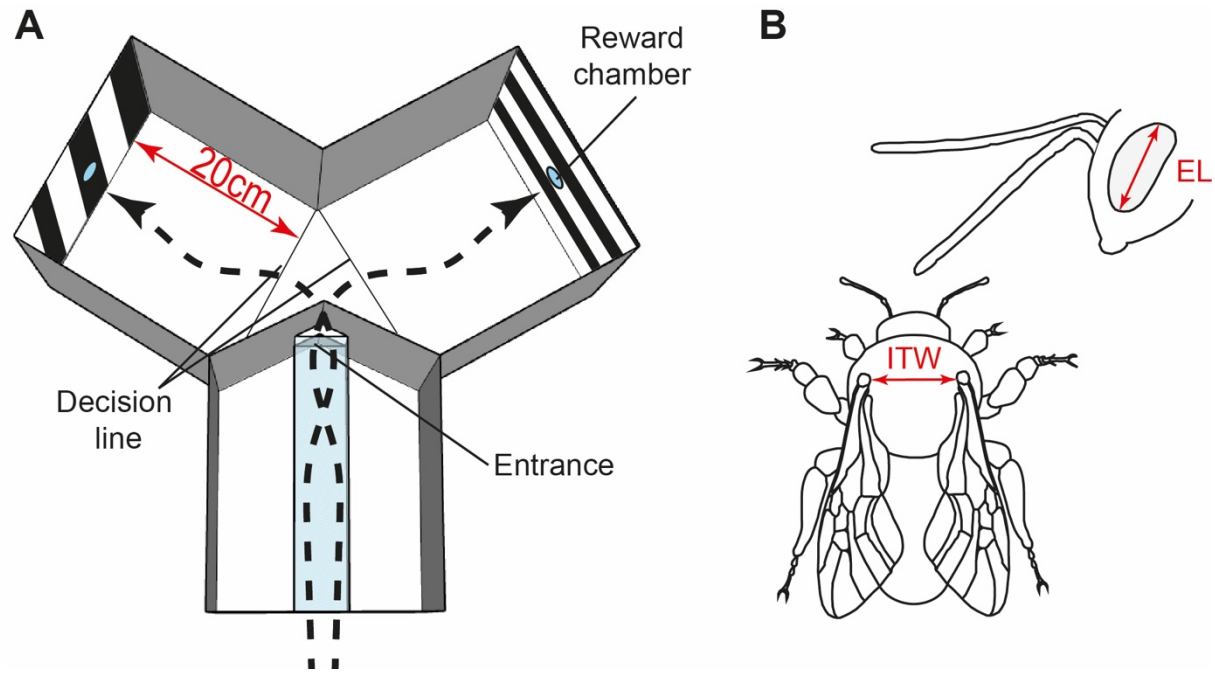

Figure 1. Experimental setup and morphological measurements. **A)** Experimental setup and stimuli. Bees accessed the decision arms through the entrance at the end of the tunnel. Both decision arms (20 x 20 x 20 cm) contained openings that led to a rewarding chamber which bees had to enter in order to sample its content. During training and testing trials, achromatic sinusoidal gratings were positioned on the back wall of each decision arm. The choice was determined by the first crossing of the 20 cm decision line in either of the two decision arms. **B)** Morphological measurements included the length of the compound eye (EL) and the intertegular width (ITW).

Stimuli

The stimuli were identical to those in Chakravarthi et al., 2016 and consisted of achromatic sinusoidal gratings with pattern wavelengths of 6.6, 5, 2.5, 1.53, 1.25, 1.0, and 0.5 cm, corresponding to spatial frequencies of 0.035, 0.070, 0.140, 0.280, 0.437, and 0.699 cycles deg^{-1} of visual angle at the 20 cm decision line (see Fig. 1A). Patterns with spatial frequency of 0.070 cycles deg^{-1} and 87% contrast were used for training because they have previously been shown to be the easiest for bees to resolve (Chakravarthi *et al.*, 2016). For the contrast sensitivity experiments, I used gratings with a fixed spatial frequency of 0.070 cycles deg^{-1}, and varying in Michelson contrasts, specifically 89%, 68%, 54%, 39%, and 22%. For the spatial resolution experiment, I used gratings of varying spatial frequencies of 0.035, 0.070, 0.140, 0.280, 0.437, and 0.699 cycles deg^{-1} at a fixed contrast, 87%.

Training and testing procedure

Pre-training

Bees foraging on the feeder were marked with a paint marker (Edding 750, Japan) and later recruited for training. The initial training step involved familiarising the bees with the two reward chamber locations. To do so, I used a cup to capture a bee at the entrance to the maze and aligned the opening of the cup with the entrance to the reward chamber. This manipulation forced the bee to enter the reward chamber and discover a sugar solution droplet for the first time (0.2 ml, 50% w/w). This procedure was repeated twice for each reward chamber for a total of four trials. During pre-training, bees were presented with two gratings for the first time: one positioned horizontally and the other vertically, with one grating in each arm (see Fig. 1A). Through the experiment, the horizontal grating was always associated with a reward, and the vertical grating with water. During pre-training, the initial presentation of the rewarding horizontal grating was randomised across bees. Subsequently, the side of this grating in the three following pre-training trials was alternated based on the initial presentation sequence (e.g., L-R-L-R or R-L-R-L).

Training

After the forced-choice pre-training, the subsequent training phase began by allowing bees to access the reward chamber freely. As before, on each training trial bees were presented with horizontal and vertical gratings positioned in the decision arms. The reward chamber on the arm with the horizontal grating contained a sugar reward (0.2 ml, 50% w/w), while the reward chamber on the side of the vertical grating contained water (0.2 ml). The side of the rewarding horizontal gratings was pseudorandomised across trials, with no more than two consecutive presentations of horizontal gratings on the same side. A new pseudorandom sequence was generated for each bee. In both training and test phases, reward chambers were changed on each trial to provide bees with fresh sucrose reward/distilled water and to control for the presence of any pheromones or scent marks remaining when bees entered the chamber. In preparation for the next experimental day, all used chambers were washed in 70% ethanol and hot water and left to dry.

In previous visual acuity experiments (Chakravarthi *et al.*, 2016), a stationary point at the entrance to the maze was used as the decision point. However, I observed that some bees crawled instead of taking off immediately upon entering the tunnel. Therefore, this stationary point was considered ineffective as a decision point for my study. To avoid inaccurate measurements, and following some previous studies (e.g. (Spaethe and Chittka, 2003), the choice point was defined as the first crossing of the 20 cm decision line in either of the two decision arms. This adjustment allowed bees to navigate a small triangular area after entering but before making their choice. Bees that successfully learned to distinguish between horizontal and vertical gratings and met the learning criterion (80% accuracy in the last 20 trials) proceeded to the testing phase.

Testing

Each bee underwent a total of 14 test trials. Each test trial consisted of the presentation of one vertical and one horizontal grating with identical spatial frequencies and contrast levels. In separate test trials, each bee was tested on six distinct spatial frequencies, all at a fixed maximum of 87% contrast. The six spatial frequencies used were 0.035, 0.070, 0.140, 0.280, 0.437, and 0.699 cycles deg^{-1}. Of these spatial frequencies, three (0.280, 0.437, and 0.699 cycles deg^{-1}) were tested twice for each bee, as they are less effectively resolved by bees (Chakravarthi *et al.*, 2016). To assess contrast sensitivity,

bees were presented with gratings featuring a fixed spatial frequency of 0.070 cycles deg^{-1} but varying contrasts of 87%, 68%, 54%, 39%, and 22%. As before, the bee's choice was recorded when it crossed the decision line for the first time. Additionally, the final decision of the bee, i.e., entering a reward chamber, was also recorded. Bees failing to enter either reward chamber within a 120-second period were categorised as "omissions" from making a final choice.

All test trials were unrewarded, with both reward chambers containing 0.2 ml of distilled water. Following each test trial, bees were given a minimum of two refresher trials, which required them to perform the same task as during training and were rewarded for making the correct choice.

Predatory attack simulation

Prior to testing, individual bees were randomly allocated to one of two groups: shaking (n = 20) or an unmanipulated group that served as a control (n = 20). The bees in the shaken group were individually subjected to 60 seconds of shaking at 1200 rpm using a Vortex-T Genie 2. Before entering the flight arena, I allowed the bee to enter a custom-made tagging cage softened by the sponge to prevent physically harming animals while shaking (40 mm diameter, 7.5 cm length). After entering, the bee was gently nudged down with a soft foam plunger until the distance between the plunger and the bottom of the cage was reduced to ~3 cm. Once the plunger was secured, the cage with the bee was placed inside the vortex cup head. I then ran the Vortex at 1200 rpm for 60 seconds to shake the bee. After the shaking, performed before each test trial, I released the bee into the tunnel connected to the Y-maze via an opening on the top of the tunnel. The bee was allowed to enter the Y-maze for testing as soon as it was ready to initiate a foraging bout (up to a maximum time of 60 seconds).

Video analysis

All test trials were recorded using a Huawei Nexus 6P phone with a 1440 × 2560 pixels resolution, capturing video at 120 frames per second (fps). Subsequently, I used the video analysis program BORIS 7.10.2107 (Friard and Gamba, 2016) to analyse the recorded videos and extract information about bee choices. Specifically, the following

time points were coded during analysis: 1) the bee entering the Y-maze, 2) the bee crossing the decision line, and 3) the bee entering the reward chamber. These coded timepoints were used to determine which arm was entered when the decision line was crossed, and which reward chamber was entered. They were also used to calculate latencies for both decisions.

Statistical analysis

My hypothesis and statistical analyses of the main active choice experiment were preregistered at aspredicted.com (#132314). The data were plotted and analysed using RStudio v.3.2.2 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org) and custom-written scripts. To determine the thresholds for contrast sensitivity and spatial resolution, I fit a logistic psychometric function (*quickpsy*, "quickpsy" package (Linares and López-Moliner, 2016) to the binomial choice data of the bees. In this model, a choice of 1 indicated crossing the decision line in the arm with the rewarding stimulus (hereafter rewarding arm), while a choice of 0 indicated crossing in the arms with the unrewarding stimulus (hereafter non-rewarding arm). The psychometric function is defined as:

$$\psi\,(x) = \gamma + (1 - \gamma - \lambda)\left(1 + e^{\left(\frac{a-x}{b}\right)}\right)^{-1}, \quad (1)$$

here $\psi\,(x)$ represents the proportion of correct choices across different spatial frequencies or contrasts. The parameter γ represents chance performance and was held constant at 0.5, while the parameter λ accounts for stimulus-independent error or lapse rate, allowing for variability (set as "TRUE") (Wichmann and Hill, 2001). The function was fit using a maximum likelihood method and parametric bootstrapping to estimate confidence intervals.

I fit a generalized mixed effect model (GLMM) with a binomial error distribution and a logit link function (*glmmTMB*, "glmmTBM" package (Magnusson *et al.*, 2017) to assess if shaking modulated a bee's ability to discriminate between horizontal and vertical patterns with decreasing contrasts and varying spatial frequencies. While the effects of spatial frequency and contrast were analysed separately, the response variable in all sets of models was choice accuracy (coded as 1 for crossing the decision line in the positive arm with horizontal grating and 0 for crossing the decision line in the

negative arm with vertical grating). For the analysis of the effects of contrast, the explanatory variables were Michelson contrast ("Contrast", coded as a continuous variable and included all five contrasts: 22%, 39%, 54%, 68%, and 87%), treatment ("Treatment", coded as a factor with two levels: control and shaking) and bee eye width ("Eye width", coded as a continuous variable). For the analysis of the effect of spatial frequency, the explanatory variables were spatial frequency ("Frequency", coded as a factor of six levels: 0.699, 0.437, 0.280, 0.140, 0.070, and 0.035 cycles deg^{-1}), treatment ("Treatment": coded as a factor with two levels: control and shaking) and bee eye width ("Eye width", coded as a continuous variable). In all models, the identity of the bee ("ID") was included as a random intercept variable.

Both initial choice (crossing the decision line) and final choice (entering the reward chamber) latency was modelled using a linear mixed effect model (LMEM) (*lmer*, lme4 package (Bates *et al.*, 2015). As before, the effects of spatial frequency and contrast were analysed separately. In all models, the response variable, latency, was log-transformed to normalise the right-skewed nature of the data. To assess if shaking affected the latency (either initial or final) on the trials with decreasing contrast, the explanatory variables were Michelson contrast ("Contrast", coded as a factor of five levels: 22%, 39%, 54%, 68%, and 87%), treatment ("Treatment", coded as a factor with two levels: control and shaking). To analyse the effect of varying spatial frequencies, the explanatory variables were spatial frequency ("Frequency", coded as a factor of six levels: 0.699, 0.437, 0.280, 0.140, 0.070, and 0.035 cycles deg^{-1}), treatment ("Treatment": coded as a factor with two levels: control and shaking) and bee eye width ("Eye width", coded as a continuous variable). As before, in all models, the identity of the bee ("ID") was included as a random intercept variable.

The final choice of reward chamber in each test was categorised according to three mutually exclusive choice categories: choosing a reward chamber on the side of horizontal gratings (correct), choosing a reward chamber on the side of vertical gratings (incorrect), and omission of response (omission). Response omission was recorded if the bee did not enter either reward chamber for longer than the cut-off criterion of 120 seconds. Each choice was transformed into binary response variable for each choice category. Thus, if a bee entered a reward chamber in the correct arm with horizontal grating, the choice was recorded as (1, 0, 0) for a choice of the correct, incorrect, and omission, respectively.

Choices were analysed using generalized mixed linear models (GLMM) using the *glmer* function of the *lme4* package with binomial errors and a logit link function (Bates *et al.*, 2015).The independent variables (fixed factors) were the treatment group (Treatment) and the choice category (Choice). As before, the identity of the bee (ID) was included as a random factor. For best-fitting models with interaction, the significance for each fixed factor and interaction term were estimated using Anova() ("car" package (Fox *et al.*, 2012)). The Tukey method was used for multiple comparisons (*emmeans*, "emmeans" package (Lenth *et al.*, 2019) within the model with a significant interaction.

For each response variable, the model selection process comprised comparing models with and without all possible interactions between explanatory variables. The most appropriate model was selected based on the Akaike information criterion (AIC) scores. I considered the model with the lowest AIC score the best model, i.e., the model that provides a satisfactory explanation of the variation in the data (Johnson and Omland, 2004). Following accepted convention, models with an AIC difference of less than 2 were considered not significantly better than the model it is being compared to (Burnham and Anderson, 2004). In such case, anova() was used to determine whether adding interaction term significantly improved model fit. I used the package DHARMa (Hartig, 2020) for residual testing of all models.

3.3. Results

A total of eight bees were excluded either due to ceasing foraging or being unable to overcome side bias, resulting in a final sample size of 40 bees. Half of the bees that successfully learned to discriminate between vertical and horizontal gratings were randomly allocated to the shaking group and the other half were allocated to the control treatment group. Bees in the shaking group were subjected to 60 seconds of vigorous shaking before each test trial, while control bees were left unmanipulated. The test consisted of the presentation of vertical and horizontal gratings at varying spatial frequencies and decreasing contrasts. Each bee was tested on six different spatial frequencies, with three of them repeated twice, all at a fixed contrast of 87%. Bees were also tested with gratings with decreasing contrasts (68%, 54%, 39%, and 22%) at a fixed spatial frequency of 0.070 cycles deg^{-1}.

Morphometric measurements of shaken and control bees

The intertegular width of the bees ranged from 3.62 mm to 4.27 mm for control bees (mean ± standard deviation: 3.99 ± 0.156 mm) and 3.65 mm to 4.19 mm for shaken bees (mean ± standard deviation: 3.93 ± 0.202 mm). The eye length fluctuated between 2.52 mm to 2.79 mm for control bees (mean ± standard deviation: 2.65 ± 0.085 mm) and 2.42 mm to 2.90 mm for shaken bees (mean ± standard deviation: 2.72 ± 0.112 mm). In line with previous studies (Spaethe and Chittka, 2003), the correlation between these two measures was statistically significant (Control: $r = 0.75$, $p < 0.00001$; Shaking: $r = 0.70$, $p < 0.00001$). Given the high correlation between these measures, I used eye length to assess whether bee response accuracy was influenced by bee size across treatments and tests. Consequently, eye length was included as an explanatory factor in all subsequent models assessing bee performance.

Shaken bees have higher contrast thresholds and a shift of the spatial resolution threshold towards higher frequencies

Contrast thresholds

The contrast threshold was determined by fitting a logistic function to the proportion of correct choices made at a decision line. The data included trials where gratings were expressed at five contrasts (87%, 68%, 54%, 39%, and 22%) with a fixed spatial frequency of 0.070 cycles deg^{-1}. A value of 0.8 correct choices was chosen to measure the threshold for both the control and shaken treatment groups, as this value was exceeded at most contrasts (see Fig. 2A). The control group had a contrast threshold of 22.10% Michelson contrast, while the shaken group had a lower contrast sensitivity, with higher contrast threshold of 35.93%. These results suggest that shaken bees have worse discriminability at lower contrasts, as they require a higher contrast threshold to achieve the same level of performance as the control bees.

Spatial resolution thresholds

To determine the spatial resolution threshold, I again fit a logistic function to the proportion of correct choices made at a decision line. Here, data included trials with gratings of varying spatial frequencies (0.035, 0.070, 0.140, 0.280, 0.437, and 0.699 cycles deg^{-1}), and fixed contrast (87%). Due to the relatively poor performance, especially at higher spatial frequencies (see Fig. 3A), the value of 0.65 correct choices was set to measure the threshold for both the control and shaken treatment groups. Although lower than the one used for contrast sensitivity, this value remained significantly above the chance level (binomial test, $X^2 = 8.41$, df = 1, p-value = 0.0037).

For control bees, the spatial resolution threshold for discriminating between vertically and horizontally oriented sinusoidal grating patterns was determined to correspond to a spatial frequency of 0.235 cycles deg^{-1}. However, the spatial resolution for shaken bees was higher, with a spatial frequency of 0.344 cycles deg^{-1}. Therefore, while control bees were able to distinguish gratings expressed in three out of six tested spatial frequencies (0.035, 0.07, 0.140 cycles deg^{-1}) with an accuracy above 65%, shaken bees demonstrated the capacity to discriminate patterns expressed at even higher spatial frequencies; including all three that control bees could (0.035, 0.07, 0.140 cycles deg^{-1}), in addition to 0.280 cycles deg^{-1}. A spatial resolution of 2.91° in shaken bees suggests they can distinguish finer details and patterns at a smaller angular size than the control group.

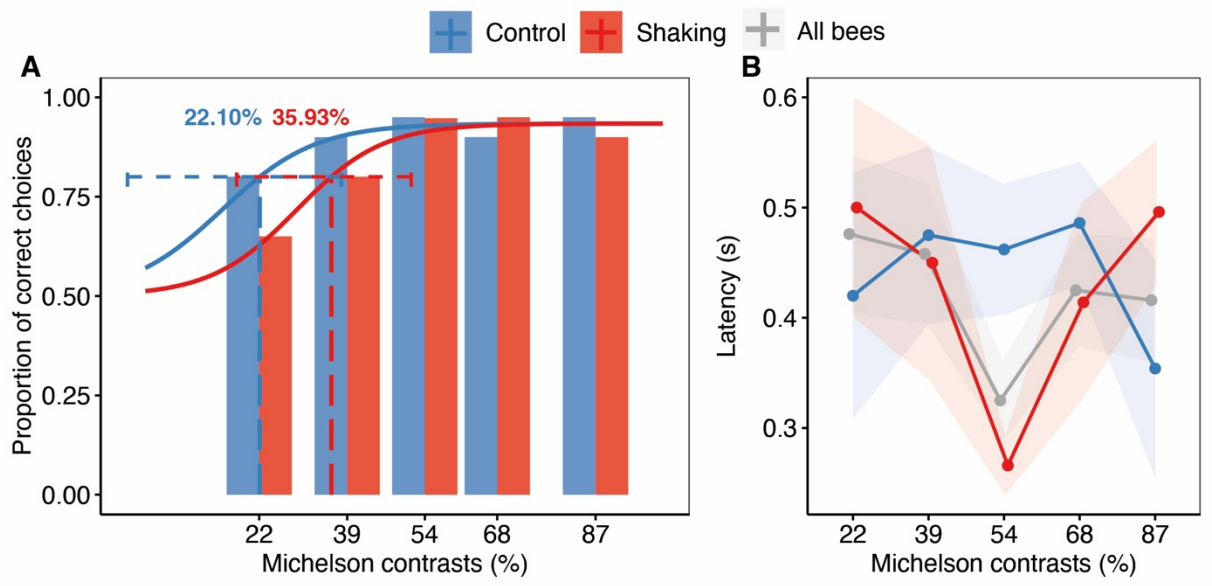

Figure 2. Shaking reduces the contrast sensitivity threshold and choice latency but only at contrast of 54%. A) Proportion of correct choices made by control (blue bars) and shaken (red bars)

bees at the decision line. The dotted line represents the thresholds at 0.80 proportion of correct choices for both groups, derived from the fitted logistic function (blue solid line control, red solid line shaking). Error bars represent the 95% confidence interval for the threshold. **B)** Average latency to cross the decision line for control (blue) and shaken (red) bees. The solid grey line indicates the cumulative latency averaged across all bees. The averages are presented as a median with the 95% confidence interval (shaded area).

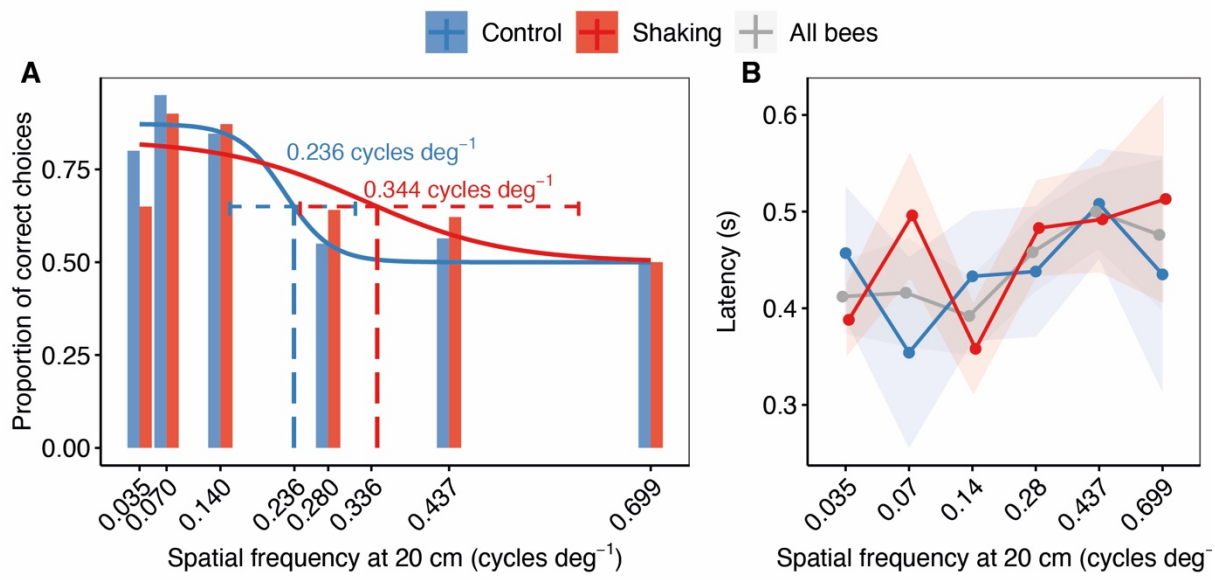

Figure 3. Shaking increases spatial resolution without affecting latency. A) Proportion of correct choices made by control (blue bars) and shaken (red bars) bees at the decision line. The dotted line represents the thresholds at 0.65 proportion of correct choices for both groups, derived from the fitted logistic function (blue solid line control, red solid line shaking). Error bars represent the 95% confidence interval for the threshold. **B)** Average latency to cross the decision line for control (blue) and shaken (red) bees. The solid grey line indicates the cumulative latency averaged across all bees. The averages are presented as a median with the 95% confidence interval (shaded area).

Shaking does not affect initial choice accuracy or choice latency at the decision line

The effect of contrast sensitivity

Surprisingly, bees demonstrated remarkable accuracy in distinguishing between horizontal and vertical gratings, even when tested at lower contrast levels (see Fig. 2A). Despite the high performance, the contrast threshold for shaken bees was found to be higher (Fig. 2A). I, therefore, wanted to determine whether this higher contrast threshold had a substantial impact on their overall choice accuracy.

The model for evaluating grating discrimination accuracy at the decision line, which included treatment, contrast, and eye width as fixed predictors without any interaction terms, had the lowest AIC (149.34). The closest competing models, which included interaction terms, had slightly higher AIC values (150.25 for the model with an interaction between contrast and eye width; 150.88 with an interaction between treatment and eye width; 150.99 with an interaction between treatment and contrast). A delta AIC lower than 2 units indicates no strong support for these alternative models over the best-fitting model. ANOVA comparisons further confirmed that adding any of the interaction terms does not provide a statistically significant improvement (Appendix Table 1). Therefore, the best-fitting model included treatment, contrast, and eye width as fixed predictors without any interaction terms (see Appendix Table 1).

As contrast levels increased, there was a corresponding increase in the likelihood of bees making the correct choice (Model Estimate ± standard error = 0.030 ± 0.011, $z = 2.761$, $p < 0.01$). Importantly, this trend was consistent across both treatment groups, as shaken bees showed no significant difference in their likelihood of making the correct choice compared to control bees (Model Estimate ± standard error = -0.410±0.505, $z = -0.811$, $p = 0.417$). Eye width also was not a significant predictor of the response accuracy (Model Estimate ± standard error = -0.498 ± 2.309, $z = -0.022$, $p = 0.8291$). This finding demonstrates the ability of bees to maintain discrimination accuracy even as contrast levels decreased, irrespective of their treatment group.

While the accuracy of bee choices remained unaffected by shaking across different contrast levels, I also explored whether there were variations in the time it took for bees to make a choice and cross the decision line. The model estimated the choice latency included treatment and contrast as fixed factors and an interaction term had the lowest AIC (350.70). The closest competing model, which excluded an interaction term, had a slightly higher AIC (348.94). With a delta AIC of 1.76, indicating no strong support for one model over the other. The ANOVA comparison shows that adding an interaction term to the model does indeed provide a statistically significant improvement ($\chi^2(2) = 12.83$, $p < 0.05$). The best-fitting model, therefore, included treatment and contrast as fixed factors and an interaction term (Appendix Table 2). Fixed effects of contrast ($X^2 = 8.84$, df: 4, $p = 0.065$) and treatment ($X^2 = 0.025$, df: 1, $p = 0.874$) were not significant. However, the effect of treatment was contrast dependent ($X^2 = 12.69$, df: 4, $p = 0.008$), with shaken bees making significantly faster choices only at contrast of 54% ($t = 2.697$, $p = 0.008$) (Fig. 2B).

The effect of spatial frequency

When examining the accuracy of bee performance in making a choice and crossing the decision line on the trials with varying spatial frequencies, the model which included treatment, spatial frequency and eye width as fixed predictors without any interaction between factors had the lowest AIC (396.01). (see Appendix Table 5). The closest competing model, which included interaction term between treatment and eye width, had slightly higher AIC values (397.49). With a delta AIC of 1.5, indicating no strong support for one model over the other. The ANOVA comparison shows that adding an interaction term to the model does indeed provide a statistically significant improvement ($\chi^2(2) = 1.5$, $p = 0.221$). (Appendix Table 5). Therefore, the best-fitting model included treatment, spatial frequency, and eye width as fixed predictors without any interaction terms. This model coded spatial frequency as a categorical variable with six levels. Unlike the linear relationship observed with contrast levels (as contrast decreases, the accuracy also decreases), the response to varying frequencies displayed a different pattern. The highest accuracy is expected at 0.07 cycles deg^{-1}, and it gradually declines as we move away from this point toward both higher and lower frequencies. Precisely this response was observed across bees (Fig. 3A). When keeping a spatial frequency of 0.07 cycles deg^{-1} (trained resolution) as a reference group, the performance of bees decreases at the lowest (Frequency 0.035 cycles deg^{-1}, Model Estimate ± standard error = -1.480 ± 0.717, z = -2.064, p = 0.039) as well as the three highest spatial frequencies (Frequency 0.28 cycles deg^{-1}, Model Estimate ± standard error = -2.200 ± 0.658, z = -3.342, p < 0.001; Frequency 0.437 cycles deg^{-1}, Model Estimate ± standard error = -2.210 ± 0.660, z = -3.347, p < 0.001; Frequency 0.699 cycles deg^{-1}, Model Estimate ± standard error = -2.682 ± 0.700, z = -3.383, p < 0.001). At the same time, bees showed no decrease in accuracy when presented with gratings at a spatial frequency of 0.14 cycles deg^{-1}, which is double their trained frequency of 0.07 cycles deg^{-1} (Model Estimate ± standard error = -0.723 ± 0.693, z = -1.043, p = 0.297). This suggests that bees can generalize to higher frequencies to some extent. Once again, shaking did not affect choice accuracy (Model Estimate ± standard error = 0.031 ± 0.307, z = 0.102, p = 0.918), or the likelihood of crossing the correct decision line (Model Estimate ± standard error = 0.298 ± 0.328, z = 0.906, p = 0.3650). As before, eye width did not significantly affect bee accuracy either (Model Estimate ± standard error = -2.890 ± 1.579, z = -1.830, p = 0.0672).

I further examined whether the decreased choice accuracy observed at lower and higher spatial frequencies also led to an increase in choice latency at the decision line and whether shaking impacted this latency. The best-fitting model for assessing the latency to cross the decision line included treatment and spatial frequency as fixed predictors without considering their interaction (see Appendix Table 6). Similar to the findings with decreasing contrasts, shaking also had no statistically significant effect on choice latency during trials involving varying spatial frequencies (Model Estimate ± standard error = 0.048 ± 0.122, t = 3.96, p = 0.6941). Interestingly, bees took longer to make their choice and cross the decision line at higher spatial frequencies when compared to the trained spatial frequency of 0.07 cycles deg^{-1} (Fig. 3B), which is easily resolvable. Choice latency was significantly different when the grating had a spatial frequency of 0.699 cycles deg^{-1} (Model Estimate ± standard error = 0.237 ± 0.109, t = 2.175, p = 0.003). Notably, these were also the spatial frequencies at which bees were less likely to choose the correct arm (Fig. 3B). Therefore, the increase in choice latency on these trials may indicate a lack of certainty at these specific spatial frequencies.

The effect of shaking on final choices

The crossing of the first decision line reflects the early perception-driven choices of bees. I also wanted to explore if shaking affected their final decision – choosing which reward chamber to enter. As the arms in the Y-maze were not separated, bees could freely change their flight trajectory, exit the initially chosen arm, and enter an alternative one. Thus, a longer latency to choose to enter one of two reward chambers indicated a lack of certainty in the bees' decision. If shaking induced a higher arousal state, bees should be faster in their decision-making and thus show shorter latencies to enter the reward chamber. To account for cases where bees did not choose even after inspection of both gratings at a close, I set a time limit of 120 seconds. Bees that took longer than this cut-off I considered to be omitting a choice, and thus, the latencies exceeding this threshold were not included in the choice latency analysis. I was also interested in evaluating whether there were differences in the likelihood of making correct or incorrect final choices, and omissions between the groups.

Fixed spatial frequency and varying contrast

The model for assessing the latency to enter the reward chamber included contrast and treatment as fixed factors and choice accuracy but not their interaction had the lowest AIC (562.05) (see Appendix Table 3). The closest competing model, which included interaction term between treatment and choice accuracy, had slightly higher AIC values (563.19). A delta AIC of 1.14 indicating no strong support for one model over the other. The ANOVA comparison shows that adding an interaction term to the model does indeed provide a statistically significant improvement ($\chi^2(2) = 0.68$, $p = 0.41$). (Appendix Table 3). Therefore, the best-fitting model included contrast and treatment as fixed factors and choice accuracy but not their interaction. As anticipated, with decreasing contrast levels, bees took longer to enter one of the two reward chambers (Model Estimate ± standard error = -0.012 ± 0.003, $t = -4.023$, $p < 0.001$). However, there was no effect of shaking on the latency (Model Estimate ± standard error = -0.321 ± 0.193, $t = -1.662$, $p = 0.171$), and choosing the correct reward chamber was not faster than choosing the incorrect chamber (Model Estimate ± standard error = -0.010 ± 0.215, $t = -0.465$, $p = 0.642$).

The model for the likelihood of choices included choice category and treatment as factors (see Appendix Table 4). Overall, bees were less likely to make incorrect choices (Model Estimate ± standard error = -3.245 ± 0.274, $z = -11.855$, $p < 0.0001$) or omit from choosing (Model Estimate ± standard error = -6.149 ± 0.736, $z = -8.355$, $p < 0.0001$) compared to making correct choices. The likelihood of making any choice (correct, incorrect, or omission) was also unaffected by shaking (Model Estimate ± standard error < 0.0001 ± 0.269, $z < 0.0001$, $p = 1$).

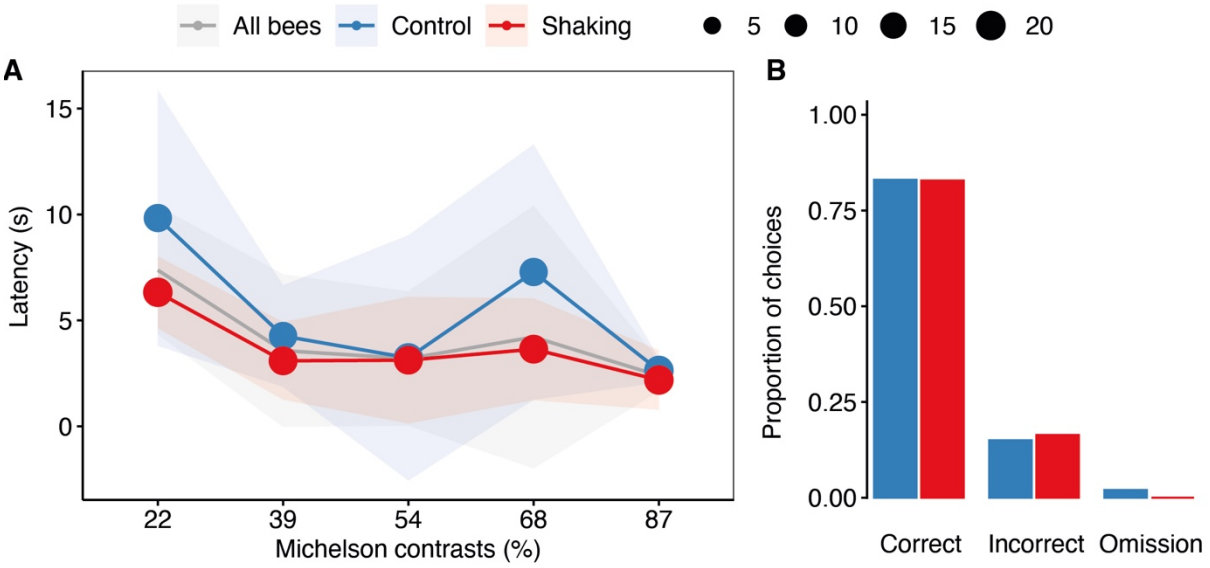

Figure 4. Shaking does not affect the latency of correct choices or the probability of choices across contrasts. A) Latency to make correct choices as a function of contrast. For Control (blue) and Shaken (red) bees. The solid grey line represents the cumulative latency averaged across all bees. Dots represent median values with 95% confidence intervals (shaded area). The size of each dot represents the number of bees. **B)** Proportions of correct choices, incorrect choices, and omissions for Control (blue bars) and Shaken (red bars) bees.

Fixed contrast and varying spatial frequency

To investigate the effect of varying spatial frequency I also analysed a set of trials with fixed contrast and varying spatial frequencies. As above, the cut-off criterion of 120 seconds was applied to the latency of a correct choice to determine choices and omissions. The best-fitting model for the latency data included treatment, frequency, and accuracy as fixed factors with an interaction between treatment and accuracy had the lowest AIC (881.54) (see Appendix Table 7). When the spatial frequency of 0.07 cycles deg^{-1} (resolution used in training) was used as a reference group, the latency to enter the reward chamber significantly increased for all other spatial frequencies (Frequency 0.035 cycles deg^{-1}, Model Estimate ± standard error = 0.782 ± 0.197, t = 3.964, $p < 0.0001$; Frequency 0.140 cycles deg^{-1}, Model Estimate ± standard error = 1.154 ± 0.170, t = 6.798, $p < 0.0001$; Frequency 0.280 cycles deg^{-1}, Model Estimate ± standard error = 1.770 ± 0.174, t = 10.165, $p < 0.0001$; Frequency 0.437 cycles deg^{-1}, Model Estimate ± standard error = 1.915 ± 0.179, t = 10.719, $p < 0.0001$; Frequency 0.699 cycles deg^{-1}, Model Estimate ± standard error = 2.163 ± 0.212, t = 10.218, $p < 0.0001$). As in the tests with varying contrasts, there was no fixed effect of shaking

(Model Estimate ± standard error = -0.009 ± 0.197, t = -0.045, p = 0.965). However, the effect of treatment was accuracy dependent (X^2 = 7.14, df: 1, p = 0.008), with shaken bees making significantly faster correct choices (t = 4.176, p = 0.0001) but not incorrect choices (t = 0.044, p = 0.965).

When assessing the likelihood of making correct or incorrect choices, or omissions, the best-fitting model included choice category, treatment, and an interaction between these factors (see Appendix Table 8). Unlike with the varying contrast trials, the likelihood of a particular choice depended on whether the bee had been shaken or not (X^2 = 10.404, df: 2, p = 0.006). Shaken bees were more likely to enter the correct reward chamber compared to control bees (t = -2.154, p < 0.032), as well as less likely to omit responding (t = 2.218, p = 0.027).

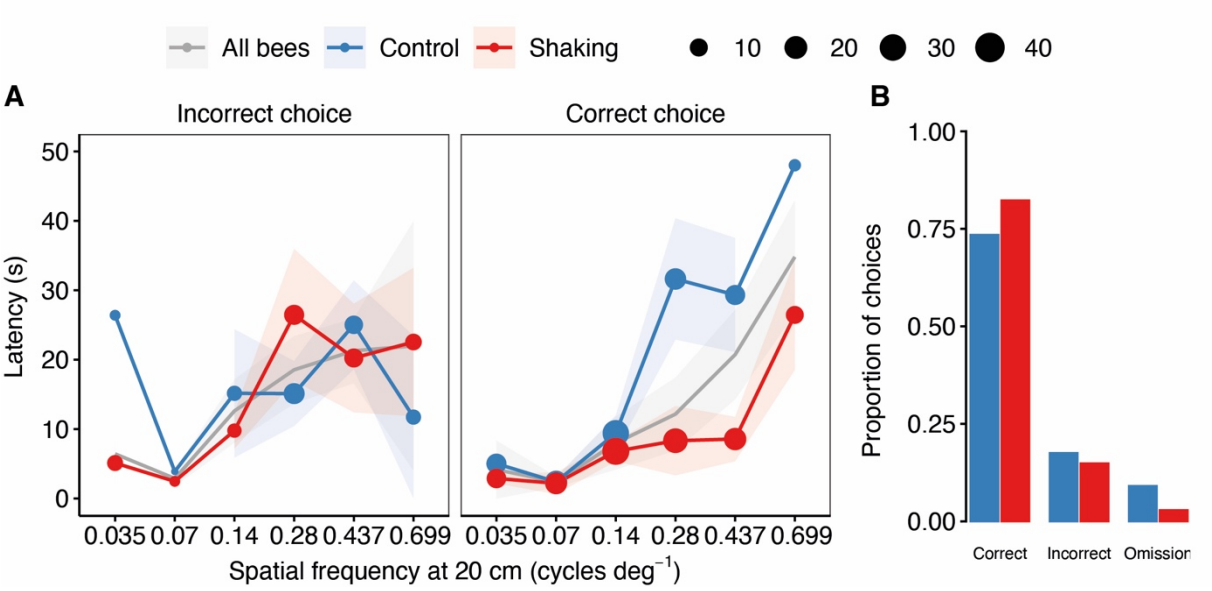

Figure 5. Shaken bees are faster to make correct choices, more likely to choose correctly and less likely to omit to choose. A) Latency to make incorrect (left) and correct (right) choices for Control (blue) and Shaken (red) bees. The solid grey line represents the cumulative latency averaged across all bees; Dots represent median values with 95% confidence intervals (shaded area). Each dot's size represents the number of bees contributing to the median. **B)** Proportions of correct and incorrect choices, and omissions for Control (blue bars) and Shaken (red bars) bees.

3.4. Discussion

Shaking affects spatial frequency generalisation

Bumblebees exposed to acute stress through simulated predatory attacks, i.e., shaking, displayed reduced contrast sensitivity, with their threshold rising to 36% Michelson contrast. Unmanipulated bees demonstrated higher sensitivity to lower contrasts, with a low contrast threshold of 22%. This is somewhat opposite to the response observed in human subjects, where exposure to a stimulus inducing fear enhanced low-level vision by improving contrast sensitivity thresholds (Phelps, Ling and Carrasco, 2006). While there is a decrease in contrast sensitivity with shaking, there is also a shift in spatial resolution that enhances the perception of fine-grained spatial features. This shift results in a spatial frequency threshold of approximately 0.34 cycles deg^{-1}, compared to the coarser perception observed in control bees, where the spatial resolution threshold was at around 0.24 cycles deg^{-1}.

Importantly, as with contrast sensitivity, these effects are somewhat opposite to what has been observed in human studies. Specifically, in humans, following the presentation of a fear-inducing facial expression, the response is enhanced for perceiving low spatial frequencies while impaired for perceiving high spatial frequencies (Bocanegra and Zeelenberg, 2009). Thus, fear in humans leads to a prioritisation of processing broader, global configurations at the expense of fine visual details. Results presented here show that in bees, just like in humans, negative emotions facilitate spatial resolution. However, in contrast to humans, this enhancement is not due to improved processing of low spatial frequencies but rather high spatial frequencies.

Interestingly, Bocanegra and Zeelenberg's (2009) study demonstrates that emotions do not simply change, but rather inverse perceptual prioritisation. The detection ability of individuals in a neutral state was highest for the patterns expressed in higher and poorest for those in lower spatial frequencies. This was opposite to what was observed in individuals exposed to fearful faces: their detection was best at lower and worst at higher spatial frequencies. Thus, emotions shifted the peak sensitivity from high to low spatial frequencies. Contrary, the control and shaken groups in my study, maintained peak sensitivity at the trained spatial frequencies (see Fig. 5A). However, there was a clear difference in how the response curves attenuated; the

shaken bees exhibited a smoother “flatter” response as similarity with conditioned resolution decreased, while the control bees displayed a steeper decline (Fig. 4A, 5A). Consequently, shaking did not shift the maximum response point but rather facilitated further generalisation, extending bee responses to higher spatial frequencies. Stimulus generalisation may occur after absolute conditioning, and implies that a learned conditioned response goes beyond the trained stimuli, specifically to novel stimuli that share similarities with the trained ones (Shepard, 1987). In a seminal work, Guttman and Kalish (1956) first trained pigeons to associate specific wavelengths of light with a reward (Guttman and Kalish, 1956). When tested with a series of unfamiliar light wavelengths, the pigeons exhibited generalisation of their responses to these novel stimuli; the pigeon greatly responded to those wavelengths that were most similar to the learned one, and as the similarity decreased, so did the response. Overall, such stimulus generalisation that leads to a broadening response curve reflects an animal’s ability to react adaptively to new stimuli based on past experience (Shepard, 1987). This ability is even more crucial in the context of threat-related responses. Since animals are unlikely to encounter the exact same threats, the capacity to generalise from prior experiences to similar but novel contexts becomes vital for survival. The generalisation of threat-related stimuli to conceptually or perceptually similar stimuli has been also previously demonstrated (Dunsmoor, White and LaBar, 2011; Dymond *et al.*, 2015). Moreover, excessive fear generalisation is also linked to conditions such as anxiety-related disorders (Cooper *et al.*, 2022). However, the influence of task-irrelevant affective states on response generalization of non-affect-related stimuli remains unknown. Based on the results presented here, bees could be a good model system to address these questions.

Taken together, the results presented here show that in bumblebees, exposure to acute stress reduces contrast sensitivity while enhancing spatial frequency sensitivity. This suggests the possibility of a trade-off, where the bee’s vision sacrifices contrast detection in favour of better resolution of fine details. This shift likely reflects an adaptive response, prioritising the perception of fine spatial features over contrast in stressful situations. While these results may seem to contrast with those in humans, where stress enhances contrast sensitivity but reduces fine detail perception, considering the different ecologies, it is possible to suggest that the direction of the trade-off is species-specific, supporting the unique needs of each species. Future studies could explore the possible neural mechanisms underlying such a trade-off.

Negative affect potentially prepares the visual system for a predatory response

Early studies in honeybees demonstrate that while both local (finer details, such as inner pattern textures) and global (broader spatial characteristics, like shape or size) features can be used in pattern recognition, bees exhibit an overall preference for global information (Dafni, Lehrer and Keyan, 1997; Avarguès-Weber *et al.*, 2015). However, while the global preference might come from favouring low spatial frequencies, their ability to change toward finer details with prior experience shows the flexibility of the visual system (Dafni, Lehrer and Keyan, 1997; Avarguès-Weber *et al.*, 2015).

In humans, rapid threat detection has been proposed to rely on fast and automatic processing of coarse visual features (Öhman, 2005; Lojowska *et al.*, 2019). For example, studies have shown that the response to snake images (stimuli known to induce fear) was much faster than to neutral images like frogs or rabbits. However, this was true only when the images were filtered using low-spatial and not high-spatial frequency filters (Mermillod *et al.*, 2018). Similarly, compared to individuals in a safe state, those in a threatened state (i.e., anticipating an electric shock) exhibit faster detection of a subliminally presented grating orientation when the grating has low, but not high, spatial frequencies (Lojowska *et al.*, 2019). These examples show how the visual system in humans can be fine-tuned for better processing of specific spatial resolutions. It, however, remains an open question whether similar mechanisms drive the shift in spatial resolution threshold towards a finer resolution observed in the present study. If so, shaking could indeed fine-tune bee visual system to prioritise high spatial resolution over low spatial resolution, which is typically used to guide bee decisions (Dafni, Lehrer and Keyan, 1997; Avarguès-Weber *et al.*, 2015).

Despite the differences in the architecture of the visual systems, it is safe to assume that bees, just as mammals, have evolved to maximize survival. This would imply that, in a state of heightened alertness, both mammals and bees should prioritise the processing of spatial information critical for a fast response. However, the exact characteristics of information gaining priority will be shaped by a species ecology, and thus may differ. To detect and escape threats, humans, for example, would benefit from perceiving low spatial features, such as a more global environment configuration. Bees, however, could gain an advantage from perceiving finer information to detect their natural predators, such as crab spiders *Misumena vatia* (Dukas and Morse,

2003). This spider can camouflage among the flowers and become almost indistinguishable from the environment. One mechanism that enables bumblebees to enhance their ability to detect cryptic spiders is side-to-side scanning (Ings, Wang and Chittka, 2012). These movements improve edge detection by gradually amplifying the cryptic spider shape. Thus, resolving fine details is crucial for bees to detect predators.

While side-to-side scanning can facilitate predator detection, it is time-consuming and thus costly (Ings, Wang and Chittka, 2012). However, if the risk of predation increases, the ability to quickly notice fine details like edges and textures becomes crucial. In this study, I show that being in a negative state itself may facilitate this process, as bees that experienced adverse events simulating a predatory attack, show a response generalisation towards higher spatial frequencies. If shaking indeed simulates a predation attempt, which typically indicates high-risk situations and imminent dangers, it is possible that being in the resulting heightened fear-like state automatically sharpens bee perception for detecting fine features in their surroundings. This increased perception of finer details could then potentially be an adaptive mechanism that enhances bees' capacity to spot the features of their sit-and-wait predators.

Shaken bees make faster and more accurate final choices with increasing spatial frequency

In my experiments, the final choices of bees, which involved entering the reward chamber, were also affected by shaking but only in the trials with varying spatial frequencies. Shaken bees outperformed control bees in choosing the correct reward chamber and did so significantly faster. While resolving high spatial frequencies to make a correct entry required investing more time in control bees and hence trading speed for accuracy, this was not the case with shaken bees. The faster accurate final choices in shaken bees are somewhat opposite to the expected psychophysical speed-accuracy trade-off that postulates increasing accuracy with increasing decision time (Chittka *et al.*, 2003; Marshall *et al.*, 2006; Heitz and Schall, 2012).

The principle of the speed-accuracy trade-off is based on two factors: sensory evidence accumulation and decision time. Decision-making involves gradually accumulating sensory evidence until a certain threshold is reached, at which point a

choice is made. When dealing with noisy signals, such as those associated with increasing spatial frequencies, investing more time in gathering evidence may improve accuracy (Chittka *et al.*, 2003; Marshall *et al.*, 2006; Heitz and Schall, 2012). Therefore, longer decision times may indicate weaker sensory evidence. This looks exactly the case for the control bees in my experiment. As spatial frequency increases, the choice latency becomes longer as bees spend more time scanning both gratings until the spatial frequency is resolved and the correct choice can be made. Some control bees did not reach the decision threshold even after 120 seconds of close inspection of both gratings. Control bees therefore were more likely to omit responses when unsure.

When making a correct choice, shaken bees choose the reward chamber faster than control bees. Despite a tendency to slow down as spatial frequency increases, the difference in latencies when making correct choice remained across all frequencies (see Fig. 5A). Importantly, the latencies on trials with gratings of frequency 0.14, 0.28, and 0.437 cycle deg^{-1} are similar to those for the trained spatial frequency, suggesting that bees may be generalising to higher spatial frequencies, which is also supported by the shift in thresholds (Fig. 3A). Therefore, these results suggest that shaken bees needed less time to accumulate evidence and make correct choices. Possibly, shaking reduced the perceptual noise as a result of increased in visual acuity. These findings are in line with findings in humans that demonstrate that exposure to emotionally arousing auditory cues (e.g., growling dogs and fire alarms) reduced search times without compromising accuracy (Asutay and Västfjäll, 2017). However, the effect was observed only on the trials where distinguishing between targets and distractors was harder.

Interestingly, recent studies however demonstrate that honeybees can make accurate decisions fast, arguing against the speed-accuracy trade-off (Maboudi *et al.*, 2023). Authors point out that when considering the speed-accuracy trade-off, usually a fixed evidence threshold for making decisions is used. However, this is not an ecologically accurate assumption, as there are associated costs with longer sampling (e.g., predation, energy). Therefore, the authors suggest that one way to avoid these costs is to adjust the threshold and accept only those options that gain high confidence after short sampling. The costs of sampling are likely to increase after experiencing shaking, for example, due to stress-induced energy losses (Even, Devaud and Barron, 2012) or an increased risk of predatory attacks (Mobbs *et al.*, 2018). Applying Maboudi et al. (2023) proposed rationale to the observed fast and accurate choices in shaken

bees suggests the possibility that shaking resulted in shifting the decision threshold towards the acceptance of options when the evidence for being correct is high and accumulates in a short scanning period. However, under this strategy, Maboudi et al. (2023) also suggest that the rate of rejections (both false positives and correct) will increase. Assessing rejection rates in the present study would require a more in-depth video analysis that measures scanning behaviour around the grating and reward chambers. Unfortunately, this is not available within the scope of the current thesis but is worth investigating in the future.

From the physiological viewpoint, an alternative explanation may be that shaking could increase tonic arousal, making bees move faster without perceptual effects. Exposure to acute stressful events induces a flight-or-fight response, making animals, including bees, more aroused (Even, Devaud and Barron, 2012). However, simply being aroused would most likely result in desire to escape. If so, bees would likely be flying erratically and not engaging in the task that requires making energetically costly choices. Similarly, increasing choice speed without perceptual effects would not make shaken bees more accurate. Alternatively, increased arousal can also temporarily modulate alertness, increase neuronal responsiveness and boost attention and perception (Phelps and LeDoux, 2005; LeDoux, 2012). This could explain the result observed in my experiment.

The absence of an effect in trials with high saliency reported in Asutay and Västfjäll (2017) may suggest why shaking bees did not affect bee responses in trials with decreasing contrast in the present study. The contrasts selected for this experiment were rather salient to bees, with all bees performing well (proportion of correct choices ≥75%). This high salience, and therefore easy discriminability between horizontal and vertical patterns, is likely influenced by the high luminance of the setup. Nevertheless, as the contrast decreased both control and shaken bees were slower to choose (see Fig. 4A). It is, therefore, possible to speculate that a more significant difference in choice latency could occur at much lower contrasts. Lower contrast levels (e.g., 3%) should be included in future research.

Unmanipulated bees show similar spatial resolution but greater contrast sensitivity than previously characterised

Finally, it is worth discussing the results of the control group and comparing them to previous work on bumblebees. Chakravarthi at el. (2016) reported a relatively low contrast sensitivity threshold, 63.6%, for *Bombus terrestris*, whereas, in our study, control bees exhibited higher sensitivity with a threshold of 22%. Although the setup and stimuli used in the present study are identical to those in Chakravarthi et al. (2016), some differences remain that may explain the significant variation between my results and theirs.

The first and most apparent is a difference in the luminance condition of both studies. Chakravarthi et al. (2016) conducted their experiments in relatively dim conditions, with just 500 lux. The setup illumination in the present study, however, was much brighter with 1100 lux. Visual acuity is heavily influenced by luminance; at low light levels, there may not be sufficient photons for reliable signal processing, leading to reduced contrast sensitivity. The visual acuity in bees is particularly reduced under low illumination conditions, and it improves logarithmically with increasing light levels until reaching a peak (Hecht and Wolf, 1929). Testing bees in a set-up with luminance that was twice as bright as the previous study could therefore easily explain the improved contrast perception I observed.

Another contributing factor to the difference in contrast sensitivity could be the behavioural aspect of the experiments. In our study, bee choice was defined as crossing a line positioned 20 cm from the grating in either decision arm for the first time. This gave the bees the freedom to move before making their decisions. In contrast, Chakravarthi et al. (2016) required bees to make decisions from a fixed point. Consequently, because of the movement allowed in the present study, stimuli appeared to be in motion for the bees, potentially resulting in higher sensitivity. Previous research in birds has shown increased contrast sensitivity for moving stimuli (Haller *et al.*, 2014).

When analysing the spatial frequency results, I found that the spatial resolution threshold of 0.24 cycles deg^{-1} in control bees closely aligns with what was reported earlier by Chakravarthi et al. (2016). The reported resolution threshold of 0.21 cycles deg^{-1} was determined for a proportion of 0.75 correct choices. In the present study, however, the threshold was lower, 0.65. If the criterion of 0.65 correct choices were used in this earlier study, like I did, their threshold would approximate at 0.26 cycles deg^{-1}, somewhat close to the threshold observed in the present study. The difference

could also be due to the different orientations used as the rewarding cue: horizontal in the current study and vertical in Chakravarthi et al. (2016). It has been shown, that the interommatidial angle of bees in the vertical and horizontal plane are different (Spaethe and Chittka, 2003). It is possible that the difference in the angle between orientations could play a role in the variations in acuity found in both current and earlier study. However, this seems unlikely since a previous study on *Bombus impatiens* demonstrated similar results in detecting targets with both horizontal and vertical orientations (Macuda *et al.*, 2001). This suggests that the orientation of the conditioned grating might not have an impact on visual acuity.

The current results suggest that a previously discovered emotion-like state (here and elsewhere (Bateson *et al.*, 2011; Solvi, Baciadonna and Chittka, 2016; Schlüns *et al.*, 2017; Strang and Muth, 2023) may potentially modulate bee vision in a way that facilitates task performance. Shifts in both contrast and spatial frequency thresholds indicate that this modulation is a fine-tuning of bee vision for better fine-detail discrimination. Moreover, the observed correct, and fast final choices, further suggest that shaking reduced noise in bee visual perception. Arguably, the effects observed in the present study may be the first insight into the adaptive function of emotion-like states in insects. Considering the potential benefits of emotion, such as fear, when it comes to detecting and responding to relevant information, it should not perhaps be surprising that mechanism analogous to those known in humans may be conserved across species or evolved independently through convergent evolution.

3.5. Appendix

Table 1

Summary of model selection to analyse the impact of stress treatments (shaking/trapping) on performance in the visual acuity experiments

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	AIC	ΔAIC
Accuracy at 20 cm	**Treatment+Contrast+Eye width**	**ID**	**5**	**149.340**	
	Treatment+Contrast*Eye width	ID	6	150.255	0.915
	Treatment*Eye width+Contrast	ID	6	150.883	1.542
	Treatment*Contrast+Eye width	ID	6	150.990	1.650
	Treatment*Contrast*Eye width	ID	9	154.391	5.051

Model pairwise comparison: Assessing the significance of interaction terms using anova()

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	χ2	Pr(>χ2)
Accuracy at 20 cm	**Treatment+Contrast+Eye width**	**ID**	**5**		
	Treatment+Contrast*Eye width	ID	6	1.086	0.298
	Treatment+Contrast+Eye width	**ID**	**5**		
	Treatment*Eye width+Contrast	ID	6	0.458	0.499
	Treatment+Contrast+Eye width	**ID**	**5**		
	Treatment*Contrast+Eye width	ID	6	0.350	0.554

The table presents the model selection procedure. Models induced “Accuracy at decision line” (briary variable, 1 for crossing decision line in the correct arm, 0 for crossing in the incorrect arm) as response variable; treatment group (two level factor: control and shaking), contrast (fixed factor Contrast) , and “Eye width” (continuous ascending variable) as explanatory factors. The table includes specifications for both fixed and random explanatory variables, along with corresponding degrees of freedom (d.f.) and Akaike’s information criterion (AIC) values. The preferred model for each dependent variable, as determined by the lowest AIC value, is denoted in bold. More complex models—with an interaction term—that had a ΔAIC < 2 were considered not significantly different from the simplest model with only fixed factors. In such cases, the significance of the interaction term was further assessed using anova().

Table 2

Summary of model selection to analyse the impact of stress treatments (shaking/trapping) on performance in the visual acuity experiments

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	AIC	ΔAIC
log(latency) at 20 cm decision point	Treatment+Contrast	ID	8	348.944	
	Treatment*Contrast	**ID**	**12**	**350.702**	**1.758**

Model pairwise comparison: Assessing the significance of interaction terms using anova()

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	χ2	Pr(>χ2)
log(latency) at 20 cm decision point	Treatment+Contrast	ID	8		
	Treatment*Contrast	**ID**	**12**	**12.830**	**0.012**

The table presents the model selection procedure. Models induced "log(Latency)" at decision line (log-transformed time taken to cross decision line) as response variable; treatment group (two level factor: control and shaking), and contrast (fixed factor Contrast) as explanatory factors. The table includes specifications for both fixed and random explanatory variables, along with corresponding degrees of freedom (d.f.) and Akaike's information criterion (AIC) values. The preferred model for each dependent variable, as determined by the lowest AIC value, is denoted in bold. More complex models—with an interaction term—that had a ΔAIC < 2 were considered not significantly different from the simplest model with only fixed factors. In such cases, the significance of the interaction term was further assessed using anova().

Table 3

Summary of model selection to analyse the impact of stress treatments (shaking/trapping) on performance in the visual acuity experiments

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	AIC	ΔAIC
log(latency) to enter reward chamber	**Treatment+Contrast+Accuracy**	**ID**	**6**	**562.054**	
	Treatment*Accuracy+Contrast	ID	7	563.196	1.142
	Treatment+Contrast*Accuracy	ID	7	571.425	9.371
	Treatment*Contrast+Accuracy	ID	7	572.093	10.039
	Treatment*Contrast*Accuracy	ID	10	590.741	28.687

Model pairwise comparison: Assessing the significance of interaction terms using anova()

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	χ2	Pr(>χ2)
log(latency) to enter reward chamber	**Treatment+Contrast+Accuracy**	**ID**	**6**		
	Treatment*Accuracy+Contrast	ID	7	0.682	0.409

The table presents the model selection procedure. Models induced "log(Latency) to enter" (log-transformed time taken to enter reward chamber) as response variable; treatment group (two level factor: control and shaking), contrast (fixed factor Contrast), and "Accuracy" (binary variable, 1 for entering reward chamber in the correct arm, 0 for entering reward chamber in the incorrect arm) as explanatory factors. The table includes specifications for both fixed and random explanatory variables, along with corresponding degrees of freedom (d.f.) and Akaike's information criterion (AIC) values. The preferred model for each dependent variable, as determined by the lowest AIC value, is denoted in bold. More complex models—with

an interaction term—that had a ΔAIC < 2 were considered not significantly different from the simplest model with only fixed factors. In such cases, the significance of the interaction term was further assessed using anova().

Table 4

Summary of model selection to analyse the impact of stress treatments (shaking/trapping) on performance in the visual acuity experiments

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	AIC	ΔAIC
Choice	**Treatment+Category**	**ID**	**5**	**391.497**	
	Treatment*Category	ID	7	392.765	1.268

Model pairwise comparison: Assessing the significance of interaction terms using anova()

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	χ2	Pr(>χ2)
Choice	**Treatment+Category**	**ID**	**5**		
	Treatment*Category	ID	7	2.732	0.255

The table presents the model selection procedure. Models induced "Choice" (binary variable, see Materials and methods) as response variable; treatment group (two level factor: control and shaking), and "Category" (three level factor: correct, incorrect and omission) as explanatory factors. The table includes specifications for both fixed and random explanatory variables, along with corresponding degrees of freedom (d.f.) and Akaike's information criterion (AIC) values. The preferred model for each dependent variable, as determined by the lowest AIC value, is denoted in bold. More complex models—with an interaction term—that had a ΔAIC < 2 were considered not significantly different from the simplest model with only fixed factors. In such cases, the significance of the interaction term was further assessed using anova().

Table 5

Summary of model selection to analyse the impact of stress treatments (shaking/trapping) on performance in the visual acuity experiments

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	AIC	∆AIC
Accuracy at 20 cm	**Treatment+Frequency+Eye width**	**ID**	**9**	**396.005**	
	Treatment*Eye width+Frequency	ID	10	397.498	1.492
	Treatment*Frequency+Eye width	ID	14	403.668	7.663
	Treatment+Frequency*Eye width	ID	14	404.174	8.168
	Treatment*Frequency*Eye width	ID	25	418.684	22.678

Model pairwise comparison: Assessing the significance of interaction terms using anova()

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	$\chi 2$	Pr(>$\chi 2$)
Accuracy at 20 cm	**Treatment+Frequency+Eye width**	**ID**	**9**		
	Treatment*Eye width+Frequency	ID	10	1.498	0.221

The table presents the model selection procedure. Models induced “Accuracy at decision line” (briary variable, 1 for crossing decision line in the correct arm, 0 for crossing in the incorrect arm) as response variable; treatment group (two level factor: control and shaking), spatial frequencies (six level fixed factor Frequency), and “Eye width” (continuous ascending variable) as explanatory factors. The table includes specifications for both fixed and random explanatory variables, along with corresponding degrees of freedom (d.f.) and Akaike’s information criterion (AIC) values. The preferred model for each dependent variable, as determined by the lowest AIC value, is denoted in bold. More complex models—with an interaction term—that had a ∆AIC < 2 were considered not significantly different from the simplest model with only fixed factors. In such cases, the significance of the interaction term was further assessed using anova().

Table 6

Summary of model selection to analyse the impact of stress treatments (shaking/trapping) on performance in the visual acuity experiments

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	AIC	∆AIC
log(latency) at 20 cm decision point	**Treatment+Frequency**	**ID**	9	572.529	
	Treatment*Frequency	ID	14	588.676	16.147

The table presents the model selection procedure. Models induced “log(Latency)” at decision line (log-transformed time taken to cross decision line) as response variable; treatment group (two level factor: control and shaking), and spatial frequencies (six level fixed factor Frequency) as explanatory factors. The table includes specifications for both fixed and random explanatory variables, along with corresponding degrees of freedom (d.f.) and Akaike’s information criterion (AIC) values. The preferred model for each dependent variable, as determined by the lowest AIC value, is denoted in bold. More complex models—with an interaction term—that had a ∆AIC < 2 were considered not significantly different from the simplest model with only fixed factors. In such cases, the significance of the interaction term was further assessed using anova().

Table 7

Summary of model selection to analyse the impact of stress treatments (shaking/trapping) on performance in the visual acuity experiments

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	AIC	∆AIC
log(latency) to enter reward chamber	**Treatment*Accurcy+Frequency**	**ID**	**11**	**881.541**	
	Treatment+Accurcy+Frequency	ID	10	885.405	3.863
	Treatment*Accurcy*Frequency	ID	26	885.655	4.114
	Treatment+Accurcy*Frequency	ID	15	892.062	10.521
	Treatment+Frequency+Accurcy	ID	15	892.963	11.422

The table presents the model selection procedure. Models induced "log(Latency) to enter" (log-transformed time taken to enter reward chamber) as response variable; treatment group (two level factor: control and shaking), and spatial frequencies (six level fixed factor Frequency), and "Accuracy" (binary variable, 1 for entering reward chamber in the correct arm, 0 for entering reward chamber in the incorrect arm) as explanatory factors. The table includes specifications for both fixed and random explanatory variables, along with corresponding degrees of freedom (d.f.) and Akaike's information criterion (AIC) values. The preferred model for each dependent variable, as determined by the lowest AIC value, is denoted in bold. More complex models—with an interaction term—that had a ∆AIC < 2 were considered not significantly different from the simplest model with only fixed factors. In such cases, the significance of the interaction term was further assessed using anova().

Table 8

Summary of model selection to analyse the impact of stress treatments (shaking/trapping) on performance in the visual acuity experiments

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	AIC	∆AIC
Choice	**Treatment*Category**	**ID**	**7**	**819.644**	
	Treatment+Category	ID	5	826.971	7.327

The table presents the model selection procedure. Models induced "Choice" (binary variable, see Materials and methods) as response variable; treatment group (two level factor: control and shaking), and "Category" (three level factor: correct, incorrect and omission) as explanatory factors. The table includes specifications for both fixed and random explanatory variables, along with corresponding degrees of freedom (d.f.) and Akaike's information criterion (AIC) values. The preferred model for each dependent variable, as determined by the lowest AIC value, is denoted in bold. More complex models—with an interaction term—that had a ∆AIC < 2 were considered not significantly different from the simplest model with only fixed factors. In such cases, the significance of the interaction term was further assessed using anova().

Table 9

Summary of statistical model results analysing the impact of stress treatments (shaking/trapping) on performance in the visual acuity experiments

Response variable		Estimate	Std. Error	z value	Pr(>|z|)
Accuracy at decision line	(Intercept)	2.083	6.165	0.338	0.7354
	TreatmentShaking	-0.410	0.505	-0.811	0.4175
	Contrast	**0.030**	**0.011**	**2.761**	**0.0058**
	Eye width	-0.498	2.309	-0.022	0.8291

The table provides an overview of the results obtained from statistical analyses aimed at investigating the effects of shaking, on the likelihood of making a correct first choice at the decision line. The response is a binary variable "Accuracy at decision line"; fixed factors are Treatment (two level fixed factors: control and shaking), Contrast (ascending continuous fixed factor) and Eye width (ascending continuous fixed factor). The data is presented in terms of estimated coefficients, standard errors, z-values, and associated p-values for response variables and predictor variables.

Table 10

Summary of statistical model results analysing the impact of stress treatments (shaking/trapping) on performance in the visual acuity experiments

Response variable	Factor	Chisq	df	Pr(>Chisq)
log(latency) at 20 cm decision point	Treatment	0.03	1	0.874
	Contrast	8.84	4	0.065
	Treatment*Contrast	**12.69**	**4**	**0.013**

The table provides an overview of the results obtained from statistical analyses aimed at investigating the effects of shaking, on the latency of making a correct first choice at the decision line. The response is a log-transformed "(log)Latency"; fixed factors are Treatment (two level fixed factors: control and shaking), Contrast (six level factor: 22%, 39%, 54%, 68%, 87%), and their interaction term.

Table 11

Summary of statistical model results analysing the impact of stress treatments (shaking/trapping) on performance in the visual acuity experiments

Response variable		Estimate	Std. Error	t value	Pr(>|t|)
log(latency) to enter	**(Intercept)**	**2.528**	**0.259**	**9.762**	**0.0000**
	TreatmentShaking	-0.321	0.193	-1.662	0.1050
	Contrast	**-0.012**	**0.003**	**-4.023**	**0.0001**
	AccuracyCorrect	-0.010	0.215	-0.465	0.6420

The table provides an overview of the results obtained from statistical analyses aimed at investigating the effects of shaking, on the latency of making a correct first choice at the decision line. The response is a log-transformed "(log)Latency"; fixed factors are Treatment (two level fixed factors: control and shaking), Contrast (ascending continuous fixed factor) and Accuracy (two level fixed factor: correct and incorrect). The data is presented in terms of estimated coefficients, standard errors, t-values, and associated p-values for response variables and predictor variables.

Table 12

Summary of statistical model results analysing the impact of stress treatments (shaking/trapping) on performance in the visual acuity experiments

Response variable		Estimate	Std. Error	z value	Pr(>|z|)
Choice	(Intercept: Control; Correct)	1.585	0.231	6.854	**0.000**
	CategoryIncorrect	-3.245	0.274	-11.855	**0.000**
	CategoryOmission	-6.149	0.736	-8.355	**0.000**
	TreatemntShaking	0.000	0.269	0.000	1.000

The table provides an overview of the results obtained from statistical analyses aimed at investigating the effects of shaking, on the likelihood of making a correct, incorrect of omitting from choosing the reward chamber to enter. The response is a binary variable "Choice"; fixed factors are Category (three level fixed factors: correct, incorrect and omission) and Treatment (two level fixed factors: control and shaking). The data is presented in terms of estimated coefficients, standard errors, z-values, and associated p-values for response variables and predictor variables.

Table 13

Summary of statistical model results analysing the impact of stress treatments (shaking/trapping) on performance in the visual acuity experiments

Response variable		Estimate	Std. Error	z value	Pr(>|z|)
Accuracy at decision line	(Intercept: Control; Frequency 0.070)	10.255	4.267	2.403	**0.0163**
	TreatmentShaking	0.298	0.328	0.906	0.3650
	Frequency 0.035	-1.480	0.717	-2.064	**0.0391**
	Frequency 0.14	-0.723	0.693	-1.043	0.2970
	Frequency 0.28	-2.200	0.658	-3.342	**0.0008**
	Frequency 0.437	-2.210	0.660	-3.347	**0.0008**
	Frequency 0.699	-2.682	0.700	-0.383	**0.0001**
	Eye width	-2.890	1.579	-1.830	**0.0672**

The table provides an overview of the results obtained from statistical analyses aimed at investigating the effects of shaking, on the likelihood of making a correct first choice at the decision line. The response is a binary variable “Accuracy at decision line”; fixed factors are Treatment (two level fixed factors: control and shaking), Frequency (six level factor: 0.07, 0.035, 0.14, 0.28, 0.437 and 0.699 cycle deg^{-1}) and Eye width (ascending continuous fixed factor). The data is presented in terms of estimated coefficients, standard errors, z-values, and associated p-values for response variables and predictor variables.

Table 14

Summary of statistical model results analysing the impact of stress treatments (shaking/trapping) on performance in the visual acuity experiments.

Response variable		Estimate	Std. Error	t value	Pr(>|t|)
log(latency) at decision line	(Intercept: Control; Frequency 0.070)	-0.876	0.112	-7.849	**0.0000**
	TreatmentShaking	0.048	0.122	0.396	0.6941
	Frequency 0.035	-0.048	0.109	-4.441	0.6596
	Frequency 0.14	-0.060	0.095	-0.630	0.5293
	Frequency 0.28	0.043	0.095	0.457	0.6482
	Frequency 0.437	0.169	0.095	1.775	**0.0770**
	Frequency 0.699	0.237	0.109	2.175	**0.0304**

The table provides an overview of the results obtained from statistical analyses aimed at investigating the effects of shaking, on the latency of making a correct first choice at the decision line. The response is a log-transformed “(log)Latency”; fixed factors are Treatment (two level fixed factors: control and shaking) and Frequency (six level factor: 0.07, 0.035, 0.14, 0.28, 0.437 and 0.699 cycle deg^{-1}). The data is presented in terms of estimated coefficients, standard errors, t-values, and associated p-values for response variables and predictor variables.

Table 15

Summary of statistical model results analysing the impact of stress treatments (shaking/trapping) on performance in the visual acuity experiments

Response variable		Estimate	Std. Error	t value	Pr(>|t|)
log(latency) to enter	(Intercept)	1.127	0.205	5.496	**0.0000**
	TreatmentShaking	-0.009	0.197	-0.045	0.9645
	AccuracyCorrect	0.171	0.161	1.063	0.2888
	Frequency 0.035	0.782	0.197	3.964	**0.0001**
	Frequency 0.14	1.154	0.170	6.798	**0.0000**
	Frequency 0.28	1.770	0.174	10.165	**0.0000**
	Frequency 0.437	1.915	0.179	10.719	**0.0000**
	Frequency 0.699	2.163	0.212	10.218	**0.0000**
	TreatmentShaking*AccuracyCorrect	-0.581	0.217	-2.672	**0.0079**

The table provides an overview of the results obtained from statistical analyses aimed at investigating the effects of shaking, on the latency of making a correct first choice at the decision line. The response is a log-transformed "(log)Latency"; fixed factors are Treatment (two level fixed factors: control and shaking), Frequency (six level factor: 0.07, 0.035, 0.14, 0.28, 0.437 and 0.699 cycle deg^{-1}) and Accuracy (two level fixed factor: correct and incorrect). The data is presented in terms of estimated coefficients, standard errors, t-values, and associated p-values for response variables and predictor variables

Table 16

Analysis of Deviance Table (car::Anova, Type II Wald chi-square tests) summarises the statistical significance of each factor and interaction in the statistical model, analysing the impact of shaking on the on latency to enter reward chamber in the trials with varying spatial frequencies

	Chisq	df	Pr(>Chisq)
Treatment	11.03	1	0.001
Accuracy	1.33	1	0.248
Frequency	**173.05**	**5**	**0.000**
Treatment*Accuracy	**7.14**	**1**	**0.008**

The table provides an overview of the results obtained from statistical analyses aimed at investigating the effects of shaking, on the latency of making a correct first choice at the decision line. The response is a log-transformed "(log)Latency"; fixed factors are Treatment (two level fixed factors: control and shaking), Frequency (six level factor: 0.07, 0.035, 0.14, 0.28, 0.437 and 0.699 cycle deg^{-1}) and Accuracy (two level fixed factor: correct and incorrect).

Table 17

Analysis of Deviance Table (car::Anova, Type II Wald chi-square tests) summarises the statistical significance of each factor and interaction in the statistical model, analysing the impact of shaking on the likelihood of a final choice being correct, incorrect, or omitted

	Chisq	df	Pr(>Chisq)
Treatment	0.02	1	0.896
Category	323.41	2	0.000
Treatment*Category	10.40	2	0.006

The table provides an overview of the results obtained from statistical analyses aimed at investigating the effects of shaking, on the type of final choice. Individual choices were translated into a response variable of binomial format (0 or 1) within each choice category; fixed factors are Treatment (two level fixed factors: control and shaking) and Category of choice (three level fixed factor: correct, incorrect, omission).

CHAPTER FOUR

Acute stress enhances reversal learning in bees for contrasting but not similar outcome values

4.1 Introduction

In a changing world, the ability to adjust behaviour is key to survival. Cognitive flexibility is the ability to flexibly adapt to occurring changes in the environment (Kehagia, Murray and Robbins, 2010). Behavioural flexibility, on the other hand, represents the observable outcome of these cognitive processes. In humans, both emotional regulation and behavioural flexibility involve the same brain structure – the orbital prefrontal cortex (Rolls *et al.*, 1994; Bechara, Damasio and Damasio, 2000; Rudebeck *et al.*, 2013). Research has demonstrated that dysfunction in this brain area can result in difficulties with regulation and behavioural flexibility (Rolls *et al.*, 1994). In other mammals, behavioural flexibility is also affected by emotions. Individuals who suffer from emotional dysregulation disorders, such as anxiety or depression, often exhibit impairments in overall cognitive function (Goldstein and Mcewen, 2002; Holmes and Wellman, 2009; Gagnon and Wagner, 2016; Xia *et al.*, 2021) and lack behavioural flexibility (Holmes and Wellman, 2009; Izquierdo *et al.*, 2017). As stress, both acute and chronic, leads to negative emotions, research has focused on how stress impacts behavioural flexibility.

Reversal learning is a behavioural assay that measures behavioural flexibility (Kehagia, Murray and Robbins, 2010). Specifically, it measures the ability to adapt behaviour when previously rewarded contingencies are reversed. In the classic reversal learning paradigm, subjects are first trained to associate one stimulus with a reward and the other with punishment. Once the association is learned, the stimulus-outcome contingency is reversed. The previously rewarding stimulus is now punishing and vice versa. To succeed in reversal learning, subjects must suppress previously rewarded responses and learn a new response.

Using a reversal learning task, the effect of stress on behavioural flexibility has been shown to be more intricate than initially believed. Exposure to unpredictable chronic stress not only increases anxiety-like behaviour in rats, but rats also consistently demonstrate reduced behavioural flexibility (Bondi *et al.*, 2008; Jett and Morilak, 2013; Naegeli *et al.*, 2013). On the other hand, when animals experience short-term stress, it helps improve their ability to learn from reversals. For example, rats exposed to acute stress delivered by 30 min of restraint stress before the reversal learning task, required fewer trials to reverse the learned association (Thai, Zhang and

Howland, 2013). Similar effects were observed in mice that underwent 10 minutes of swim stress (Graybeal *et al.*, 2011), or rats exposed to acute elevated-platform stress (Dong *et al.*, 2013). Other stresses, e.g., tail pinching, however, do not show such facilitating effects (Butts, Floresco and Phillips, 2013). Thus, the effect of acute stress appears to depend on various factors, including the type, intensity, and repetition of the stressor (for a more detailed discussion, see (Hurtubise and Howland, 2017).

Social bees live in a naturally complex, ever-changing environment. As the availability of nectar and pollen can change over time, bees must be able to adapt their decisions in line with these changes (Harder, 1990; Chittka, Gumbert and Kunze, 1997). It is, therefore, crucial for bees to maintain the capacity for behavioural flexibility. Behavioural flexibility using reversal learning tasks has been demonstrated in bees in both the olfactory (Ben-Shahar *et al.*, 2000; Komischke *et al.*, 2002; Hadar and Menzel, 2010; Mota and Giurfa, 2010) and visual domains (Chittka, 1998; Raine and Chittka, 2012; Strang and Sherry, 2014). A recent study investigated the neural substrate for reversal learning, showing that the mushroom bodies are required for reversal learning but not for initial differential conditioning (Devaud *et al.*, 2007). The fact that reversal learning requires higher-order brain structures, such as the mushroom bodies, suggests, that these tasks are more demanding and involve conflict resolution.

While most studies focused on intra-colony differences in reversal learning abilities (Ben-Shahar *et al.*, 2000; Carr-Markell and Robinson, 2014; Cabirol *et al.*, 2018), to my knowledge, no study has investigated the effects of stress exposure on reversal learning in bees. Bees are subjected to multiple environmental stressors (Klein *et al.*, 2017), and they also tend to develop negative emotion-like states in response to acute stress exposure (see Chapter 2). It is, therefore, unknown if being in this negative state modulates the ability to maintain behavioural flexibility in bees. I therefore aimed to test whether exposure to an acute stress, a predatory attack simulated by vigorous shaking, would impair, or improve behavioural flexibility in bees.

Successful performance in reversal learning tasks heavily relies on reward processing. The individual must consistently track outcome values and detect changes, disengage from unrewarding stimuli, and develop a new rewarding stimulus-response association. I therefore used two experimental conditions to gain better insights. In both experimental conditions, bumblebees, *Bombus terrestris*, learned to forage in a flower patch with yellow and green artificial flowers. In the first experimental

condition (*Experimental Condition 1*), I specifically examined how shaking would impact learning when the value of possible outcomes was highly contrasting. Here, flowers of one colour provided a high-value reward, while flowers of another colour offered no reward. Therefore, the feedback bees received in this condition is highly informative. In the second condition, *Experimental Condition 2* both flower types were rewarding but differed in value (high vs low reward value). This second condition was designed to test if shaking would modulate fine reward perception. In *Experimental Condition 2*, I predicted that stress would reduce the sensitivity of bees to differences in reward. As a result, bees would be more likely to accept low rewards. In the reversal learning phase, flowers that the bees had earlier learnt as high rewarding would now be low rewarding, but stressed bees would still be willing to accept these flowers. If so, the acceptance of low reward would result in taking longer to switch their choices to the flowers that were now highly rewarding. I therefore hypothesised that in this experimental condition, shaking would slow down or impair reversal learning.

4.2. Materials and methods

Animal housing and preparation

I used five commercially raised bumblebee (*Bombus terrestris*) colonies (Koppert B.V., The Netherlands) in this experiment. After receiving bumblebees from the commercial breeder, I transferred bees to bipartite plastic nest boxes (28.0 × 16.0 × 12.0 cm) connected a flight arena with a transparent acrylic tunnel (56.0 × 5.0 × 5.0 cm) with a UV-transparent Plexiglas® lid and lit by a lamp (HF-P 1 14-35 TL5 ballast, Philips, The Netherlands) fitted with daylight fluorescent tubes (Osram, Germany). Animals were kept under standardised temperature conditions (23 ± 2℃). Housing, maintenance, and experimental procedures were non-invasive and were kept as close as possible to the natural living conditions of the animals. Through the experimental period, colonies were fed with ~ 3g commercial pollen daily (Koppert B. V., The Netherlands) and provided with sucrose solution (20% w/w) *ad libitum* outside the experimental work. Before the onset of the experiment, bees were food-deprived by removing the feeder for the arena. Food deprivation increases foraging motivation, as colonies with reduced food show higher activity and a more significant number of foraging bouts (Molet *et al.*, 2008). Although invertebrates do not fall under the Animals (Scientific Procedures) Act,

1986 (ASPA), the experimental design and protocols were developed incorporating the 3Rs principles.

Experimental set-up

All experiments were conducted with individual free-flying bees in a contained flight arena (110.0 × 61.0 × 40.0 cm). All experiments consisted of a pre-training phase followed by an initial conditioning phase and reversal learning phases. All phases included bees feeding on artificial flowers consisting of square chips (24 x 24 mm) with a well in which rewarding solutions could be placed. Each chip was placed on a glass vial (7 ml, 10 mm inner diameter) that elevated them 3 cm above the floor of the testing arena. In total, there were 30 flower positions, equally spaced at 11 cm from each other (Fig. 1). I recorded flower choices in each foraging bout; it began when a bee entered the arena and ended when the bee filled her crop and left the arena. The spatial arrangement of flowers was randomised between successive foraging bouts. After each foraging bout, I cleaned all flowers with 70% ethanol and hot water to eliminate any potential residual pheromonal cues left by the bee and then dried them with paper before reusing the flowers.

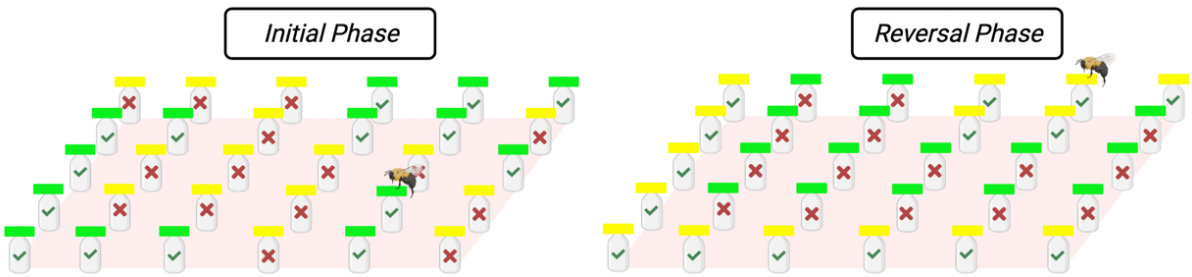

Figure 1. Reversal learning experimental set-up. The experiment consisted of two phases: the initial conditioning phase, *Initial Phase*, and the reversal learning phase, *Reversal Phase*. In the *Initial Phase*, all bees had to perform a discrimination task to learn the initial association between colours and rewards. Bees that reached the learning criterion (80% accuracy in the last 20 choices) proceeded to *Reversal Phase*, where the colour-reward association was reversed. The total number of flowers and the colours used were the same in both experimental conditions. The picture here depicts green flowers as rewarding in the initial phase and yellow flowers as unrewarding, but this was counterbalanced across all the bees.

Bee identification and pre-training

Before conducting the main experiments, the bees were given unrestricted access to the flight arena with 30 transparent flowers (24 x 24 mm) filled with 15 µl of a 50% (w/w) sugar solution placed at the centre of the flower. Motivated foragers were identified as ones that repeatedly filled their crop and returned to the nest. These foragers were individually marked with number tags for later identification (provided by Christian Graze KG, Weinstadt-Endersbach, Germany).

The individually marked bees were then trained to forage from 15 artificial flowers with two halves, each measuring 12x24 mm. One half was yellow (Perspex® Yellow 260), and the other green (Perspex® Green 6205). All bicoloured flowers were filled with 15 µl of a 50% (w/w) sugar solution placed at the centre of the flower. Therefore, during the pre-training phase, colour-naïve bees were exposed to both yellow and green colours for the first time simultaneously. This exposure allowed them to associate both colours used in the subsequent reversal learning task with the sugar reward. Pre-training continued for at least four foraging bouts to ensure that the bees had adequately learned to associate the colours with the sucrose reward.

Experimental Condition 1: Initial Phase

During training, all bees foraged individually in our set-up with yellow and green flowers. *Experimental Condition 1* aimed to assess if agitating bees impairs their ability to reverse their learning when there is a large difference in rewards between the two flower types. Bees in this condition had to learn to distinguish between rewarding and non-rewarding flowers based on colour. In the initial phase, I trained 24 individual foragers on 15 yellow and 15 green flowers, each measuring 24×24 mm. The rewarding flowers provided 15 µl of 50% (w/w) sugar solution, while the non-rewarding flowers had 15 µl of distilled water. The rewarding colours (green or yellow) were counterbalanced across bees for both treatment groups.

I recorded the choice sequence for each bee in each foraging bout until the bee made at least 120 choices. A choice was recorded when a bee probed a flower by either extending its proboscis or touching it with her antennae. Bees that reached the

learning criteria (80% accuracy in the last 20 choices) within 120 choices continued to the reversal phase.

Experimental Condition 1: Reversal Phase

All bees were allowed to forage overnight from feeders containing 20% sugar solution. The following day, bees that had been trained in the initial phase proceeded to the reversal learning phase, where I reversed the previously learned reward contingency. Prior to the reversal, individual learners were allowed to forage in an arena with transparent artificial flowers. The purpose of this was to reintroduce them to the availability of 50% sugar solution and boost their foraging motivation. The reversal phase began after they completed three foraging bouts.

In the reversal phase, flowers of the previously rewarding colour contained 15 µl of water, while previously unrewarding flowers contained a sugar reward of 15 µl of a 50% (w/w) sugar solution. As in the initial phase, I recorded bee choices until the learning criterion was met (80% accuracy in the last 20 choices) with a minimum of 120 choices. Upon the completion of the reversal phase, the bee was sacrificed by freezing and stored at -20°C. I measured bee body size by taking intertegular width under a dissection microscope with a digital calliper (RS PRO Digital Caliper, 0.01 mm ± 0.03 mm) (Hagen and Dupont, 2013).

In this experiment, five bees were excluded in the reversal phase as they either ceased foraging before completing 120 choices or failed to probe rewarding flowers within 60 min of being let in the flight arena.

Experimental Condition 2: Initial and reversal phase

The purpose of *Experimental Condition 2* was to determine if a negative state affected the bees' ability to accurately assess reward value and detect a change in colour-reward contingency. Here, both flower types contained a reward that varied in its quality. For example, if green flowers were high-rewarding, they contained 15 µl of 50% (w/w) sucrose solution, and the alternative yellow flowers 15 µl of 30% (w/w) sucrose solution, thus were the low-rewarding option. The rest of the procedure in *Experimental*

Condition 2 was the same as *Experimental Condition 1*. In the reversal phase, in addition to recording flower choices as described in *Experiment Condition 1*, I also recorded if a lower-quality reward was consumed or rejected. Bees are sensitive to reward concentration and will reject poor rewards in the presence of better ones. I was therefore interested in whether bees in a negative affective state would modulate their sensitivity to reward concentration, making shaken bees more likely to accept low rewards and fully deplete the sugar solution. The rest of the procedure in this experiment followed the steps described earlier in *Experimental Condition 1*.

In this experiment, two bees were excluded in the reversal phase as they either ceased foraging before completing 120 choices or failed to probe rewarding flowers within 60 min of being let in the flight arena.

Predatory attack simulation

In my previous work, I confirmed that shaking induces a negative affective state, as measured through a judgment bias test (see Chapter Two). Therefore, this treatment was chosen to examine whether a negative affective state could influence reversal learning in bees.

In each experiment, individual bees were randomly allocated to one of two groups: shaking (Group 1, n=12) or an unmanipulated group that served as a control (Group 2, n=12). The bees in the Shaken group were individually subjected to 60 seconds of shaking at 1200 rpm using a Vortex-T Genie 2. Before entering the flight arena, I allowed the bee to enter a custom-made tagging cage softened by the sponge to prevent physically harming animals while shaking (⌀ 40 mm, 7.5 cm length). After entering, the bee was gently nudged down with a soft foam plunger until the distance between the plunger and the bottom of the cage was reduced to ~3 cm. Once the plunger was secured, the cage with the bee was placed inside the vortex cup head. I then ran the Vortex at 1200 rpm for 60 seconds to shake the bee. The bee was shaken before each foraging bout during the reversal phase in both experimental conditions. After the shaking, I released the bee into the tunnel connected to the testing arena via a top opening. All bees initiated their foraging bouts in under 60 seconds.

Statistical analysis

Our hypothesis and statistical analyses of the main active choice experiment were preregistered at aspredicted.com (#82555). The data were plotted and analysed using RStudio v.3.2.2 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org) and custom-made scripts. All models were fit using maximum likelihood estimation. The nlminb optimiser was used where models failed to converge (glmmTMB function in the R glmmTMB package, (Brooks, Kristensen, van Benthem, *et al.*, 2017). In each analysis, several models were run and compared to identify significant fixed factors as well as possible interactions. The Akaike information criterion (AIC) scores for all models were calculated. I considered the model with the lowest AIC score the best model, that is, the model that provides a satisfactory explanation of the variation in the data (Johnson and Omland, 2004). Following accepted convention, models with an AIC difference of less than 2 were considered not significantly better than the model it is being compared to (Burnham and Anderson, 2004). In such case, anova() was used to determine whether adding interaction term significantly improved model fit. I used the package DHARMa (Hartig, 2020) for residual testing of all models.

To analyse the total choices and errors made before reaching the learning criterion, I fit generalised linear mixed models (Poisson distribution) to the data. Treatment (Control, Shaking) and Experimental Condition (*Experimental Condition 1* (50% vs water), *Experimental Condition 2* (50% vs 30%) were included as fixed factors and bee identity as a random factor to correct for repeated measurements. Separate models were run with the total number of choices and the total number of errors as dependent variables. To analyse the number of low-reward depletions in *Experimental Condition 2*, I also fit a generalised linear mixed model adjusted for zero-inflated count data (Brooks, Kristensen, Benthem, *et al.*, 2017). This model was fit using a Poisson distribution and the logarithm (log) link function. The models were fitted to a subset of choice data that included only incorrect choices. This is because, in the reversal phase, low-reward flowers are incorrect flowers only. Here treatment (Control, Shaking) and the order of flower choices were included as fixed factors and bee identity as a random factor. The order of flower choices was used to investigate how bee behaviour changed as they made more choices.

Some earlier studies have found the effect of stress is greater on late reversal learning. I therefore split the data into early and late phases (Graybeal *et al.*, 2011; Bryce and Howland, 2015). To do this, I first calculated a 20-choice moving average across all flower choices for each bee (i.e., choices 1–20, 2–21, 3–22, …). Next, I compared the moving averages sequentially to identify the point at which the bees achieved an accuracy of 50% in the last 20 flower choices. The early reversal learning phase was defined as the phase before bees achieved this criterion. The late phase of reversal learning was defined as the phase after achieving the criterion. I then ran separate generalized linear models with the choices to criterion and errors as dependent variables. I ran the models as detailed above with treatment (Control, Shaking) and the stage of reversal learning (early, late) as fixed factors and bee identity as a random factor. I modelled data with and without interaction between fixed factors and compared the models using the AIC.

4.3 Results

The current study aimed to investigate the impact of shaking on the ability of the bees to adjust their behaviour in response to the changed reward contingencies. Half of the bees that successfully learned the reward-colour discrimination in the Initial phase were subjected to 60 seconds of vigorous shaking repeated before each foraging bout. I compared the shaken group's performance with an unmanipulated control group of bees.

Initial phase: No difference in learning between treatment groups

The best model included both treatment and experimental condition as explanatory factors. The model estimated that neither Treatment nor Experimental Condition had any apparent effect on the initial discrimination learning (for details see Appendix Table 1) On average, bees in the initial phase took longer (mean ± sd: 136 ± 34.9 vs 117 ± 33.8) and made more errors (mean ± sd: 71.4 ± 23.1 vs. 64.1 ± 17.2) in *Experimental Condition 2* compared to *Experimental Condition 1*. However, these differences were not significant (Appendix Table 1 and Table 2). In summary, bees in all treatments and

experimental conditions demonstrated comparable learning abilities in the initial colour discrimination task (Fig. 2).

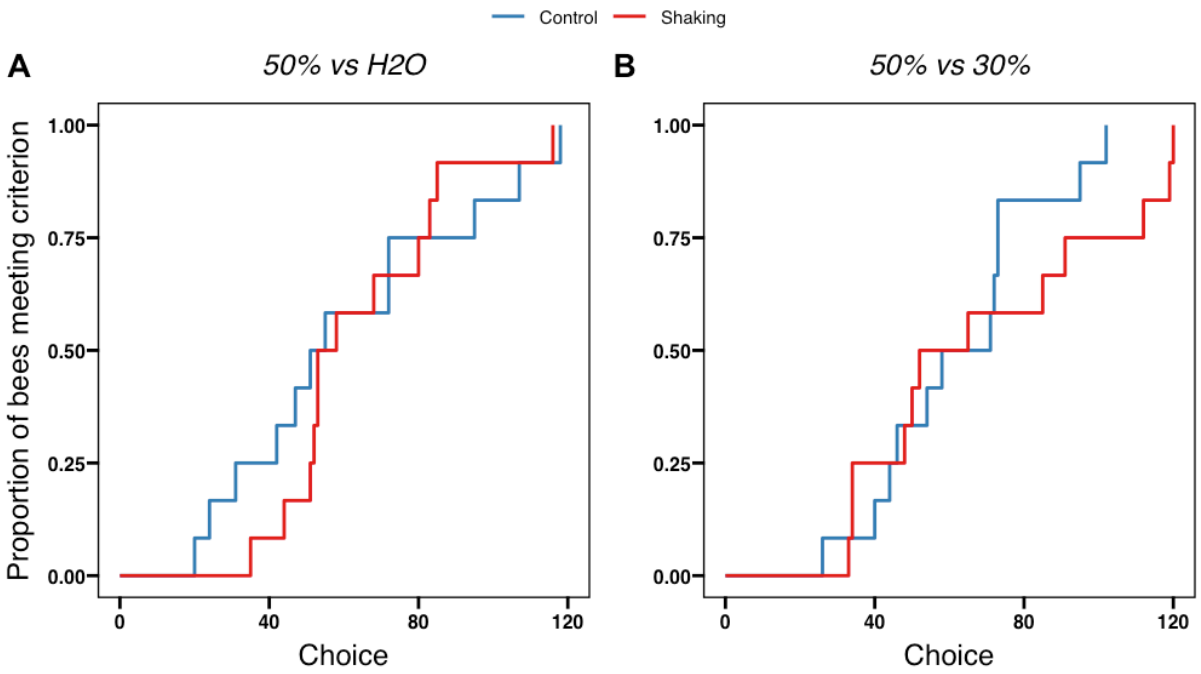

Figure 2. Bees show no differences in initial learning acquisition. The plot shows bees in different experimental conditions that were later assigned to different treatments performed equally well in the initial colour discrimination task. On average, bees in *Experimental Condition 2* took longer to meet the learning criterion than those in *Experimental Condition 1* (mean ± sd: 71.4 ± 23.1 vs. 64.1 ± 17.2, respectively). However, this difference was not statistically significant. Lines show the proportion of bees reaching the learning criterion (80% in the last 20 choices) over the course of the 120 choices in the initial phase of the experiments. Red lines depict the performance of bees that later went on to the Shaking treatment (n=12). Blue lines depict the performance of bees that were later included in the Control treatment (n=12). The left panel depicts *Experimental Condition 1* with rewarding flowers containing 50% sucrose solution and non-rewarding flowers that only contained water. The right panel depict *Experimental Condition 2* with high rewarding flowers containing 50% sucrose solution and lower rewarding flowers containing 30% sucrose solution.

Reversal phase: Shaking facilitates reversal learning

To investigate if acute stress affects bee reversal learning, half of the bees were shaken for 60s before each foraging bout. Bees in *Experimental Condition 1* received between 4-12 stress events, with an average of 7.33 ± 2.71 stressors. While different bees

therefore received a different number of stress events, there was no correlation between the number of stress events and the number of errors made in the reversal phase (r = -0.045, p= 0.889). This suggests that the acute negative impact caused by shaking, in conjunction with the rewarding nature of foraging, enabled the bees to restore their homeostasis following each stress occurrence.

The model that best fit the choices to criterion data included two fixed factors, the treatment and the experimental condition, and an interaction between factors. While there were no significant main effects of either Treatment (Model Estimate ± SE = 0.064 ± 0.108, z = 0.60, p = 0.551) or Experimental Condition (Model Estimate ± SE = -0.011 ± 0.108, z = -0.11, p = 0.913) on the total number of choices, the interaction between factors was significant (Model Estimate ± SE = -0.323 ± 0.0.154, z = -2.09, p < 0.05). This significant interaction implies condition-dependent effect of shaking. Specifically, shaken bees in *Experimental Condition 1* needed fewer choices to complete reversal (Fig.3A).

The model for the total number of errors before reaching the learning criterion that included Treatment and Experimental Condition as fixed predictors with an interaction had the lowest AIC (428.51). The closest competing model, which excluded interaction term, had a slightly higher AIC (429.23). A delta AIC of 0.72, indicating no strong support for one model over the other. The ANOVA comparison shows that adding an interaction to the model does indeed provide a statistically significant improvement ($\chi^2(2) = 2.72$, p = 0.05). The best-fitting model, therefore, included Treatment and Experimental Condition as fixed predictors and an interaction between them (Appendix Table 3). As with the number of choices, the main effects of Treatment and Experimental Condition were not significant (*Treatment:* Model Estimate ± SE = 0.055 ± 0.119, z = 0.47, p = 0.640; *Condition:* Model Estimate ± SE = 0.023 ± 0.119, z = 0.19, p = 0.849). The interaction effect between Treatment and Experimental Condition approached significance (Model Estimate ± SE = -0.281 ± 0.168, z = -1.67, p = 0.0948). There is therefore perhaps weak evidence suggesting that the impact of shaking on the number of errors may also depend on the experimental condition bee is exposed to (Fig.3B).

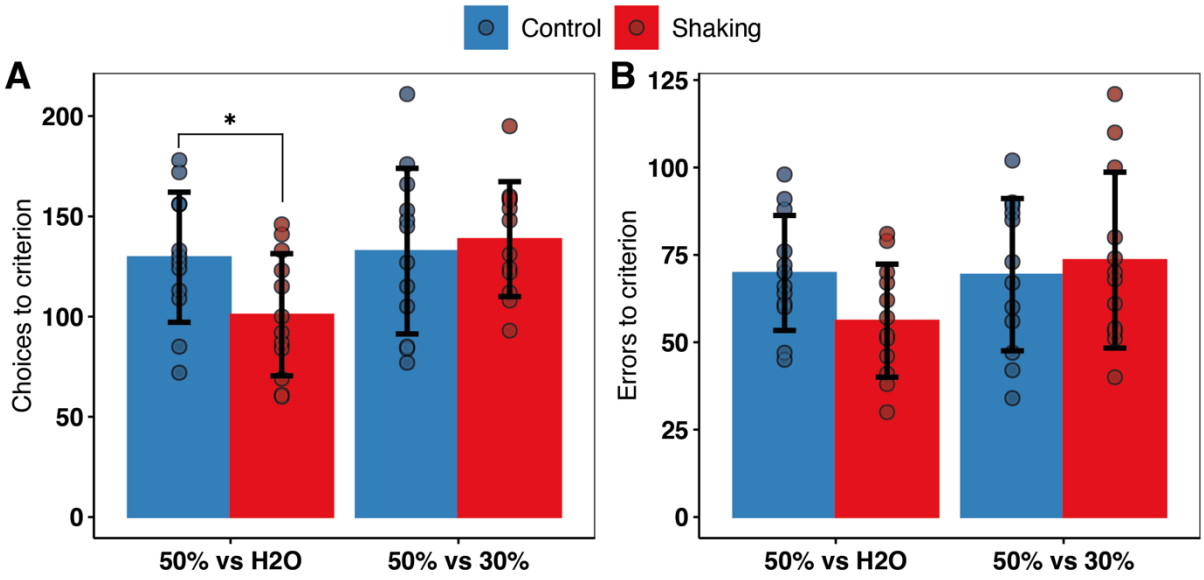

Figure 3. The impact of shaking on reversal learning. A) The total number of choices to reach the learning criterion (80% accuracy in the last 20 choices). In Experimental Condition 1 (50% vs H2O), bees subjected to shaking (red, n=12) required significantly fewer flower choices to reach the learning criterion compared to the control (blue, n=12). However, this pattern did not replicate in Experimental Condition 2 (50% vs 30), showing no significant difference between control (blue, n=12) and shaken bees (red, n=12). **B)** The total number of errors to reach the learning criterion (80% accuracy in the last 20 choices). In Experimental Condition 1 (50% vs H2O), shaken bees made fewer errors compared to the control bees. However, the observed difference, while showing a tendency, was not yet statistically significant. Similar patterns were not replicated in Experimental Condition 2 (50% vs 30), where both control (blue, n=12) and shaken bees (red, n=12) made a similar number of errors to reach the criterion. The bars indicate the mean, and the error bars represent the standard deviation. The asterisk (*) indicates significance at $p < 0.05$.

Experiment Condition 1: Early vs late reversal

For the analysis of bee performance in the early and late reversal phases, the model that included the treatment and the stage of reversal learning as fixed factors and the interaction between them had the lowest AIC (461.02). The closest competing model, which excluded interaction term, had a slightly higher AIC (462.63). A delta AIC of 1.6, indicating no strong support for one model over the other. The ANOVA comparison shows that adding Treatment to the model does not provide a statistically significant improvement ($\chi^2(2) = 3.60$, $p = 0.05$). The best-fitting model, therefore, included the treatment and the stage of reversal learning as fixed factors and the interaction between them (Appendix Table 4). While there was no

significant main effect of treatment (Model Estimate ± SE = 0.024 ± 0.190, z = 0.12, p = 0.897) or learning stage (Model Estimate ± SE = 0.237 ± 0.182, z = 1.302, p = 0.193), the interaction was marginally significant (Model Estimate ± SE = -0.526 ± 0.273, z = -1.927, p = 0.054). The best-fitting model for the total number of errors made before reaching the criterion also included treatment and the stage of reversal learning as fixed factors and the interaction between them. As with choice to criterion, there was no significant main effect of treatment (Model Estimate ± SE = 0.012 ± 0.195, z = 0.063, p = 0.949) or learning stage (Model Estimate ± SE = -0.298 ± 0.211, z = -1.409, p = 0.158), but the interaction was significant (Model Estimate ± SE = -0.674 ± 0.331, z = -2.034, p = 0.0419). Therefore, the number of choices and errors to criterion was lower in the later reversal learning in shaken bees (Fig.4A, B).

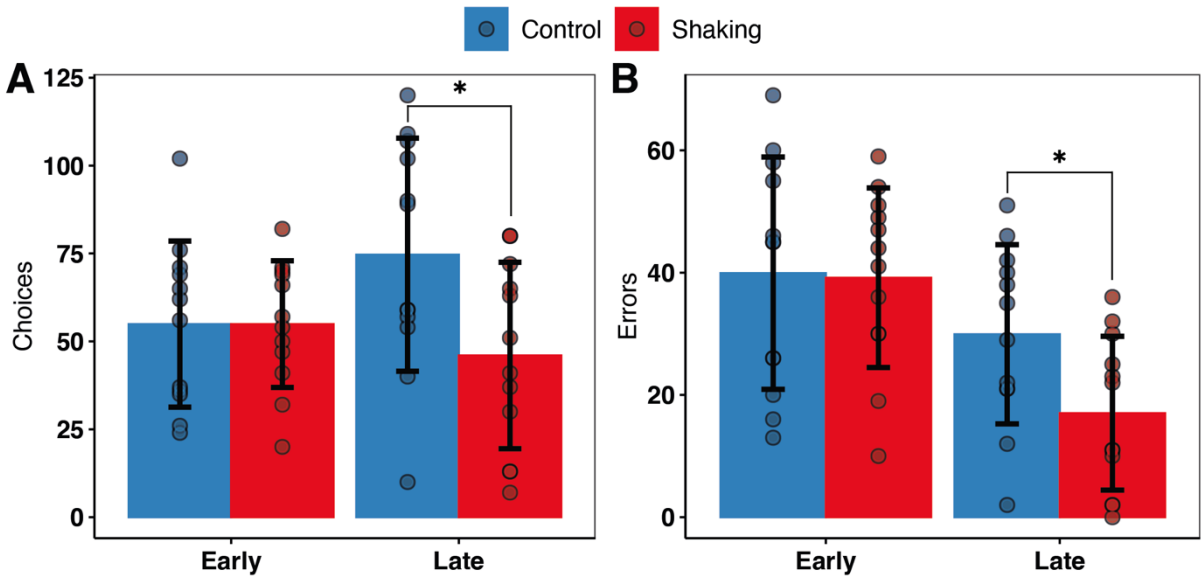

Figure 4. *Experimental Condition 1*: shaking facilitates late but not early reversal learning. A) No difference in the total number of choices during the early stage of reversal learning (50% correct choices in the last 20 choices, "*Early*") was observed. However, in the later stage of reversal learning (>50% correct choices in the last 20 choices, "*Late*"), shaken bees (red, n=12) required fewer choices than the control group (blue, n=12). **B)** During the early stage of reversal learning, there was no significant difference in the total number of errors made between treatment groups. In the later stage, however, shaken bees (red, n=12) made fewer errors compared to the control (blue, n=12). The bars indicate the mean, and the error bars represent the standard deviation. The asterisk (*) indicates significance at $p < 0.05$.

Experiment Condition 2: Early vs late reversal

As in *Experimental Condition 1*, bees in *Experimental Condition 2* received between 5-12 stress events, with an average of 8.67 ± 2.02 stress events. Despite the difference in the number of stress events experienced by each bee, here too there was no correlation between the number of stress events and the number of errors made in the reversal phase ($r = 0.31$, $p = 0.329$).

Analysis of early and late reversal learning further confirmed that there was no effect of shaking on reversal learning in *Experimental Condition 2* (Fig.5A, B). The model of total choices to criterion that included treatment (Control vs Shaking) and stage (Early vs Late) as fixed factors only without an interaction had the lowest AIC (465.75). The closest competing model, which included an interaction term, had a slightly higher AIC (467.41). A delta AIC of 1.65, indicating no strong support for one model over the other. The ANOVA comparison shows that adding Treatment to the model does not provide a statistically significant improvement ($\chi^2(2) = 0.35$, $p = 0.56$). The best-fitting model, therefore, included treatment (Control vs Shaking) and stage (Early vs Late) as fixed factors only without an interaction (Appendix Table 4). While the overall choices to criterion were significantly larger in the later phase of reversal learning (Model Estimate ± SE = 0.384 ± 0.124, $z = 3.08$, $p < 0.0021$), shaking had no significant effect on the total choices to criterion (Model Estimate ± SE = 0.023 ± 0.122, $z = 0.19$, $p = 0.850$). The model with the lowest AIC (420.18) for the total number of errors made before reaching the learning criterion included treatment and the stage of reversal learning as fixed factors. A competing model, which had a slightly higher AIC but with a delta AIC of less than 2 units, also included the interaction term between the fixed factors (Appendix Table 4). As with choices to criterion, further ANOVA comparison confirmed that including the interaction term did not significantly improve the model fit ($\chi^2(2) = 0.52$, $p = 0.47$), thus simpler model was selected. There was no significant main effect of treatment (Model Estimate ± SE = -0.099 ± 0.198, $z = -0.501$, $p = 0.617$) or stage (Model Estimate ± SE = -0.263 ± 0.206, $z = -1.277$, $p = 0.202$) and the interaction term was also not significant (Model Estimate ± SE = 0.209 ± 0.291, $z = 0.719$, $p = 0.472$).

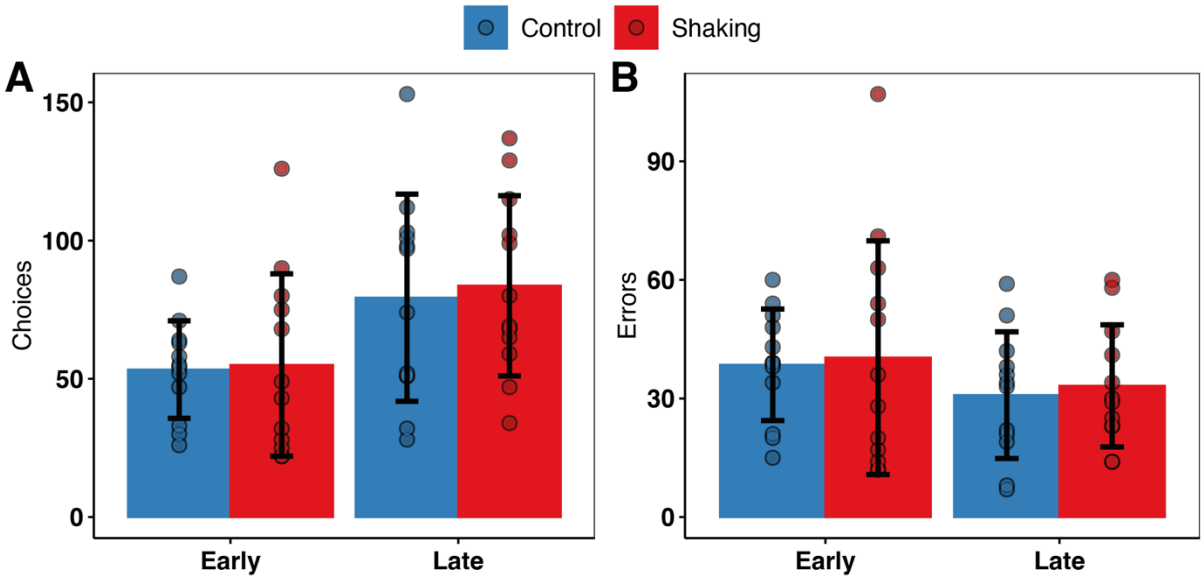

Figure 5. *Experimental Condition 2*: Shaking had no effect on either early or late reversal Learning. A) The number of choices was not statistically different between the control (blue, n=12) and shaken (red, n=12) bees, either in the early stage (50% correct choices in the last 20 choices, "*Early*") or in the later stage of reversal learning (>50% correct choices in the last 20 choices, "*Late*"). **B)** Similarly, no difference in the number of errors was observed in either the early or late stages of reversal. The bars represent the mean, and the error bars indicate the standard deviation. Blue bars correspond to the Control treatment, while red bars correspond to the Shaking treatment.

Impact of shaking on low-reward depletions

In the reversal phase of *Experimental Condition 2*, bees faced two possibilities when landing on previously correct but now incorrect flowers: they could either probe and reject the low-reward (30% sugar solution) or deplete it. Compared to control bees, shaken bees were more likely to consume the low-quality sugar reward when landing on incorrect flowers (167 occurrences vs 20, Fig. 6). The number of depletions had a strong and significant positive correlation with the number of choices ($r = 0.753$, $p < 0.01$) and errors ($r = 0.833$, $p < 0.001$) required to complete the reversal in shaken bees, but not in control bees (Choices: $r = 0.294$, $p = 0.354$; Errors: $r = 0.110$, $p = 0.733$).

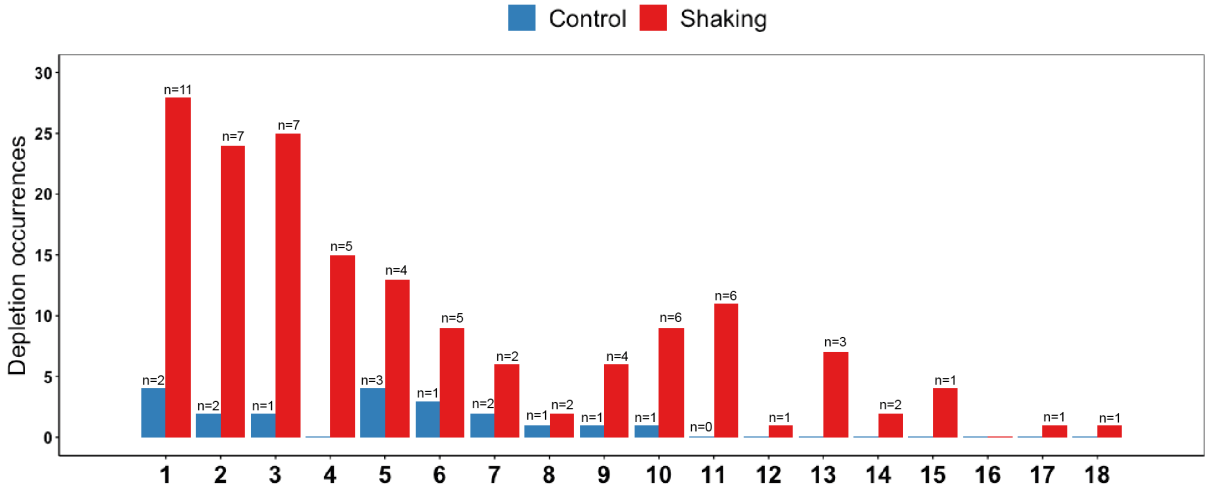

Figure 6. Low-reward depletions made by bees in the reversal phase of *Experimental Condition 2*. Each bar represents the cumulative number of depletions for individual treatment groups over consecutive bins of 10 flower choices. The number of bees contributing to the cumulative total of depletions per bin is indicated by a sample size above each bar.

I further investigated if these low-reward depletions were treatment-dependent and if they were more likely to happen at the beginning of the reversal phase when bees lacked experience with the new high-rewarding flower. To do so I fit generalized linear mixed models using a Poisson distribution and a log link function. The best-fitting model included Treatment and Choice Order as fixed predictors without their interaction.

As expected, the choice order significantly affected the low-reward depletion rate (Model Estimate ± standard error = -0.009 ± 0.002, $z = -4.596$, $p < 0.001$). As bees continued to make choices, the number of low-reward depletions decreased, indicating that bees chose to reject, not deplete, incorrect flowers. Shaking also had a significant positive effect (Model Estimate ± standard error = 2.222 ± 0.443, $z = 5.015$, $p < 0.001$), showing that the depletion rate was treatment-dependent and increased with shaking.

Bee morphology did not affect the learning performance

I measured the intertegular width of all bees that completed reversal learning successfully. The intertegular width ranged from 3.25 to 4.33 mm for all bees, regardless of treatment and experimental conditions. The average intertegular widths

for shaken bees in *Experimental Conditions 1* and *2* were 3.42 ± 0.269 mm and 3.50 ± 0.288 mm, respectively, while the average intertegular widths of control bees were 3.56 ± 0.237 mm and 3.25 ± 0.262 mm, respectively. All bees were therefore highly similar in size.

While treatment-dependent differences in reversal learning were observed, the performance in both groups also varied greatly (see Fig.3). I, therefore, wanted to investigate if there was a correlation between bee body size and learning performance across treatments in each experimental condition. Although the size of a bee has been previously linked to learning and memory (Worden, Skemp and Papaj, 2005; Riveros and Gronenberg, 2009), I did not find any correlation with the number of choices or errors to criterion in our experiments (Appendix Table 3).

I also explored whether the observed differences in reversal performance could be attributed to certain bees inherently having better learning abilities. To address this, I again performed Pearson Correlation between bee performance in the initial and the reversal phase. There was no correlation observed between the number of choices or total errors to criterion during both the Initial and Reversal Phases for any of the experimental conditions (Appendix Table 1). This indicates that the variability in learning performance cannot be attributed to individuals being better or worse in learning.

4.4 Discussion

In this study, I investigated how subjecting bumblebees to shaking modulates their behavioural flexibility. To do this, I used a reversal learning task. Although reversal learning has been previously studied in social bees (Chittka, 1998; Ben-Shahar *et al.*, 2000; Komischke *et al.*, 2002; Hadar and Menzel, 2010; Mota and Giurfa, 2010; Raine and Chittka, 2012; Strang and Sherry, 2014), to my knowledge, this is the first study looking at the effects of stress on their behavioural flexibility.

Shaking facilitates late reversal learning when choosing between rewarding and non-rewarding flowers

Shaking can induce a negative state in bees. This was earlier demonstrated using the judgment bias test paradigm (see Chapter Two). Applying shaking treatment immediately before bees began foraging significantly affected reversal learning in *Experimental Condition 1*, where flowers contained reward or no reward (water). Here, bees in a negative state reached the learning criterion with significantly fewer flower choices and fewer total errors. Thus, the stress appears to facilitate reversal learning in this experimental condition.

These results are consistent with some previous findings in the mammalian literature, which show that acute stress can indeed improve reversal learning. Thai et al. (2013) demonstrated that exposing rats to acute restraint stress just before the reversal learning test significantly enhanced their performance. Stressed rats completed reversal with fewer trials and errors. Similarly, acute elevated-platform stress delivered for 30 min immediately before the reversal trials enhanced spatial reversal learning in rats (Dong *et al.*, 2013).

Just as with any discrimination learning, reversal learning is a two-process phenomenon. It consists of excitatory learning and extinction learning (Hadar and Menzel, 2010; Nilsson *et al.*, 2015). For example, in a two-stimuli-discrimination task, the excitation learning driven by positive reinforcement promotes the approach of stimuli associated with a reward, while no reward associated with alternative stimuli gradually suppresses the approach via extinction learning. However, classic discrimination learning starts with stimulus-naïve individuals. This is not true for reversal learning. Here, learning unfolds based on existing knowledge and rules associating a stimulus with specific outcomes; it poses a challenge for acquiring new excitatory and new extinction learning. The previously formed stimulus-outcome association results in a tendency to persevere in approaching the initial stimulus that was rewarded and avoiding the initial stimulus that was not rewarded, despite the change in contingencies (Nilsson *et al.*, 2015). This is especially true at the start of the reversal. It is, therefore, assumed that early errors reflect the strength of the initial stimulus-reward association (perseverance), whereas later errors indicate the ability to acquire a new stimulus-reward association (Boulougouris, Dalley and Robbins, 2007; Bryce and Howland, 2015; Nilsson *et al.*, 2015). Investigating early and later reversal can thus provide attentional insight.

Some earlier studies applied this logic to their analyses and showed that animals exposed to acute stress make fewer errors than unstressed animals but only during later stages of reversal learning (Graybeal *et al.*, 2011; Bryce and Howland, 2015). Later in reversal, the performance is no longer dominated by persevering on the previously rewarded stimulus but is primarily driven by learning the new rewarding association. Thus, acute stress seems to affect new excitation learning but not extinction learning. In line with these findings, I also observed that the facilitating effect of shaking on reversal learning in *Experimental Condition 1* was mainly driven by the improved performance during late stages (after accuracy increased above 50%). Shaken bees made fewer choices and made fewer errors to criterion. This suggests that shaking may indeed facilitate excitatory learning of a new stimulus-outcome association but without simultaneously affecting extinction learning of the initial stimulus-response associations. When taken together, these findings suggest that shaking has a delayed effect on reversal learning in bees. Stress did not interfere with the early stages of reversal learning, where the extinction of the initial association is needed. However, after the high rates of persevering on previously rewarding flowers had ceased, experiencing acute stress facilitated the acquisition of new excitatory learning.

Why does stress facilitate reversal learning?

In the present study, bees were exposed to mild stress delivered at the beginning of each foraging bout. Although shaking was administered several times during the reversal phase, the short-lived nature of the stress, coupled with the positive effect of receiving a reward during successful foraging, likely allowed bees to restore homeostasis and recover after each shaking event. This assumption is confirmed as the number of stresses administrated did not correlate with learning performance during reversal learning. Thus, shaking can be considered a repeated yet acute stressor.

The fight-or-flight response is a well-known coping mechanism that is initiated by acute stress exposure. In mammals, such response is mediated by activation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis. The unfolding cascade of neurological, physiological, and behavioural changes support

response to threats, and later, help an organism to return to a state of balance (De Kloet, Joëls and Holsboer, 2005). A similar stress response model has been proposed in bees (Even, Devaud and Barron, 2012).

The facilitating effect of acute stress on behavioural flexibility is attributed to a sudden release of stress hormones (Hurtubise and Howland, 2017). In bees, however, this function could be achieved by octopamine. Octopamine is thought to act as a major stress hormone within the bee nervous system (Roeder, 2005, 2020), with increased levels of this biogenic amine observed during energetically demanding situations (Davenport and Evans, 1984; Roeder, 2005; Adamo and Baker, 2011; Adamo, Kovalko and Mosher, 2013). In the peripheral nervous system, octopamine plays a role in stress-related physiological responses (Even, Devaud and Barron, 2012). In the central nervous system, however, octopamine acts as a neuromodulator that mediates sensory and cognitive processes associated with feeding (Giurfa, 2006; Rein *et al.*, 2013). Given the latter, octopamine plays a key role in appetitive learning and memory (Hammer and Menzel, 1998; Unoki, Matsumoto and Mizunami, 2005). Octopamine is also known for its role in regulating reward sensitivity. For example, honeybees (Scheiner *et al.*, 2002) and bumblebees (Muth, Breslow and Leonard, 2023) show increased sensitivity to sucrose after receiving higher doses of octopamine. By fine-tuning the sensory system, octopamine increases animals' "preparedness to learn", which in turn facilitates appetitive learning (Scheiner *et al.*, 2002).

Shaking is an acute stressor. Therefore, it is reasonable to speculate that shaking may increase the levels of biogenic amines, especially octopamine. If so, the increase in octopamine levels may in turn have led to increased responsiveness to reward, thereby, facilitating learning. Similar speculations were made in the earlier study showing improved appetitive learning in honeybees exposed to stress delivered by formic acid exposure (Bachert and Scheiner, 2023). The authors explained their observed improvement in appetitive learning by the possibility of increased release of octopamine in the mushroom bodies initiated by stress. Octopamine, however, is not the only biogenic amine that is modulated by stress; serotonin and dopamine are also affected (Davenport and Evans, 1984; Harris and Woodring, 1992; Even, Devaud and Barron, 2012). As a result, the observed facilitating effect of stress on reversal learning in my study may be caused by the cumulative effect of several biogenic amines modulating different cognitive functions. For example, serotonin may facilitate

disengaging from previously reinforced stimuli (Hurtubise and Howland, 2017), while increased dopamine levels may further contribute to better learning by increasing arousal (Mustard, Pham and Smith, 2011), "wanting" (Huang *et al.*, 2022), and perhaps attentional processes (Raza *et al.*, 2022). A quantitative assessment of biogenic anime levels would be needed to confirm this hypothesis.

Shaking has no effect when both flowers are rewarding

Shaking facilitates reversal learning only in *Experimental Condition 1* but not *Experimental Condition 2.* In the reversal phase, shaken bees showed no differences in the number of choices or errors made till the criterion was met. The conditions differed only in the rewards offered by flowers. While in *Experimental Condition 1* flowers were either highly rewarding or unrewarding, both flower types in *Experimental Condition 2* carry a reward, although of different quality (50% vs 30%). Therefore, when landing on the incorrect flower in the second condition, bees had to choose to reject or accept the low-quality reward. As the results show, the tendency to accept low rewards was higher in the shaken group. Speculatively, these depletions might explain why shaking has no effect on reversal learning in *Experimental Condition 2.*

When a stimulus in association with a reward continues to fail to provide the expected outcome, the reward associated with such stimuli gradually decreases (Izquierdo *et al.*, 2017). However, sporadic acceptance of low rewards, as seen in *Experimental Condition 2*, may have delayed this extinction process. This is because the occasional depletion of the low reward could have reinforced the association with incorrect choices and thus interfered (at least to some extent) with a gradual decrease in the value associated with stimuli. This is further supported by a positive correlation between the number of total depletions and the total number of choices and errors to criterion. Thus, bees that depleted low rewards took longer to reverse. Therefore, the sporadic acceptance of low rewards may prolong the perseverance of the association of the initial stimulus with a high reward. This could have potentially counteracted any beneficial effect of shaking on reversal learning observed in *Experimental Condition 1*.

The occasional low reward depletion in shaken bees is likely due to a combination of factors. For example, depletions could have occurred due to a sudden increase in energetic demands caused by shaking**.** This idea finds some support in an

earlier study, which showed that shaking not only lowered glycogen levels in muscles but also increased fructose levels in the haemolymph (Božič and Woodring, 1997). Changes in energy reserves observed in this study indicate an energy-demanding state induced by shaking. The consumption of the low reward by shaken bees in my study may represent a desperate attempt to restore energy losses. This would imply that when shaken, bees perceive the low reward as more valuable, as in times of need, any form of energy is considered beneficial. Similarly, as mentioned earlier, shaking may result in elevated octopamine levels and, therefore, an increased sensitivity to reward. The depletions of the low reward were occasional and did not persist throughout the entire reversal learning period. This suggests that the lower reward acceptance threshold induced by shaking is short-lived, just like the shaking-induced negative affective state. Once shaken bees have compensated for the energy loss and potentially returned to homeostasis (i.e., a neutral affective state), the shaken bees would begin to reject low rewards and not perform worse than control bees.

Limitations of the study

One great limitation of this study is that I did not measure levels of biogenic amines. Nonetheless, it is intriguing to consider the possibility that octopamine could play a role in enhancing the learning performance of stress bees. While the octopamine-driven explanation is supported by some earlier invertebrate literature (Davenport and Evans, 1984; Harris and Woodring, 1992; Božič and Woodring, 1997; Even, Devaud and Barron, 2012), one particular study showed the opposite effect of shaking.

A previous study showed reduced levels of octopamine, dopamine, and serotonin in the haemolymph of shaken honeybees (Bateson *et al.*, 2011). Although the duration of shaking in Bateson et al. (2016) and my study was the same, 60 seconds, the way in which shaking was administered was different. Bees were placed in harnesses in the Bateson et al. (2016) study while they were free-moving in mine. Shaking could potentially have a different effect on biogenic amine levels in these different settings.

Firstly, harnessing could have itself induced a negative state. Thus, the effect of later shaking on the levels of these amines would be modulated. Bateson et al. (2016) did not specify the exact time between harnessing and haemolymph collection.

However, assuming it followed the protocol used in the main experiment, it could have included a 24-hour rest period. Therefore, being harnessed for such a long period of time could act as a chronic stressor. Indeed, the depression model in rodents uses exactly such treatment - chronic immobilisation (Kim and Han, 2006; Grissom and Bhatnagar, 2009). If so, prolonged harnessing could indeed cause a depression-like state in bees.

Secondly, if considering such a depression-like state in harnessed bees, the reduced response to the subsequent stress can be expected. It is known that chronic stress can lead to stress habituation. For instance, the longer a bee experiences leg pinching, the less profound her stress response behaviour becomes (Harris and Woodring, 1992). As chronic stress gradually exhausts organisms, the ability to respond to subsequent acute stress can also be reduced (Vallès, Martí and Armario, 2003; Rich and Romero, 2005). If the same is true with prolonged harnessing, it could have indeed induced a depression-like state in bees that resulted in reduced response to subsequent shaking.

Lastly, prolonged harnessing could have reduced biogenic amine levels prior to shaking. One reason to think so is that, unlike Bateson et al. (2016), other studies that used shaking reported elevated, not reduced, levels of biogenic amines (Davenport and Evans, 1984; Harris and Woodring, 1992). Commonly, in these studies bees were freely moving before, during (somewhat), and after the stress administration. This has substantial similarity to my stress administration protocol. In my study, bees were placed in the shaker as soon as they entered the testing area. Despite the limited space in the shaker, the bee remained active throughout the shaking period, evident from a vigorous escape response during shaking and high reactivity afterwards. Increased levels of biogenic amine, therefore, could be expected with my treatment.

Although this is speculative, shaking could thus possibly have had a different effect on the levels of biogenic amines in bees in my study and Bateson et al. (2016). Harnessing bees may act as a chronic stressor, generating a low-arousal- negative-valence state, similar to depression in mammals. In such a state, lower levels of biogenic amines are expected. Therefore, acute stress, such as shaking could result in either further reduction in biogenic amine levels or an increase of lesser magnitude. In contrast, shaking “free-flying” bees in my experiment is more likely to induce a high-arousal- negative-valence state. Therefore, elevated levels of biogenic amines are

expected. Methods like high-performance liquid chromatography could be used to confirm these speculations in the future.

In this research, using reversal learning, I investigated how acute stress affects the ability of bees to adapt their behaviour to a change. I proposed that being in a negative emotion-like state would influence the bees' ability to learn new stimulus-reward associations. The results showed that when bees experienced acute stress induced by shaking, the ability to reverse previously learned behaviour was facilitated, and this facilitating effect occurred later in the reversal. The effect was however observed only when distinct reward outcomes were presented. These findings are consistent with what has been observed in studies on mammals that suggest the delayed impact of acute stress on behavioural flexibility. I speculate, that this improvement in behavioural flexibility in bees may be driven by stress-induced modulations of biogenic amine levels, such as octopamine. Shaking could also increase energy demands, enhance responsiveness to rewards and greater readiness for learning. Further investigations are needed to unravel the true underlying cause. Importantly facilitation effect was not observed when the outcome values were less contrasting. I suggest that observed occasional depletion of low rewards could have reinforced the initial associations between stimuli and rewards, thus obstructing the facilitating effects of stress. To further validate the underlying mechanisms at play it would be helpful to conduct studies that assess amine levels in free-flying bees both with and, without shaking-induced stress.

4.5. Appendix

Table 1

Summary of best-fit mixed models to analyse bee learning performance in Initial Phase of reversal learning

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	AIC	ΔAIC
Total choices	**Treatment+Experimental condition**	**ID**	**4**	**456.43**	**0.00**
	Treatment*Experimental condition	ID	5	458.38	1.95
Total errors	**Treatment+Experimental condition**	**ID**	4	396.68	
	Treatment*Experimental condition	ID	5	398.67	2.00

Model pairwise comparison: Assessing the significance of interaction terms using anova()

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	χ2	Pr(>χ2)
Total choices	**Treatment+Experimental condition**	**ID**	**4**		
	Treatment*Experimental condition	ID	5	0.05	0.83

The table presents the model selection procedure undertaken to investigate the impact of treatment group (treatment: control and shaking) and experimental condition (Experimental Condition 1 and Experimental Condition 2) on learning performance during the initial phase of reversal learning. Two distinct models were employed to analyse choices to criterion and errors to criterion separately. The table includes specifications for both fixed and random explanatory variables, along with corresponding degrees of freedom (d.f.) and Akaike's information criterion (AIC) values. The preferred model for each dependent variable, as determined by the lowest AIC value, is denoted in bold. Models that had a ΔAIC < 2 were considered not significantly different; in such cases, the significance of adding complexity (e.g., interaction term) was further assessed using anova().

Table 2
Summary of statistical model results analysing the impact of treatment group and experimental condition on bee learning performance in the initial phase of reversal learning

Response variable	Predictor variables	Estimate	Std. Error	z value	Pr(>|z|)
Total choices	(Intercept)	4.07	0.11	37.59	<0.0001
	Treatment(Shaking)	0.10	0.12	0.79	0.430
	Condition(Experimental Condition 1)	-0.06	0.12	-0.47	0.639
Total errors	(Intercept)	3.15	0.14	21.91	<0.0001
	Treatment(Shaking)	0.08	0.17	0.50	0.619
	Condition(Experimental Condition 1)	-0.03	0.17	-0.19	0.853

The table presents a summary of results derived from statistical analyses conducted to examine potential variations in learning performance among bees assigned to distinct treatment groups (control and shaking) and experimental conditions (*Experimental Condition 1*: high reward vs. water, and *Experimental Condition 2*: high reward vs. low reward). In all models, the control treatment and experimental condition 2 serve as the reference groups. The information includes estimated coefficients, standard errors, z-values, and corresponding p-values for both response and predictor variables.

Table 3

Summary of best-fit mixed models to analyse bee learning performance in Initial Phase of reversal learning

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	AIC	ΔAIC
Total choices	Treatment+Experimental condition	ID	4	482.48	0.00
	Treatment*Experimental condition	**ID**	**5**	**480.27**	**2.21**
Total errors	Treatment+Experimental condition	ID	4	429.23	0.00
	Treatment*Experimental condition	**ID**	**5**	**428.51**	**0.72**

Model pairwise comparison: Assessing the significance of interaction terms using anova()

	Explanatory variables				
Dependent variable	Fixed	Random	d.f.	χ2	Pr(>χ2)
Total errors	Treatment+Experimental condition	ID	4		
	Treatment*Experimental condition	**ID**	**5**	**2.72**	**0.05**

The table presents the model selection procedure undertaken to investigate the impact of treatment group (treatment: control and shaking) and experimental condition (Experimental Condition 1 and Experimental Condition 2) on learning performance during the initial phase of reversal learning. Two distinct models were employed to analyse choices to criterion and errors to criterion separately. The table includes specifications for both fixed and random explanatory variables, along with corresponding degrees of freedom (d.f.) and Akaike's information criterion (AIC) values. The preferred model for each dependent variable, as determined by the lowest AIC value, is denoted in bold. Models that had a ΔAIC < 2 were considered not significantly different; in such cases, the significance of adding complexity (e.g., interaction term) was further assessed using anova().

Table 4

Summary of best-fit mixed models to analyse bee learning performance in early and late stages of reversal phase of reversal learning

		Explanatory variables				
	Dependent variable	Fixed	Random	d.f.	AIC	ΔAIC
Experimental Condition 1	Total choices	Treatment+Stage	ID	5	462.63	0.00
		Treatment*Stage	**ID**	**6**	**461.02**	**1.60**
	Total errors	Treatment+Stage	ID	5	421.79	0.00
		Treatment*Stage	**ID**	**6**	**410.70**	**2.08**
Experimental Condition 2	Total choices	**Treatment+Stage**	**ID**	**5**	**465.75**	**0.00**
		Treatment*Stage	ID	6	467.41	1.65
	Total errors	**Treatment+Stage**	**ID**	**5**	**420.18**	**0.00**
		Treatment*Stage	**ID**	6	421.67	1.48

Model pairwise comparison: Assessing the significance of interaction terms using anova()

		Explanatory variables				
	Dependent variable	Fixed	Random	d.f.	χ2	Pr(>χ2)
Experimental Condition 1	Total errors	Treatment+Stage	ID	5		
		Treatment*Stage	**ID**	**6**	**3.60**	**0.05**
Experimental Condition 2	Total choices	**Treatment+Stage**	**ID**	**5**		
		Treatment*Stage	ID	6	0.35	0.56
	Total errors	**Treatment+Stage**	**ID**	**5**		
		Treatment*Stage	ID	6	0.52	0.47

The table presents the model selection procedure undertaken to investigate the impact of the treatment group (Treatment: control and shaking) and the stage of reversal learning (Stage: early and late) on bee learning performance during the reversal phase. Two distinct models were employed to analyse choices to criterion and errors to criterion separately for each experimental condition. The table includes specifications for both fixed and random explanatory variables, along with corresponding degrees of freedom (d.f.) and Akaike's information criterion (AIC) values. The preferred models for each dependent variable, as determined by the lowest AIC value, are denoted in bold. Models that had a ΔAIC < 2 were considered not significantly different; in such cases, the significance of adding complexity (e.g., interaction term) was further assessed using anova().

Table 5
Summary of statistical model results analysing bee learning performance in the reversal phase of reversal learning.

Model		Response variable	Predictor variables	Estimate	Std. Error	z value	Pr(>|z|)
Overall learning performance		Total choices	(Intercept)	4.85	0.08	62.97	< 0.0001
			Treatment(Shaking)	0.06	0.11	0.60	0.551
			Condition(Experimental Condition 1)	-0.01	0.11	-0.11	0.914
			Treatment(Shaking)*Condition(Experimental Condition 1)	-0.32	0.15	-2.09	0.036
		Total errors	(Intercept)	4.20	0.08	49.96	< 0.0001
			Treatment(Shaking)	0.06	0.12	0.47	0.640
			Condition(Experimental Condition 1)	0.02	0.12	0.19	0.849
			Treatment(Shaking)*Condition(Experimental Condition 1)	-0.28	0.17	-1.67	0.095
Early and late performance	*Experimental Condition 1*	Total choices	(Intercept)	4.04	0.14	29.40	< 0.0001
			Treatment(Shaking)	0.02	0.19	0.13	0.897
			Stage(Late)	0.24	0.18	1.30	0.193
			Treatment(Shaking)*Stage(Late)	-0.53	0.27	-1.93	0.054
		Total errors	(Intercept)	3.69	0.14	26.20	< 0.0001
			Treatment(Shaking)	0.01	0.20	0.06	0.949
			Stage(Late)	-0.30	0.21	-1.41	0.159
			Treatment(Shaking)*Stage(Late)	-0.67	0.33	-2.03	0.042
	Experimental Condition 2	Total choices	(Intercept)	3.99	0.12	34.03	< 0.0001
			Treatment(Shaking)	0.02	0.12	0.19	0.851
			Stage(Late)	0.38	0.12	3.08	0.002
		Total errors	(Intercept)	3.70	0.14	26.63	< 0.0001
			Treatment(Shaking)	-0.10	0.20	-0.50	0.617
			Stage(Late)	-0.26	0.21	-1.28	0.202
			Treatment(Shaking)*Stage(Late)	0.21	0.29	0.72	0.472

The table provides a comprehensive overview of the results obtained through statistical analyses of bee performance during the reversal phase. The analysis encompasses various response variables, including overall learning performance (total number of choices and errors to criterion), early and late performance (total choices and errors for each experimental condition separately). The predictor variables consist of the treatment group (Treatment: Control and Shaking), condition (Condition: Experimental Condition 1 and Experimental Condition 2), reversal learning stage (Stage: Early and Late). The specific reference group for each predictor variable is highlighted in bold. Statistically significant p value is highlighted in bold. Additionally, all models include information about estimated coefficients, standard errors, zvalues, and corresponding p-values for both response and predictor variables.

CHAPTER FIVE

Exploring the bee brain in virtual reality: towards an effective method to study the neural architecture of a miniature brain

5.1. Introduction

Although the study of insect emotion is still in its early stages, several successful attempts have been made. Using the judgment bias task, emotion-like states in social bees have been demonstrated in this thesis (see Chapter Two), as well as in earlier studies (Bateson *et al.*, 2011; Solvi, Baciadonna and Chittka, 2016; Schlüns *et al.*, 2017; Strang and Muth, 2023). I showed that, as in mammals, prior negative experiences modulate bee cognition and behaviour. However, it is crucial to acknowledge that merely identifying similarities in behavioural reactions is not sufficient. Exploring the mechanisms through which the brain generates and regulates these states is still an unexplored yet important field of research.

Insect brains lack the cortical structures known to be involved in emotion in mammals (Panksepp, 2011). Nevertheless, time and time again, social bees have demonstrated the capacity of their tiny brains to exhibit rather sophisticated behaviours (Giurfa *et al.*, 2001; Devaud *et al.*, 2015; Chittka, 2017; Solvi, Al-Khudhairy and Chittka, 2020). For instance, bees are capable of concept learning, such as understanding sameness and difference between stimuli (Giurfa *et al.*, 2001). In mammals, such learning depends on the prefrontal cortex (Wallis, Anderson and Miller, 2001; Miller *et al.*, 2003). Bees, however, achieve this through higher-order structures, such as mushroom bodies (Giurfa, 2013). Despite the evident structural differences between bee and mammalian brains, the mechanisms behind information processing, integration, and storage are rather similar (Hammer and Menzel, 1995; Joerges *et al.*, 1997; Wilson and Stevenson, 2003; Schultz, 2006; Szyszka, Galkin and Menzel, 2008; Menzel, 2014). Similarly, functional parallels have also been suggested – for instance, insect mushroom bodies have been cited to be analogous to the midbrain in vertebrates (Barron and Klein, 2016), while the central complex might be comparable to vertebrates' basal ganglia (Strausfeld and Hirth, 2013). These higher-order brain structures, therefore, are the best candidates for exploring the neural correlates of emotion-like states in bees.

The techniques available for *in vivo* assessments of the bee brain are rather limited. Common methods, e.g., calcium imaging or targeted inhibition of neural activity, involve injecting substances (such as calcium-sensitive dyes or blockers of voltage-gated channels) into specific brain regions or neurons of a harnessed animal. Despite obvious advantages, there are certain issues with applying these methods to

study emotion-like states. First, restricting the animals' movement can impact their emotional state. For example, chronic immobilisation is used as a treatment to induce depression states in rodents (Kim and Han, 2006; Grissom and Bhatnagar, 2009). Similar effects may be expected in bees, thereby complicating the use of existing neurophysiological methods to study affective states.

This challenge, however, can be overcome by conducting studies using virtual reality environments (VR). VR permits full control over the visual surroundings of the animals, while at the same time, allowing freedom of movement. In the past, VR has been successfully used in behavioural (Buatois *et al.*, 2017, 2018, 2020), and neuroethological studies, e.g., involving electrophysiological recordings (Paulk *et al.*, 2014; Zwaka *et al.*, 2019). Simpler neurophysiological techniques can also be explored for use in VR setups. An excellent avenue to investigate involves coupling behavioural assays in VR with transient silencing of selected brain regions using microinjections of local anaesthetics, e.g., procaine. The method has previously been applied to studying aversive learning in honeybees (Plath *et al.*, 2017) but has not yet been tested in a more complex experimental setting. Therefore, microinjection and VR could indeed become a great tool for exploring several complex questions, including investigating the neural correlates of emotion-like states in bees. Yet, before moving further, it is very important to evaluate another possible concern.

Targeted brain microinjection is a highly invasive technique (Søvik *et al.*, 2016). It is therefore crucial to investigate whether cognitive abilities remain intact in animals before applying these methods to address more complex questions. Previously, Macri, Lafon and Avargues-weber (2021) explored the effectiveness of combining targeted microinjections with behavioural assays in the VR (Macri, Avargues-weber and Lafon, 2021). The team used honeybees, *Apis mellifera*, to investigate whether the procedure required for microinjections caused significant brain damage. Two higher-order brain areas – the mushroom bodies and central complex - were injected with phosphate buffer saline (PBS), followed by subjecting bees to a colour discrimination task. Contrary to previous studies (Plath *et al.*, 2017), most saline injected bees either failed to engage with the behavioural task or were unsuccessful in learning acquisition. It was therefore proposed to repeat the experiment with another social bee species, bumblebees (*Bombus terrestris*). Specifically, the aim of my study was to adjust existing microinjection-VR protocols to bumblebees and to assess whether this bee species is a better candidate model for the procedure.

5.2. Materials and Methods

Animal housing and preparation

I used four commercially raised *Bombus terrestris* colonies (Koppert, Cavaillon, France) in this experiment. Throughout the experiment, bees were kept in commercial boxes containing a highly nutritious food supply provided by the suppliers. To increase foraging motivation and deprive them of rich commercial food, bees used in experiments were kept on a low-quality diet overnight (*at libitum* 30% (w/w) sugar solution and water). On the day before the onset of the experiment, individual bees were collected by placing a glass vial at the box's entrance. To attract bees towards the exit, the procedure was done under the red light (invisible to bees), with only the entrance illuminated with visible spectrum light. The collected bees were placed on ice for a short bout of cold anaesthesia and transferred into housing cages (10x5x7cm). I provided the bees with *ad libitum* low-quality sucrose solution (30%, w/w) and water. Overnight, I kept housing cages in the incubator under controlled conditions (50-60% humidity and 28°C temperature). I compared four different groups: the unmanipulated control group (*C1*), the 'sham' group with surgery only (*C2*), and two PBS-injected groups: with injections in the alpha lobes of the mushroom bodies (*PBSAL*) and in the central complex (*PBSCX*) respectively.

Surgery and microinjection into brain structures

The following day, bees were anaesthetised on ice and restrained in harnesses. To prepare the bee for surgery (groups *C2*, *PBSAL* and *PBSCX*), its head was immobilised with dental wax to prevent movement. The procedure was as described earlier (Søvik *et al.*, 2016). In brief, the antennae were restrained with insect pins. A window was cut into the head cuticle to access the brain for injections (*PBSCX*, *PBSAL*). Three cuts were made - one at the border of the right eye, one above the median ocellus and one below the antennal stems. This created the flap that was helped opened through the procedure with an insect pin. To permit easy access to the nanoinjector, the glands and tracheae were gently moved aside. Following this, a small incision was made into the neurolemma exposing the area of interest. As soon as the

surgery was complete, the flap was repositioned and fixed with eicosane to prevent brain from drying (Søvik *et al.*, 2016).

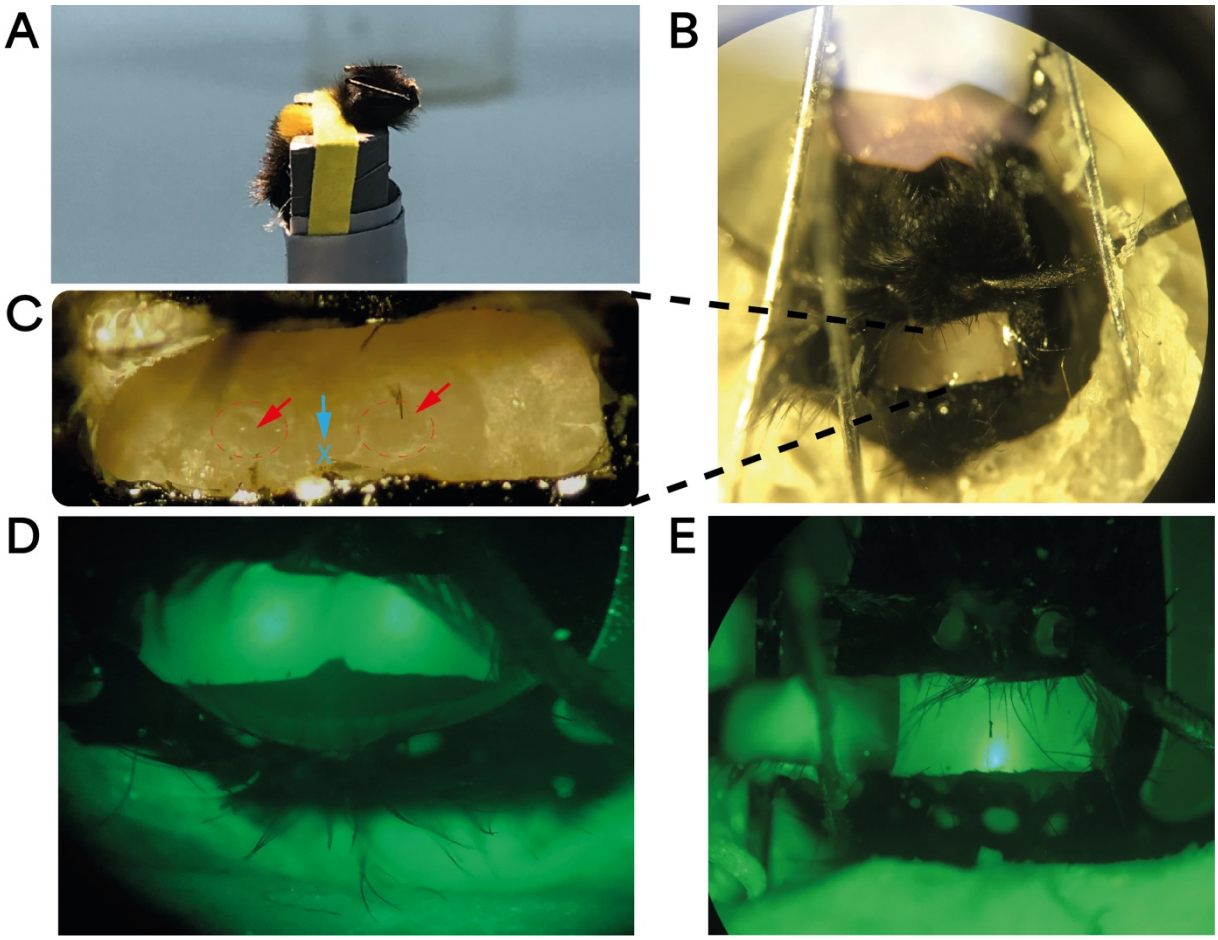

Figure 1. Microsurgery and injection sites. A. Before surgery, the bee is briefly anesthetised on ice and harnessed. **B.** The surgery involves cutting a window into the head cuticle, with three incisions: one at the border of the right eye, one above the median ocellus and one below the antennal stems. **C.** After gently moving glands and tracheae aside, a small incision is made into the neurilemma to access the structure of interest and permit a smooth entry of the nanoinjector. The arrows indicate the injection sites: bilateral injection into the alpha lobes of the mushroom bodies (*AL*, red, dotted circles) and a single injection into the central complex (*CX*, blue, marked with X). The injections into the alpha lobes, **D**, and the central complex, **E**, were later confirmed under fluorescent lighting.

Microinjections were performed 30 minutes before conditioning, a delay that is sufficient to induce a blockade of neurotransmission when procaine is administered (Devaud *et al.*, 2007). In all cases, injections were performed using a pulled glass capillary (GC 100-10; Harvard Apparatus) connected to a pressure nanoinjector

(Nanoject III, Drummond). To target the *MBs*, ~1.0 nL of PBS was injected into each lobe at a depth of 50 µm and an angle of 68–70° relative to the brain surface. To target the central complex, ~1.0 nL of PBS was injected at a depth of 400 µm, entering the midline between the dorsal part of the ventral lobes (Fig 2). Fluorescein (0.5mg/ml) was added to the PBS solution to ensure the injection hit the area of interest. The site was confirmed after injection by fluorescence microscopy (Nikon Intensilight C-HGFI for fluorescence). Under fluorescent lighting, the injection site becomes visually identifiable. When targeting the alpha lobes of the mushroom bodies, the accuracy of the injection was confirmed when both lobes were illuminated. In the case of central complex injections, the accuracy of the injection was confirmed when the illumination was centralised (Fig. 1D, E). Bees that were injected incorrectly, such as those with only one lobe illuminated in the case of bilateral mushroom body injections, were excluded from the study.

Tethering procedure

The tethering procedure was performed right after either surgery (*C2*), injection (*PBSAL*, *PBSCX*) or harnessing (*C1*). The procedure was adopted from earlier work (Lafon *et al.*, 2021). A harnessed bee was positioned vertically with a thorax exposed to a tether attachment (Fig. 3). The banded part of a tether needle was positioned on the bee's thorax and fixed with a small amount of bee wax. After securing the tether, the bee was released from the harness and placed on a Styrofoam ball (50 mm in diameter) for familiarisation with a temporary set-up for 30 minutes. The temporary setup was identical to that employed in the VR experiment. This setup served as a treadmill on which a tethered bee could move as desired.

Virtual reality set-up

After 30 minutes of familiarisation with the temporary set-up, the bee was placed in the VR setup. All experiments used a closed-loop paradigm as described in Lafon *et al.*, 2021. I used software written by Dr Gregory Lafon that is publicly available at https://github.com/G-Lafon/BeeVR. The VR set-up comprised of Styrofoam ball (50 mm in diameter, weight 1.07 g) placed in a 3-D printed cylindrical system (50 mm high, 59 mm in diameter). The ball was supported by the airflow (33 $L.min^{-1}$) continuously

supplied by the aquarium air pump (AquaOxy 2000). The cylindrical setup was fixed facing a half-cylinder vertical screen (semitransparent tracing paper, 268 mm in diameter, 200 mm in height), positioned at a distance of nine centimetres from the bee. A virtual environment was thus projected on this screen (video projector Acer K135).

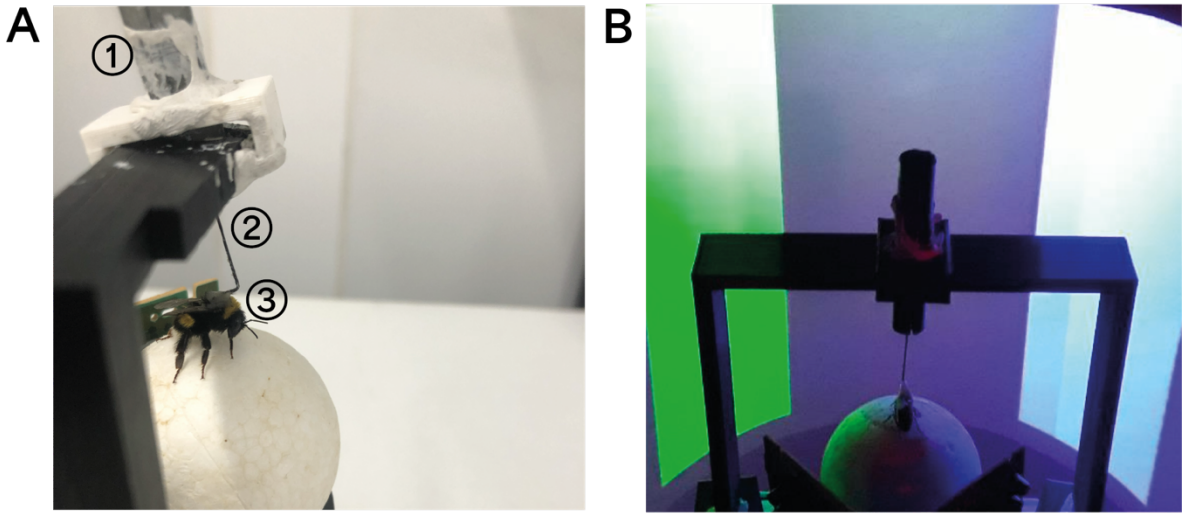

Figure 2. Experimental set-up and virtual environment. A) The tethering system consisted of a plastic cylinder (1) fitted into a holding frame. Within the cylinder, a glass cannula held the steel needle (2), with its curved end secured to the thorax of a bee using melted beeswax (3). **B)** Virtual environment. Bees were presented with a colour discrimination task involving learning the association between the colour of the cylinder and reward or punishment.

Visual stimuli

I trained bees to discriminate between two vertical-coloured virtual cylinders. The dimensions of both cylinders were the same as described earlier (Lafon *et al.*, 2021). The colours were Dark Green (RGB: 0, 51, 1, with a dominant wavelength of 528 nm) and Blue (RGB: 0, 0, 255, with a dominant wavelength of 446 nm). At the beginning of each trial, the cylinders were positioned centrally at -50° and +50° from the bee's body axis. The decision would be logged whenever a bee approached either cylinder within three centimetres and cantered it within her visual field (Lafon *et al.*, 2021).

Discrimination task and testing

The training protocol consisted of a single pre-training trial and six consecutive training trials followed by a single unrewarded test trial (Fig. 1). Pre-training was used to determine an innate colour preference and assign rewarding colours individually. I assigned the rewarding colour as the opposite of what was initially chosen. If no choice was made, the reward colour was assigned at random. A choice of the correct cylinder, *CS+,* was rewarded with 50% w/w sucrose solution, and an incorrect choice, *CS-*, of the opposite colour was associated with 1.2 $g \cdot L^{-1}$ quinine solution.

In each trial, bees were presented with a black screen for 60 seconds, followed by the presentation of two cylinders (Fig. 3). Every trial continued until a bee fixated on one of two stimuli. If no choice was made, the trial automatically terminated after 180 seconds. A dark screen was presented between trials for 60 seconds (Fig.3). Bees that did not make a choice in three or more trials were not included in the analysis. This resulted in the following percentages of bees kept for later analysis: 49% (20/41) *C1* group, 30% (21/70) *C2* group, 27% (21/79) *PBSAL* group and ~17.9% (10/56) *PBSCX* group. At the end of the experiment, all animals were frozen at -20°C.

After the last training trial and following a 60-second inter-trial interval as within the training block, bees were tested. The test consisted of a single presentation of both stimuli for a maximum of 180 seconds. Here, choices were not reinforced. As in previous work (Lafon *et al.*, 2021), I recorded the first stimulus the bee fixated on and the duration of fixation. These variables were collected during both the training and test trials and used for later analysis.

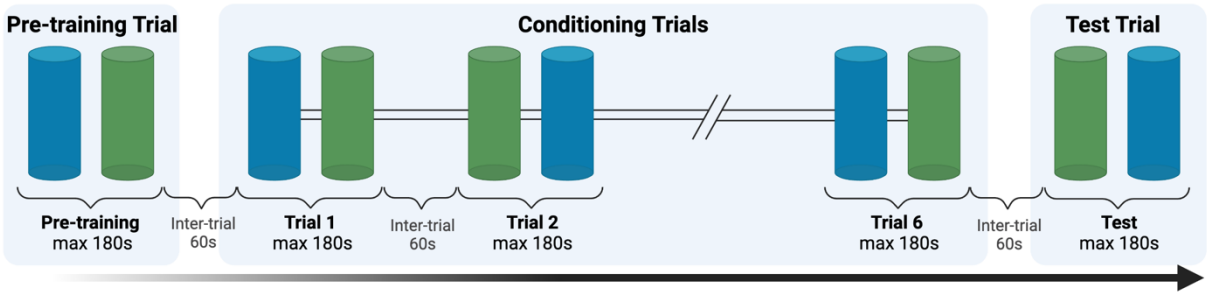

Figure 3. Conditioning protocol for colour discrimination learning. To determine a possible innate colour preference, bees were first offered a pre-training trial (unrewarded). Next, bees were trained for six conditioning trials lasting a maximum of three minutes each and separated by one minute (inter-trial interval). After the end of conditioning, bees were tested. Test consisted of a single trial during which cylinders were displayed for three minutes. Test choices were unreinforced.

Statistical analysis

The data were plotted and analysed using RStudio v.3.2.2 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org) and custom-written scripts. To analyse the learning performance, each bee's choice was categorised as either correct (*CS*+), incorrect (*CS*-) or no choice (*NC*). These categories were used to translate bee choices to a binary variable – "Score". For example, if a bee fixated on a rewarded cylinder, the variable "Score" would be recorded as "1, 0, 0" for "Choice" categories *CS*+, *CS*-, and *NC*, respectively. Learning performance data were analysed using generalised mixed linear models (GLMM) with a binomial error distribution and a logit link function (glmer function, lme4 package) (Bates *et al.*, 2015). As described earlier, "Score" was a binary dependent variable. The independent variables were the trial number ("Trial"), the choice category ("Choice") and the treatment group ("Treatment"). The identity of the bee ("ID") was included as a random intercept variable. I additionally fit a linear mixed model to analyse the walking speed of the bees during the acquisition trials (lmer function, lme4 package). Here, the treatment group ("Treatment") and trial number ("Trial") were included as fixed factors, and the identity of the bee ("ID") as a random intercept variable. Bee performance in the test was also analysed using a generalised linear mixed model (GLMM) with a binomial error distribution and a logit link function. As with the learning performance analysis, the model included the choice category (*CS*+, *CS*-, *NC*) and treatment group as fixed factors, with "Bee ID" as a random factor. The significance of each fixed factor in the model was assessed using analysis of variance tests (Anova function, *car* package), and followed by post hoc multiple comparisons when applicable (Tukey p-value adjustment method, R package emmeans (Lenth *et al.*, 2019). The time spent fixating on one of two choice types (*CS*+ vs *CS*-) was compared using the Wilcoxon single-rank test.

5.3. Results

Comparing the learning performance of different treatment groups: all bees

Bumblebees were subjected to manipulations varying in invasiveness to explore the possibility of using structure-specific procaine microinjections before testing bees under a controlled VR environment. The ability to learn colour-based discrimination was tested within four treatment groups: *C1*, unmanipulated control bees; *C2*, "sham" control bees subjected to surgery only; and two PBS-injected groups targeting the alpha lobes of the mushroom bodies, *PBSAL*, and the central complex, *PBSCX*.

During the conditioning session, bees that failed to fixate on one of two cylinders for at least three trials were excluded. Due to the difference in the degree of brain damage caused by the manipulations, I expected an increase in excluded bees with increasing invasiveness. As demonstrated in Figure 4, there is a graduated increase in the proportion of discarded bees with an increasing degree of invasiveness (*C1* being non-invasive, and *PBSCX* most invasive due to the deeper location of the central complex in the brain). However, the difference was significant only between *C1* and *PBSCX* (51.22% and 82.14%), as demonstrated by a paired proportion test (χ^2=9.73, df=1, p.adj<0.05) (Fig. 4).

I then looked at learning acquisition performance during the training session. Learning curves were constructed based on the percentage of bees choosing the rewarding cylinder (*CS*+), unrewarding cylinder (*CS*-), or making no choice (*NC*) over the course of six training trials. No significant interaction between the group, the number of trials and bee choice were observed (χ^2=5.83, df:6, p=0.44), thereby showing that learning dynamics were similar across groups (Fig. 5).

Only full control groups (without manipulations, C1) showed a significant interaction between *CS* and *Trial* (*C1*: χ^2=9.74, df:2, p<0.01). The paired trial-by-trial comparison of the likelihood of performing one of three behaviours (responding to *CS*+, *CS*- or not responding, *NC*) showed significant differences in the later trials with significantly more responses towards *CS*+ (see Table 1).

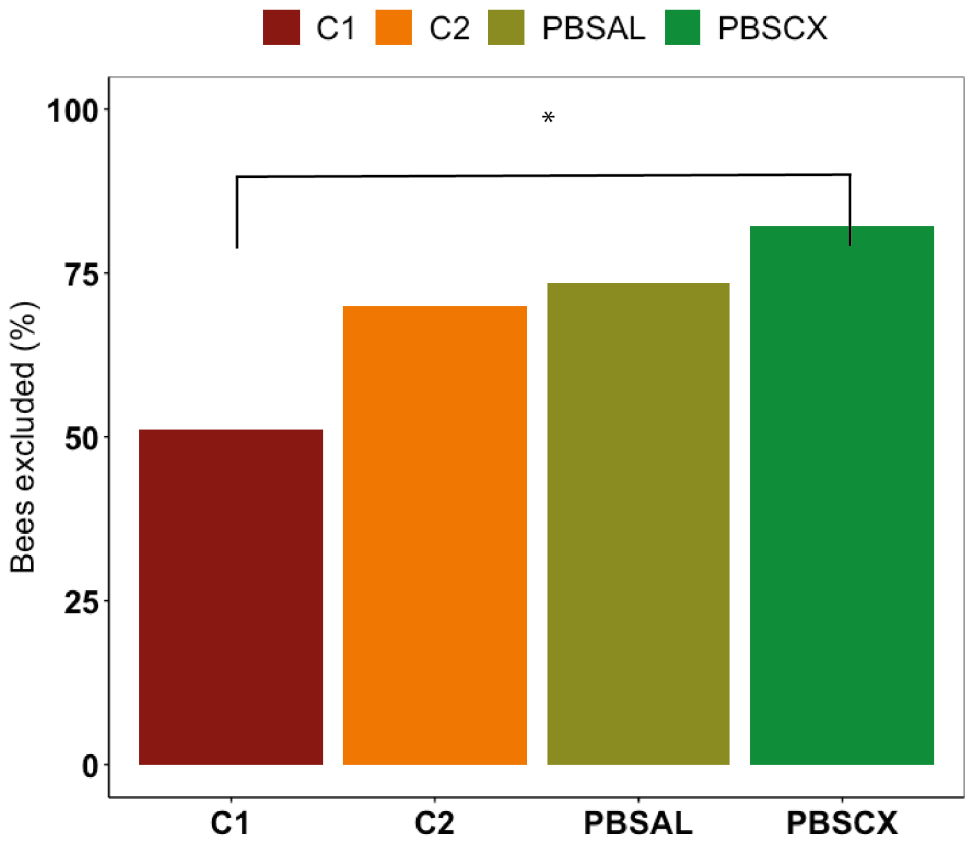

Figure 4. Percentage of excluded bees. The percentage of bees in each group that failed to make a choice in three or more trials. These bees were excluded from later testing; 51.22% of unmanipulated bees (C1, $N_{excluded}$= 21, N_{total} = 40), 70% of 'sham' control (C2, $N_{excluded}$ = 49, N_{total} = 70), 73.42% of bilaterally saline-injected (PBSAL, $N_{excluded}$ = 58, N_{total} = 79) and 82.14% of central complex saline-injected (PBSCX, $N_{excluded}$ = 46, N_{total} = 56) bees were excluded. The asterisk indicates a statistically significant difference in the percentage of discarded bees between *C1* and *PBSCX* (χ^2=9.73, df=1, p.adj < 0.05).

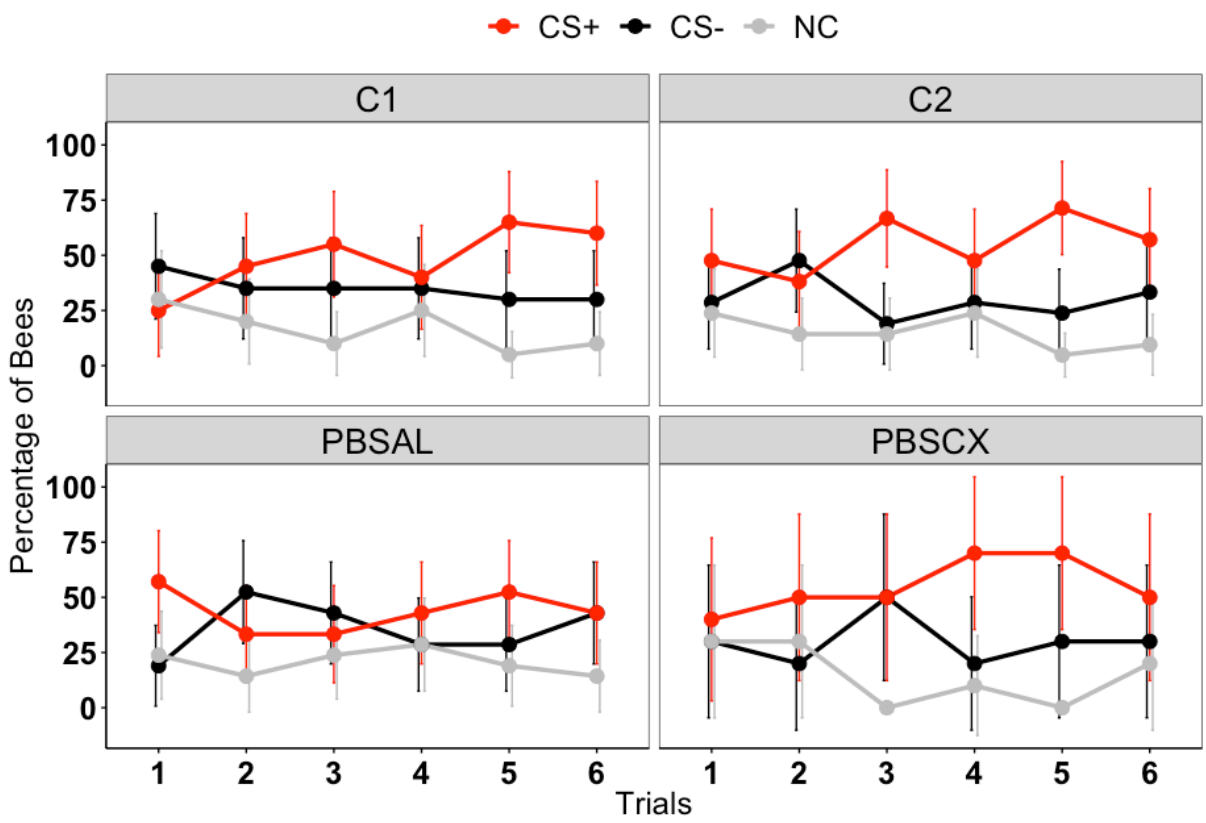

Figure 5. Performance of all bees trained under differential conditioning in VR. Panels depict learning acquisition over six training trials, expressed as the percentage of bees making a choice and 95% CI. Making a correct choice is depicted in red (CS+), incorrect in black (CS-), and not choosing in grey (NC). Each panel corresponds to one of four treatment groups: unmanipulated bees, C1, n=20; "sham" control, C2, n=21; saline injected in both MB alpha lobes, PBSAL, n=21; saline injected into central complex, PBSCX, n=10.

Walking speed as a measure of bee lethargy

I next assessed whether the walking speed of bees varied between treatment groups over the training period (Fig. 6). The analysis revealed no significant interaction term between the trial number and the treatment group (χ^2=3.5234, df:3, p=0.32). This implies that there was no difference in the progression of the walking speed across trials between all treatment groups. However, the overall walking speed was significantly affected by the treatment group (χ^2=12.7507, df:3, p<0.01), even if it did not vary significantly across trials (χ^2=2.0009, df:1, p=0.157). Paired post-hoc analysis showed that the difference was due to a significant difference between *C1* and both saline-injected groups (*C1 vs PBSAL*: t.ratio=4.37, df=68, p<0.001; *C1 vs PBSCX*:

t.ratio=4.44, df=68, p<0.001), and *C2* and the central complex injected bees (t.ratio=2.74, df=68, p<0.038).

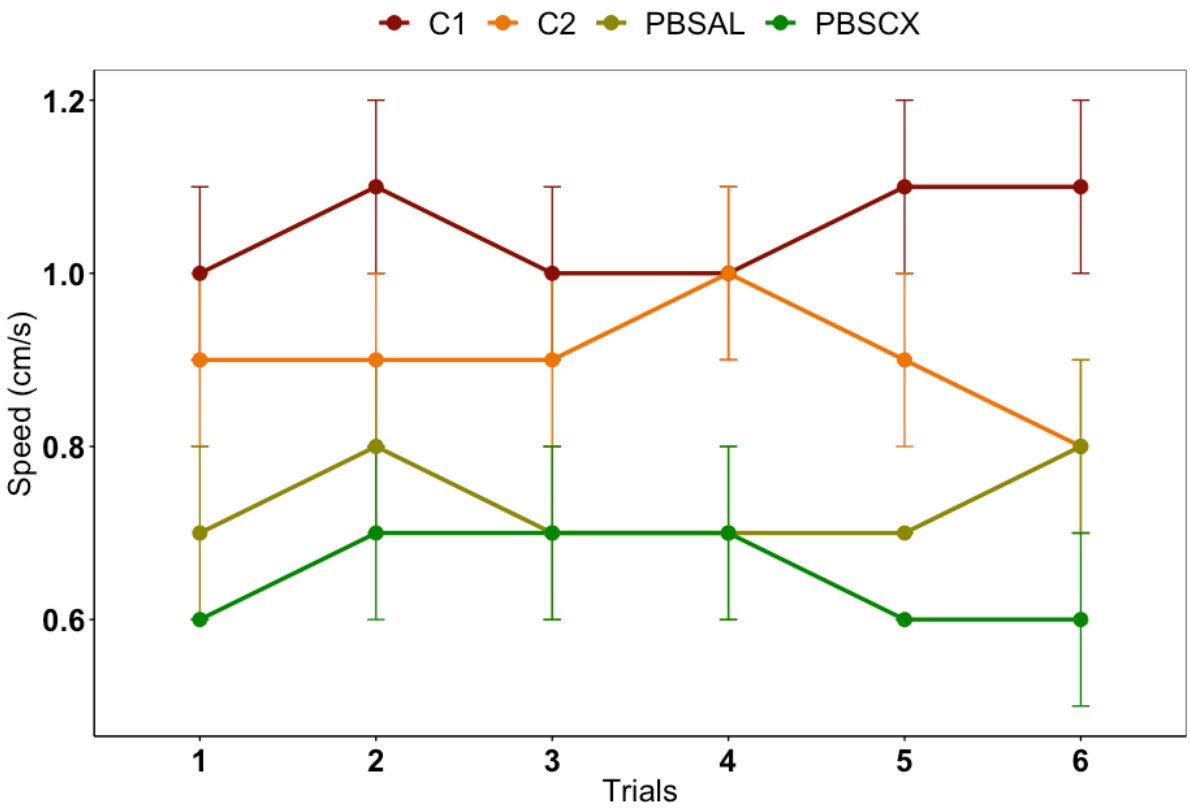

Figure 6. Walking speed during the acquisition trials. None of the treatment groups showed a significant change in their walking speed across trials. Both saline-injected groups were significantly slower compared to unmanipulated bees (emmeans: *C1* (n=20) vs *PBSAL* (n=21): p<0.001; *C1* (n=20) vs *PBSCX* (n=10): p<0.001), but only the *CX-injected* bees differed in their walking speed from sham control bees (emmeans: *C2* (n=21) vs *CX* (n=10), p<0.05). Dots depict the mean walking speed for each treatment group, error bars depict the standard error of the mean.

Test Performance

To further investigate the effect of manipulations on learning, I looked at bee performance in the test (Fig.7A). Additionally, I looked at the time bees kept the *CS+* and the *CS-* in the centre of their visual field, i.e., fixation time (Fig.7B). As in the learning phase, only unmanipulated bees were significantly more likely to choose the correct cylinder (χ^2=23.5754, df:2, p<0.0001), and fixated significantly longer on the CS+ than the CS- (CS+ vs CS-, z=-2.44, df=19, p=0.02).

Comparing learning performance of different treatment groups: learners only

Next, I assessed the learning dynamics of true learners in each group. Learners were considered bees who made correct choices in the non-reinforced test, while bees who made errors or did not make a choice were considered non-learners. The learning acquisition models were re-run including learners only. Although the curves did suggest a tendency for more learning during the training phase (Fig.8), the model did not show any significant effect of treatment (χ^2=8.7787, df:6, p=0.19). Therefore, even learners of different groups experienced similar learning dynamics.

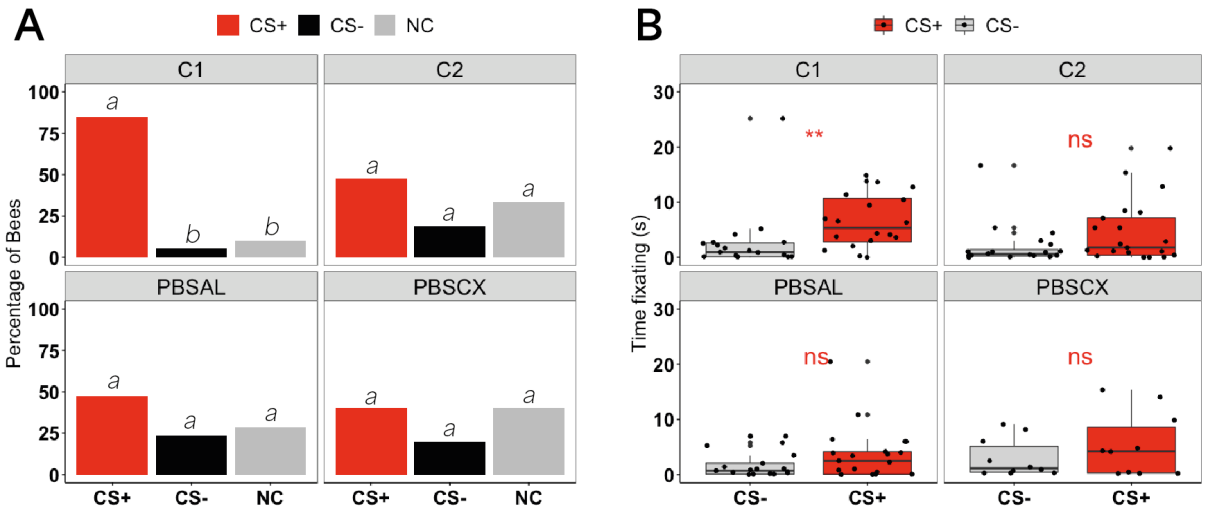

Figure 7. Test performance in a colour discrimination task. A) Percentage of bees making a choice. Correct choices are depicted in red (CS+); incorrect choices are depicted in black (CS-); making no choice is depicted in grey (NC). Each panel represents one of four treatment groups: unmanipulated control, C1, n=20; "sham" control, n=21, C2; saline injected in both alpha lobes of the mushroom bodies, n=21, PBSAL; and saline injected into the central complex, n=10, PBSCX. Within each panel, bars labelled with different lowercase letters indicate significant differences between the groups ($p < 0.05$). **B**) Time spent fixating on one of two coloured bars. Fixating on the cylinder associated with a reward is depicted in red (CS+), and the cylinder associated with punishment in grey (CS-).

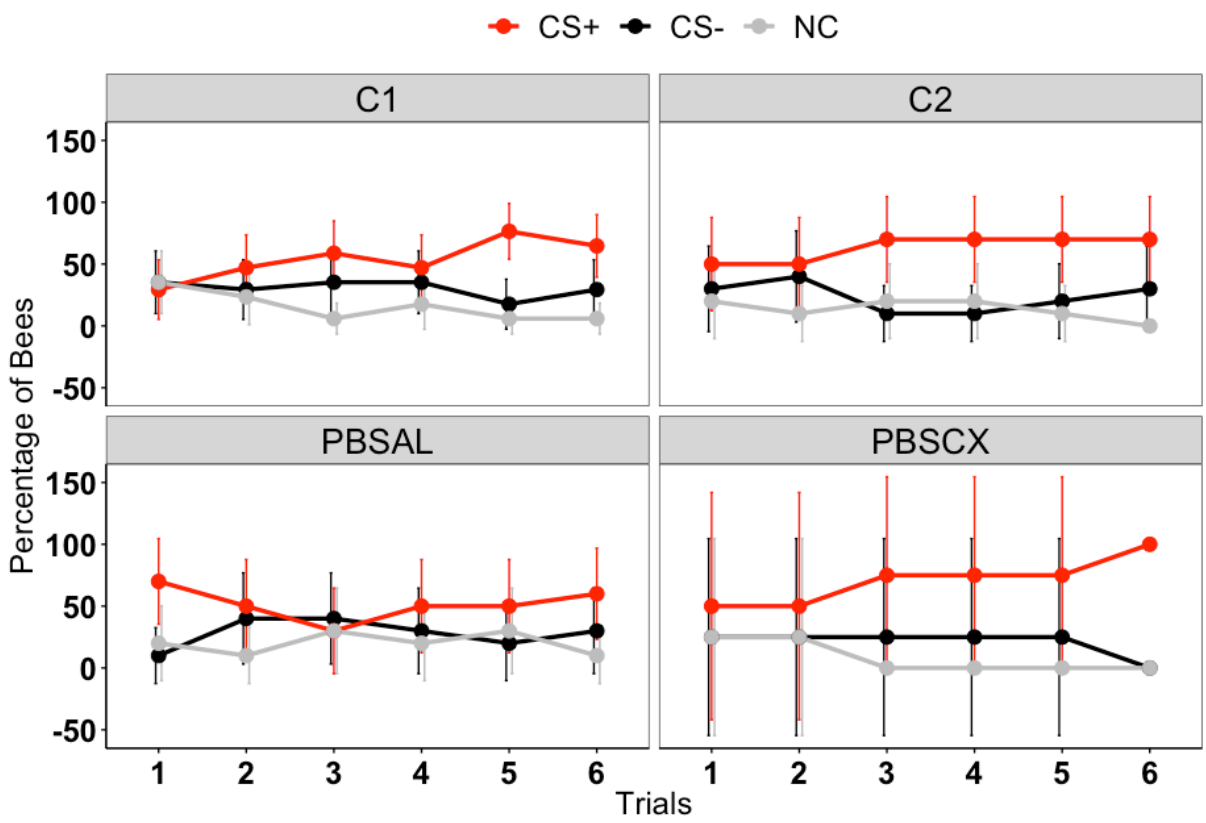

Figure 8. Performance of learner bees trained under differential conditioning in VR. Panels depict learning acquisition over six training trials, expressed as the percentage of bees making a choice and 95% CI. Making a correct choice is depicted in red (CS+), incorrect in black (CS-), and not choosing in grey (NC). Each panel corresponds to one of four treatment groups: unmanipulated bees, C1, n=17; "sham" control, C2, n=10; saline injected in both MB alpha lobes, PBSAL, n=10; saline injected into central complex, PBSCX, n=4.

5.4. Discussion

The objective of the study was to adapt existing microinjection and VR protocols for bumblebees and evaluate whether the procedure affects their cognitive abilities. To address this concern, I examined the influence of saline injections on colour learning performance in VR. I demonstrated that even with reducing the pre-VR waiting time from the standard 60 minutes to 30 minutes (Macri, Avargues-weber and Lafon, 2021), unmanipulated bumblebees (Group C1) are still able to complete a colour discrimination task. Thus, reduced time spent in the dark before the conditioning onset did not impair learning. Keeping the pre-VR time period short, becomes important when using procaine as a compound for transiently inactivating brain areas of interest in the future. After injection, the procaine remains active for a maximum of 90 minutes

(Devaud *et al.*, 2007), therefore, with waiting time reduced to 30 minutes, the discrimination learning experiment can be completed well within this period.

A significant number of bees failed to pass the exclusion criterion. Importantly, the percentage increased as the complexity of site-specific injections increased. Injecting into the central complex had the most negative impact. The central complex plays a key role in analysing sensory data and translating it into appropriate behavioural reactions, and thus is responsible for generating motor responses (Pfeiffer and Homberg, 2014; Plath and Barron, 2015). Considering the depth of its localisation within the bee brain, injecting saline into this region is likely to result in significant damage to the central complex, as well as surrounding tissue, explaining the high number of eliminated bees in the central complex injected group.

The substantial tissue damage caused by the procedure could also explain the lack of learning acquisition. Both the mushroom bodies and central complex are known to be involved in visual learning. The lobes of the mushroom bodies are the output regions of sensory signals (Menzel, 1999). The central complex not only plays a crucial role in motor function (Strauss, 2002; Neuser *et al.*, 2008; Triphan *et al.*, 2010; Homberg *et al.*, 2011; Ritzmann *et al.*, 2012) but also visual memory (Neuser *et al.*, 2008; Kong *et al.*, 2010). Impaired learning could be expected if these brain areas malfunction. Alternatively, the lack of learning acquisition in the present study could be a result of impaired motor response rather than learning *per se*. In an earlier study by Plath *et al.* (2017), where no impairments were observed with saline injections, bees only needed to avoid spending time in one of the two-coloured chambers associated with an electric shock. In our task, on the other hand, bees had to walk on a ball to approach coloured cylinders in VR. Controlling ball movements while being tethered can be considered a significantly more demanding motor task as compared to walking, which was the required behaviour in the Plath et al. (2017) study. Tissue damage is likely to have occurred in both studies. It may, however, have been, less noticeable in Plath et al. study (2017) because the motor requirements were not as demanding. The significant reduction in walking speed with increasing invasiveness of the procedures further supports these speculations. Here, just as with the exclusion criterion discussed earlier, the central complex injected bees walked slowest. Due to the role that this particular area of the brain plays in generating appropriate motor responses, exploring the central complex using microinjection techniques might present certain difficulties.

Finally, it should also be noted that the final sample size for this study was relatively small. A significant number of bees did not engage in the task, and consequently, did not meet the inclusion criteria. The percentage of bees eliminated for the least invasive treatment, “sham” control, was 51% while reaching as high as 82% for the considerably most invasive group that received a saline injection into the central complex. Given such a drastic reduction in sample size, it is therefore possible that the analysis simply lacks statistical power. A larger sample size would be necessary to define whether learning acquisition was truly impaired across treatments. Moreover, including more bees in the final analysis could also control for the observed individual variability. Specifically, it would allow one to exclude bees that failed to probe both reward and punishment. When making a correct choice, some bees were unsuccessful in extending their proboscis and probe a reward. Although these bees met the inclusion criterion and were included in the final analysis, it remains unclear whether they learned to associate colour with the correct outcome. Experiencing both rewards and punishments is crucial for associative learning tasks. Consequently, including these bees could potentially misrepresent the performance of the group by possibly masking the learning acquisition in those bees who fully completed the task (i.e., probed both reward and punisher).

In this study, I explored the possibility of combining brain microinjections with behavioural assays performed in a virtual environment. The main objective was to evaluate the potential harm of the required procedures, such as brain surgery and site-specific injection. Using a simple colour discrimination task, I demonstrate that the cognitive abilities required to perform the task in VR are negatively affected. Both the surgical procedures and saline injections resulted in an increased number of excluded bumblebees and reduced learning performance. Despite these negative results, I emphasise the need for follow-up studies. The technique has been successfully applied in past studies (Plath *et al.*, 2017). Therefore, given the relatively small final sample size, the results presented here could be misleading. Thus, more data are needed to reach firm conclusions.

CHAPTER SIX

Discussion

6.1. Summary of findings

In this thesis, I investigated the effects of stress-induced affective states on visual decision-making in the buff-tailed bumblebee (*Bombus terrestris*). The primary goal of my work was to first establish a new judgment bias test for measuring emotion-like states in bees and re-assess whether bees in a negative state are indeed "pessimistic". Next, I experimentally investigated whether being in such a state impacts bee decision-making in a way akin to mammals. Specifically, I tested whether being in a negative state lead to the modulation of information processing at early and later stages of decision-making. The former was assessed by estimating visual acuity while the latter was assessed by measuring behavioural flexibility.

Overall, my results demonstrate the capacity for states in bees that resemble emotions in mammals. Importantly, the findings not only provide evidence that negative emotion-like states in bees exist but that these states directly affect the way visual information is processed to guide bee decisions. In the first experimental chapter (Chapter Two) I revisited the judgment bias paradigm. I developed a new test that utilises active choice design to eliminate earlier confounds. My results provide strong evidence that, stressed bees do indeed exhibit a pessimistic bias. The results also provide strong evidence that a state-dependent sensory bias, particularly the ability to differentiate conditioned colours, could not explain the observed behaviour. Further validation for interpreting the observed pessimistic behaviour as a judgment rather than a sensory bias was provided by mathematical modelling. Both the behavioural and modelling results thus strongly suggest that stressed bees are truly pessimistic. Importantly, modelling also proposed that the best explanation for the observed biased responses in stressed bees was due to a drastic change in their prior expectations of reward. This suggests that when in a negative state, bees perceive higher rewards as less likely. Taken together, the results of this chapter suggest that negative manipulations do indeed affect bee decision-making under ambiguity in a way that parallels state-dependent judgment biases in mammals. In addition to providing much stronger evidence for the existence of such states, my work also provides a testable hypothesis about the mechanisms underlying judgment biases in bees.

After confirming the presence of emotion-like states in bumblebees, I next explored whether these states affect decision-making through the modulation of visual information at different processing stages (Chapters Three and Chapter Four). To

assess the effects of stress on early vision, I aimed to measure the perceptual thresholds for fundamental characteristics processed early in the visual pathway – contrast and spatial frequency. I demonstrate that, similar to humans (Becker, 2009; Bocanegra and Zeelenberg, 2009; Bocanegra, 2011), the processing of both these low-level features is modulated when in a negative state. These results therefore suggest that threat exposure, mimicked by shaking, fine-tune bee early vision to facilitate finer detail perception and increase certainty in their decision-making.

I further investigated how stress-induced states modulate later stages of information processing using a reversal learning paradigm (Chapter Four). Reversal learning assesses the capacity to adapt one's behaviour to change (Kehagia, Murray and Robbins, 2010) This ability requires accurate evaluation and integration of information. I demonstrate that being in a negative state facilitates faster adaptation by bees to changes in stimuli-outcome contingencies. Moreover, I argue that the affect-inducing manipulation specifically facilitated new excitation learning but not extinction learning. I also speculate that this positive effect is driven by a treatment-dependent increase in sensitivity to rewards. This speculation finds partial support in the results observed in the second experimental condition. When both outcomes were rewarding, no treatment-specific modulatory effects were observed. This was possibly because bees in a negative state were more accepting of lower rewards, as evidenced by greater low reward depletions, thus indicating a temporal increase in sensitivity to rewards. Therefore, when in a negative state, bees exhibit greater sensitivity to changes in rewards, enabling them to adapt more effectively to such changes.

The final aim of my thesis was to investigate possible options for future neurophysiological explorations of emotion-like states in bees (see Chapter Five). In the absence of sophisticated methods, techniques involving targeted microinjections of compounds, like procaine, that suppress neural activity are an important avenue to explore. In principle, such microinjection could be coupled with virtual reality behavioural assays. In my fifth chapter, I explored the effects of site-specific microinjections on bee cognition. Injecting bees with saline alone greatly impaired learning performance. While the injection procedures indeed damaged neural tissue, it is however unclear if the lack of learning acquisition observed in the study is due to impaired cognitive function, impaired motor function, or both. Taken together, although the results are far from promising, it remains unclear whether the combination of microinjection with VR is truly a non-viable method.

6.2. Limitations and future work

This research provides substantial evidence demonstrating that emotion-like states affect bee decision-making. Nevertheless, some limitations should be considered. The rationale behind designing the experiments presented in Chapters Three and Four was that emotion can modulate distinct stages of information processing. While the assays used to explore this hypothesis - visual acuity and reversal learning - are a good fit, they have some limitations. In bees, just as in mammals, the neurophysiological processing of visual information can be complex. Such processing is achieved at sequential stages within the brain: beginning with the transformation and segregation in the peripheral visual neuropils, followed by the integration of information in higher-order brain centres (Paulk *et al.*, 2008, 2009). Processing of achromatic cues begins within the optic lobes, where neurons in the lamina exhibit amplitude responses to varying spatial frequencies and contrasts (Ryan *et al.*, 2020). In humans, these low-level visual features are also processed in the early visual processing areas and can undergo emotional modulations (Becker, 2009; Bocanegra and Zeelenberg, 2009; Bocanegra, 2011). In Chapter Three, I aimed to address whether the same is true in bees. However, while estimating contrast and spatial resolution thresholds reflects differences in responding to these low-level visual features, it remains unknown whether the behavioural response was driven by differences in neural activity originating specifically in the peripheral neuropils. Therefore, it would be of great interest to perform neurophysiological measurements to further confirm state-induced modulations of early vision. Furthermore, it would be highly valuable to compare the visual acuity thresholds obtained through behavioural measures in my study with estimates gathered through pattern electroretinography (Ryan *et al.*, 2020). Similarly, the goal of Chapter Four was to use reversal learning – a paradigm that requires higher-order brain structures (Boitard *et al.*, 2015) – to identify whether later information processing undergoes emotional modulation in bees. As with visual acuity experiments, it could also benefit from a neurophysiological investigation in the future.

The final limitation to mention, and perhaps of greatest interest for future work, involves assessing biogenic amines in shaken bees. In the introductory section of my thesis, I presented evidence suggesting that biogenic amines are probably working together to facilitate comprehensive coordination among the subsystems of the nervous system. Given biogenic amine structural and functional commonalities, this

coordination appears to be conserved across both vertebrates and invertebrates (LeDoux, 2012). Importantly, such holistic coordination is suggested to support the transmission of emotion-evoking information to various brain regions (Lövheim, 2012). Therefore, it is not unreasonable to suggest that the difference in behaviour observed in some of the experiments (or even all) might be influenced by changes in biogenic amines, explaining the results. For example, as I discuss in Chapter Four, the facilitating effect of shaking on reversal learning could be potentially well explained by an increase in reward sensitivity mediated by an increase in octopamine. Although the proposed explanation is rooted in previous work linking octopamine with reward processing (Scheiner *et al.*, 2002; Giurfa, 2006; Rein *et al.*, 2013; Muth, Breslow and Leonard, 2023), previous work had shown that the shaking treatment decreased levels of all three systemic biogenic amines, including octopamine (Bateson *et al.*, 2011). However, the procedure by which shaking was administrated in my study and this early work is different (as I argue in Chapter Four). Nevertheless, to resolve this issue, assessing levels of biogenic amines in shaken bees using the same procedure of shaking as in my study is essential. If such a change is indeed observed, it would be interesting to take it further and estimate how the change unfolds over time. Perhaps, it would be possible to detect a time period when the levels return to the base line thus indicating the termination of the negative affective state. Taken together, such a biogenic amine study could indeed give us a better insight into the neural dynamics supporting negative emotion-like states in bees.

6.3. The adaptive value of negative emotion-like states in bees

The approach adopted in my thesis did not aim to find which human discrete emotion best matched the states observed in bees. Emotional expressions in species significantly different from ours are probably so distinct that trying to find homologous expressions might not be productive. A more valuable approach is to explore the functional properties of these states — namely, whether they support the survival goals of the species — and compare these functions to emotional states in other animals. Therefore, the key question to my thesis was not whether bumblebees have “fear” or some other human-like emotion but, rather, whether they have inner states that have functional properties analogous to those in other animals. Therefore, the remaining

question to as is: could the differences in response observed in my experiments be thought of as adaptive?

Emotions are thought to evolve from reflexes to permit a more flexible response to an environment to achieve important survival goals – approaching resources and avoiding punishments (Mendl, Oliver and Paul, 2010). An animal's response to rewards and punishments is orchestrated by emotions that are shaped by cognitive appraisals of situations necessitating such responses. For example, experiencing a predatory attack activates the flight-or-fight response, increases energetic demands, and leads to appraising the current state of the world as dangerous. To survive in a given state of the world, resources might become more valuable, and responding more efficiently to perceived threats might become more beneficial. Thus, lowering the threshold for response to both rewards and punishment might function adaptively (Nettle and Bateson, 2012). Neurophysiological, cognitive, and behavioural changes thus must take place to support such response tendencies. For example, the reduced threshold for threat could be achieved by increasing the processing of threat-related features. This was observed in the visual acuity experiment (Chapter Three) where an increase in visual acuity, specifically in processing fine details, was estimated in shaken bees. Such modulation could be an advantage as perceiving finer information may facilitate the detection of their natural predators, such as crab spiders *Misumena vatia* (Dukas and Morse, 2003). Threat avoidance therefore could be considered as one possible adaptive function carried by a negative emotion-like state in bees. Another such function could be surviving in unpredictable environments. Becoming more sensitive to reward and punishment can facilitate the ability to rapidly learn from experiences and adapt behaviour. When in a negative state this becomes crucial for survival, especially if the environmental conditions are unstable. Thus, emotion might orchestrate cognitive change to support such flexibility. This idea is supported by the results presented in Chapter Four. Bees that were subjected to a negative state were better at reversing their learning when the stimulus-outcome contingencies changed. Therefore, another functional property of negative emotion-like states in bees is to quickly adjust behaviour in a way that supports current needs.

While detecting threats and adjusting to changes in the environment are clearly advantageous adaptations, displaying pessimistic tendencies may appear contradictory. When faced with an ambiguous situation, with information equally suggesting the possibility of favourable and less favourable outcomes, why would bees

be biased toward the latter? There could be some arguments that this behaviour might indeed be beneficial. Pessimism is thought to be an adaptive “investment strategy” in an environment of scarcity, unpredictability, and dangers (Leahy, 2002). Therefore, a pessimist is more likely to avoid risky decisions that could jeopardize what they have already accumulated in pursuit of more rewarding opportunities, which, in such unfavourable environments, are considered unlikely. Similarly, experiencing negative events, e.g., predatory attacks, exhausts energetic stores available to an animal and signals that the present state of the world is rather unfavourable. Therefore, adopting a pessimistic strategy allows the individual to avoid costs associated with risky decisions, thereby facilitating survival. In other words, from an evolutionary perspective, when conditions are unfavourable, having reduced future expectations is adaptive. Similar thinking can be applied to the pessimistic-like behaviour observed in Chapter Two. Experiencing a predatory attack, which demands high energy to overcome (Even, Devaud and Barron, 2012), triggers a state in bees signalling an unfavourable environment — uncertain, scarce, and dangerous - thereby lowering expectations regarding positive outcomes. Anticipating negative outcomes, such as expecting flowers to stop providing rewards, promotes less risky behaviour in bees, as earlier studies demonstrate that these bees are more likely to terminate persisting on unfavourable flower patches (Gil, Marco and Menzel, 2007; Gil and De Marco, 2009), and thus save time and energy. Thus, it is worth speculating that in natural scenarios, a pessimistic bias induced by dangers could benefit bee survival by guiding their decisions toward less risky options.

Taken together, my results thus argue for emotion-like states in bees that enable adaptive responses to their environment.

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