LEARNING ON THE BLINK: PHYSIOLOGICAL PREDICTORS OF ADAPTIVE LEARNING IN AN EQUINE MODEL

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ABSTRACT

Domestic horses are regularly expected to demonstrate behavioural flexibility, the ability to adapt to changing environments, such as different riders and handlers. On the other hand, safe and successful equestrian activities rely on the horse to give consistent responses to important commands, demonstrating cognitive control. Striatal dopamine is a neurotransmitter involved in learning and may be related to spontaneous eye blink rate. Physiological arousal is known to influence cognitive performance in humans, but little is known about the relationship between arousal and learning in domestic horses. The aim of this thesis was to investigate novel physiological predictors of learning performance in horses. The same cohort of horses were investigated in a series of cognitive tasks, designed to challenge various aspects of cognitive flexibility and cognitive control. Spontaneous eye blink rate, heart rate variability and eye temperature were measured throughout as possible predictors of learning performance. It was revealed that horses' arousal state at baseline and during training reliably predicts cognitive performance. In addition, this thesis provided preliminary evidence that hemispheric activation may be observable through lateralised eye temperature changes Further, it was revealed that horses may have higher cognitive capabilities than previously thought. This thesis makes several novel contributions to knowledge about equine learning, cognition and welfare.

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LIST OF ABBREVIATIONS

+R	Positive reinforcement
AG	Anna Gregory
AIC	Akaike information criterion
AV	Atrioventricular
BIS/BAS	Behavioural Inhibition System/Behavioural Activation System
BRV	Blink rate variability
BWB	Belgian Warmblood
CH	Catherine Hake
CLS	Contact lens sensor
CP	Count period
CSTC	Cortico-striatal-thalamo-cortical loop
CVT	Consistent value target
ECG	Electrocardiogram
EEG	Electroencephalogram
EMG	Electromyography
ERN	Error-related negativity
FE	Further education
fMRI	Functional magnetic resonance imaging
G	Gelding
GLM	Generalised linear model
HE	Higher education
HF	High frequency band
hh	Hands high
HRV	Heart rate variability

HVT	High value target
IBI	Inter-beat interval
ID	Irish Draught
IRT	Infrared thermography of eye temperature
LE	Louise Evans
LF	Low frequency band
LVT	Low value target
M	Mare
MBL	Model-based learning
mPFC	Medial prefrontal cortex
MVH	Modified valence hypothesis
NTU	Nottingham Trent University
-P	Negative punishment
PET	Positron Emission Tomography
PFC	Prefrontal cortex
RD	Reward value discrimination phase
RL	Reward loss phase
RMSSD	Root mean square of successive differences
RPE	Reward prediction error
RVT	Reduced value target
S-	Unrewarded stimulus
S+	Rewarded stimulus
SDNN	Standard deviation of N-N intervals
SEBR	Spontaneous eye blink rate
SH/ISH	Sport Horse/Irish Sport Horse

SNC	Successive negative contrast
SPE	State prediction error
TB	Thoroughbred
VIF	Variance inflation factor
WB	Warmblood

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1. Introduction

Effective animal training relies on the ability of the trainer to understand animal cognition and identify the efficacy of the training programme for the facilitation of adaptive learning. Adaptive learning is a learning process by which an individual uses previous outcomes of behaviour to predict future outcomes of the same behaviour (Soltani and Izquierdo, 2019). This is evolutionarily adaptive because it facilitates the avoidance of pain and suffering, and the seeking of pleasure and valuable resources (Sznajder et al., 2012). Striatal dopamine is a neurotransmitter responsible for marking reward as it occurs, making it instrumental in the process of adaptive learning (Mirenowicz & Schultz, 1996; Schultz, 2002).

Operant conditioning is the process of associating actions with outcomes (Skinner, 1971) using rewards and punishments. Operant conditioning relies on an effective dopamine system to signal that a behavioural response is rewarded, so that it may be repeated for future reward seeking and punishment avoidance (Dayan, 2009). Physiological arousal can have a confounding effect on behavioural responses to operant learning (Starling et al., 2013). The level of arousal at the point of peak performance is thought to be optimal, and arousal above or below this level may be detrimental to cognitive performance (Yerkes and Dodson, 1908).

Individual differences exist in both striatal dopamine and arousal levels, which may affect an individual’s ability for adaptive learning and cognitive flexibility. This review will first outline suitable cognitive paradigms to probe such learning, then present evidence for how individual differences in adaptive learning ability may be influenced by both striatal dopamine and arousal, including a critical review of methods to measure these, before presenting the aims and objectives of this thesis.

2. Adaptive Learning Paradigms

In animal training terms, adaptive learning is the ability of the animal to alter their behaviour to reflect a reward-related change in their environment (i.e. the presence of a stimulus) (Soltani and Izquierdo, 2019). Being adaptable is a function of cognitive flexibility, the ability to balance the contradictory demands of both staying 'on-task' and being flexible in the face of an altered environment or situation (Jongkees and Colzato, 2016). Being able to identify adaptive learning as it occurs is therefore necessary for effective training, though it can be challenging in non-verbal animals, as it is difficult to identify the motivation behind behavioural adaptations in such individuals. Cognitive flexibility is a key feature of cognition, learning and creativity (Ionescu, 2012), and is required for specific higher cognitive abilities such as discrimination-reversal learning, inhibitory control and reward sensitivity. This section of the General Introduction will introduce learning paradigms used to test these cognitive abilities in animals.

2.1 Two-choice Discrimination-Reversal learning

Two-choice Discrimination-Reversal learning (referred to as Reversal learning here) involves actively suppressing the impulse to respond with previously rewarded behaviour, in favour of a newly rewarded behaviour, when either the task stimulus or parameters have changed (Izquierdo and Jentsch, 2012). Reversal learning paradigms are commonly used in studies of cognitive flexibility across a wide range of species, as they test an individual's ability to rapidly alter their behaviour in response to change (Izquierdo *et al*., 2017). An individual with good cognitive flexibility is able to transition from one stimulus response to another relatively quickly, whereas an individual with poor cognitive flexibility will repeatedly offer the previously rewarded response, even though it is no longer rewarded and, as such, is maladaptive. A typical reversal learning paradigm involves a discrimination task, where interaction with one of two targets is rewarded, until this becomes a habitual response (Izquierdo et al., 2017). This is followed by a sudden reversal, whereby the previously unrewarded target becomes the rewarded target, and vice versa. The number of attempts taken to successfully reverse the habitual response is measured as an index of cognitive flexibility (Kringelbach and Rolls, 2003; Clark *et al*., 2004; Izquierdo *et al.,* 2017).

Reversal learning is often grouped under the umbrella of extinction learning, since it requires the individual to suppress a learned behaviour completely when it no longer leads to reward. However, unlike reversal learning, there is no alternative behaviour in extinction learning, and it has been suggested that extinction and reversal learning both measure different aspects of cognitive flexibility (Izquierdo *et al*., 2017). Although both reversal and extinction require an individual to suppress the habit-led impulse to perform previously rewarded behaviours, the two skills are not entirely reflective of impulse control alone (Izquierdo *et al*., 2017). The prefrontal cortex region of the brain is where executive function is controlled, and this includes impulse control (Bechara and Van Der Linden, 2005). However, studies in a variety of species have demonstrated that structures of the striatum, including the nucleus accumbens, are also implicated in reversal learning (Clarke *et al*., 2008; Castañé *et al*., 2010; Dalton *et al*., 2014; Izquierdo *et al*., 2017). This suggests that reversal learning is not purely a marker of executive impulse control and has led to further investigation into the role of the striatum in reversal learning ability. It has been proposed that a combination of serotonin and striatal dopamine determines reversal learning success (Reynolds and Wickens, 2002; Izquierdo *et al*., 2017), and this challenges the idea that reversal learning is purely dependent on an individual's ability for impulse control.

Two-choice discrimination-reversal learning has been previously studied in horses (Warren and Warren, 1962; Fiske and Potter, 1979; Sappington et al., 1997; Martin et al., 2006; Briefer Freymond et al., 2019). These studies have shown that horses can learn to reverse conditioned responses, though with mixed performance results. Briefer Freymond et al. (2019) found no significant association between physiological arousal and reversal learning performance in horses, though the authors suggest that this may have been confounded by the locomotory demands of their study design on subjects. They suggest that future studies should design stationary reversal tasks and take additional measures of arousal (Briefer Freymond et al., 2019). Further, no studies to date have included baseline arousal in their analyses, despite a possible association between day-to-day arousal and the impact this may have on learning performance, as indicated in human studies (Maldonado et al., 2008; Stawski et al., 2019).

2.2 Inhibitory control

In addition to reversal learning, it is also important to make the distinction between impulse control in the context of an extinction learning task, and inhibitory control. Inhibitory control is the ability to actively ignore distracting stimuli (attentional inhibition) and/or suppress a behavioural response (response inhibition) (Tiego *et al*., 2018). It is used as a marker for cognition in non-human animals, as it can reveal whether an animal is giving a habitual behavioural response or using context to flexibly alter responses. Inhibitory control indicates how impulsive an individual is, making it a clinically relevant executive function for individuals with ADHD (Schachar *et al*., 2000; Ma *et al*., 2016). Distinctly, success in a Go/No-Go task is a marker of inhibitory control (Bokura *et al.,* 2001). A typical Go/No-Go task involves two trials: the Go trial, in which the participant is expected to give a response, and the No-Go trial, in which no response should be given. The two trials are presented in a randomised order, with a specific cue for each trial, signalling whether the participant should respond or not (Bokura *et al*., 2001). An everyday example of this in action is a traffic light, which gives coloured light cues to signal whether the driver should go or stop. The Go/No-Go task challenges stimulus control, an important executive function for human behaviour (Dinsmoor, 1995) and is a marker of cognitive flexibility (Flagel *et al*., 2011).

Inhibitory control is influenced by dopamine signalling in the prefrontal cortex and basal ganglia (Miller and Cohen 2001; van den Wildenberg et al. 2006; Colzato et al., 2009). High dopamine levels have been associated with impulsivity, characterised by poor inhibitory control (Logan et al., 1997; Enticott et al., 2006). Further, a higher impulsivity score (on the Barratt scale; Barratt, 1959) has been associated with increased physiological arousal in humans during a stop-signal task (Zhang et al., 2015). The stop-signal task is an alternative paradigm for measuring individual differences in response inhibition (Raud et al., 2020). Therefore, both dopamine activity and arousal may predict inhibitory control performance in a Go/No-Go or Stop-Signal task.

Go/No-Go tasks have been carried out in horses (Lansade et al., 2014; Hintze et al., 2017; 2018). However, studies in this area are limited and have focused on investigating judgement bias, rather than cognitive control *per se*. For example, in Hintze et al. (2017) a small sample of only five horses were used and both Go and No-Go responses were rewarded (Symmetrical Reinforcement; Gross & Weiskrantz, 1962). This study did reveal that horses may be capable of achieving successful Go/No-Go, and that responses to Go/No-Go may relate to judgement bias. However, the authors (Hintze et al., 2017) advise caution when drawing conclusions about their results, owing to the design of their Go/No-Go task. Lansade et al. (2014) found that horses managed in an enriched environment (n=10) performed better on a Go/No-Go task than control horses (n=9). To date, however, no studies have investigated dopamine and arousal as potential predictors of inhibitory control performance in horses.

2.3 Reward Sensitivity

Reward sensitivity is the extent to which an individual is able to rank multiple reinforced behaviours based on their relative reward values (Martin and Potts, 2004). Reward sensitivity is determined by analysing responses towards rewards of differing values (Cho et al., 2016). Reward sensitivity tests are used to better understand the motivations underlying reward-related behaviours (van der Harst and Spruijt, 2007). This is useful for ensuring that the most effective training methods are used. Sensitivity to loss of reward is thought to indicate emotion and welfare states in animals (Burman et al., 2008). High welfare individuals are thought to be more resilient to loss of reward than low welfare individuals (Paul et al., 2005; Burman et al., 2008). Therefore, an individual's response to an unexpected downgrade or loss of expected reward can be used to indicate their overall welfare state (Burman et al., 2008).

Reward value discrimination has been tentatively studied in horses (Henselek et al., 2012; Kieson et al., 2020; Brucks et al., 2022), however, reward sensitivity (the ability to appraise behavioural responses based on corresponding reward value) has not been investigated. Further, while recent studies have identified behavioural, locomotory and facial expression changes associated with disappointment and frustration in horses (Ricci-Bonot and Mills, 2023; Phelipon et al., 2024), sensitivity to reward loss has not yet been investigated.

3. Influence of Striatal Dopamine and Arousal on learning

3.1 Striatal Dopamine

Striatal dopamine is a neurotransmitter that is involved in learning, due to its role in marking reward (Mirenowicz & Schultz, 1996; Schultz, 2002). The dopamine system is complex, spanning across a variety of brain structures, and is involved in a variety of cognitive processes (Filla et al., 2018; Graybiel, 2016). Specific areas of the brain such as the substantia nigra in the midbrain are densely populated with dopaminergic nerve cells (Damier et al., 1999). These dopaminergic nerve cells use dopamine as the neurotransmitter to execute very specific actions such as reward signalling (Schultz, 1996). However, the axons from these neurotransmitters send impulses across all areas of the brain, which means that dopamine is involved in many different processes in the brain and body (Klein et al., 2019). Dopamine is often referred to as the reward hormone, which is an oversimplification (Mott *et al*., 2020). Rather than being rewarding *per se*, Dopamine is the neurotransmitter which marks reward, meaning that when reward occurs, dopamine is involved in ‘stamping’ that event into memory, so that it may be repeated in future (Schultz, 2002). This forms the basis of reward learning, which is integral to operant conditioning.

The striatum, in the basal ganglia of the brain, is where dopamine acts to mediate reward perception (Badgaiyan *et al*., 2007; Landau *et al*., 2009). The striatum is made up of the Caudate Nucleus, the Putamen and the Nucleus Accumbens (Nolte, 2002; McBride and Hemmings, 2009). These structures together make up the area of the brain where many cognitive processes occur, including reinforcement learning, decision-making, motivation and reward perception (Wickens, 1990; Nolte, 2002; Badgaiyan *et al.,* 2007; Cools *et al*., 2009; Landau *et al*., 2009). The equine and human striatum are almost identical (Hemmings *et al*., 2018), so human neuroscience has been integral to understanding these processes in the equine brain. In return, research using the horse as a model can inform our understanding of human reward learning.

Striatal dopamine release mediates reward prediction error (RPE), due to signals carried by the midbrain dopamine system (Shultz *et al*., 1997; Hollerman and Schultz, 1998; Holroyd *et al*., 2009; Jongkees and Colzato, 2016). In RPE, dopamine peaks indicate positive error, signalling that an outcome is better than expected, whereas dopamine troughs indicate that an outcome is worse than expected (Maia and Frank, 2011; Jongkees and Colzato, 2016). In other words, dopamine increases from baseline firing rate following rewards and decreases from baseline firing rate following the absence of reward, and this is what mediates reinforcement learning (Holroyd *et al*., 2009). The error-related negativity mechanism (ERN) has two components: the response ERN and the feedback ERN (Holroyd *et al*., 2009). The

ERN system reflects the expectedness or unexpectedness of a reward outcome (Holroyd *et al.*, 2009). This means that when the outcome (the feedback) is expected, feedback ERN amplitude is low, relative to feedback ERN amplitude when the outcome is unexpected (Holroyd and Coles, 2002; Holroyd *et al.*, 2009).

Further, dopamine acts to improve perceptual learning through enhancement of the signal-to-noise ratio. Tonic dopamine increases the signal-to-noise ratio of neurons in the medial prefrontal cortex (mPFC) (Frank, 2005), such that mPFC neural signals are amplified, relative to artefact noise. Artefact noise can impair visual perception, so the enhanced signal-to-noise ratio caused by dopamine receptor activation is thought to boost visual perceptual learning and performance (Yousif *et al.*, 2016). So, not only does striatal dopamine signal reward predictions, but it also improves the capacity for learning by reducing artefact noise to focus the brain on the learning stimulus.

Jocham *et al.* (2009) investigated the influence of dopamine on the way that positive and negative outcomes are received during operant conditioning, and how these outcomes shape value-based choices. While dopamine D2 receptor antagonists did not affect initial reinforcement learning, the subjects' abilities to use previous learning to inform their value-based choices in future tasks was improved, as subjects were better able to choose the highest value option between two rewarding options (Jocham *et al.*, 2009). This suggests that dopamine plays a role in the ability of an individual to make value-based choices, through their reward sensitivity.

Animal welfare studies have used reward sensitivity as an indicator of individual affective state (Spruijt et al., 2001; van der Harst and Spruijt, 2007; Burman et al., 2008). Reward sensitivity is tested under different welfare conditions, to indicate how welfare affects an individual animal's general outlook, motivation for reward and optimism (Van der Harst and Spruijt, 2007). Since dopamine plays a role in reward sensitivity (Jocham *et al*., 2009), behavioural indicators of dopamine activity may be useful for understanding how animals are learning, and this may have positive outcomes for the study of animal welfare. Further, reward sensitivity can also reveal personality trait tendency, indicating impulsivity in an individual (Martin and Potts, 2004). When given the choice, more impulsive individuals select outcomes which elicit immediate, yet lower-value rewards, rather than delayed but higher-value rewards (Martin and Potts, 2004).

It has been suggested that animals with higher welfare are motivated to work harder and wait longer for a higher-value reward (Kirkden and Pajor, 2006). By contrast, impulsivity may be exacerbated by chronic stress and anxiety in humans (Cackowski *et al*., 2014; Moustafa *et al*., 2017; Raio *et al*., 2020) and non-human animals (Binder *et al*., 2004; Coppens *et al*., 2012; Camp and Johnson, 2015). A recent study into the effects of stressful life events on impulsivity and gambling habits in the United States found that college students who had experienced stressful life events were 9% more likely to develop gambling problems (Wang *et al*., 2020). The authors attributed many of the gambling issues to increased impulsivity, citing work on addiction by Deleuze *et al*. (2017). Therefore, behavioural indicators of cognitive processes may be useful for understanding risk factors for psychological conditions such as addiction in humans.

3.2 Arousal

Arousal is a state of physiological activation, regulated by the hypothalamus and brainstem through multiple neurotransmitter pathways (Marrocco et al., 1994). Physiological effects of increased arousal include increased heart rate (Graham and Jackson, 1970; Azarbarzin et al., 2014; Davies et al., 2014; Wascher, 2021), lower heart rate variability (Appelhans et al., 2006; Scherz et al., 2020) and increased eye temperature, caused by increased blood flow to the eyes as a sympathetic nervous system response (Stewart et al., 2007; 2010; Jansson et al., 2021; Travain and Valsecchi, 2021). Increased arousal during learning has been associated with effects on storage (Eyesenk, 1976), retention (Levonian, 1972) and retrieval (Eyesenk, 1976) of learned information. Ferrari (2014) found that tadpoles who were at higher risk of predation performed better on a predator recognition and memory task than tadpoles who were at lower risk of predation. This suggests that improved cognitive performance can be an adaptive response to arousal, as it increases chances of survival in threatening situations. However, increased arousal does not always aid learning. For example, in Sage and Bennett (1973), learning was enhanced when performance-related arousal was induced (electric shock administered when learning criteria was not met). However, learning was not enhanced when unrelated arousal was induced (electric shock administered at random regardless of performance) (Sage and Bennett, 1973). Therefore, the effects of arousal on learning performance may be context dependent.

Further, it has been suggested that over-arousal, or stress, can have detrimental effects on learning performance (Fisk and Warr, 1996). Maloney et al. (2014) suggest that when students experience high levels of exam anxiety, their academic performance is impaired, due to an overstimulation of the sympathetic nervous system. As such, there appears to be an arousal sweet spot, in which performance is optimal. This was first proposed in 1908 by Yerkes and Dodson (Yerkes and Dodson, 1908) and has since been named the Yerkes-Dodson curve (Westman and Eden, 1996; Salehi et al., 2010). The Yerkes-Dodson curve is an inverted-U-shaped relationship between arousal and performance (Figure 1.1). The inverted-U-relationship between arousal and performance has famously been applied in the context of sports performance (Kerr, 1985; Jones and Hardy, 1989; Gould and Krane, 1992; Krane, 1992; 1993; Raglin, 1992; Arent and Landers, 2003; Cashmore, 2008), though its origins were in cognition. The Yerkes-Dodson Law stipulates that moderate arousal is required for optimal performance in difficult cognitive tasks. Interestingly, the Yerkes-Dodson law appears to only apply to difficult tasks. When cognitive tasks are simple, performance is positively correlated with arousal level in a linear relationship (Yerkes and Dodson, 1908; Diamond, 2005).

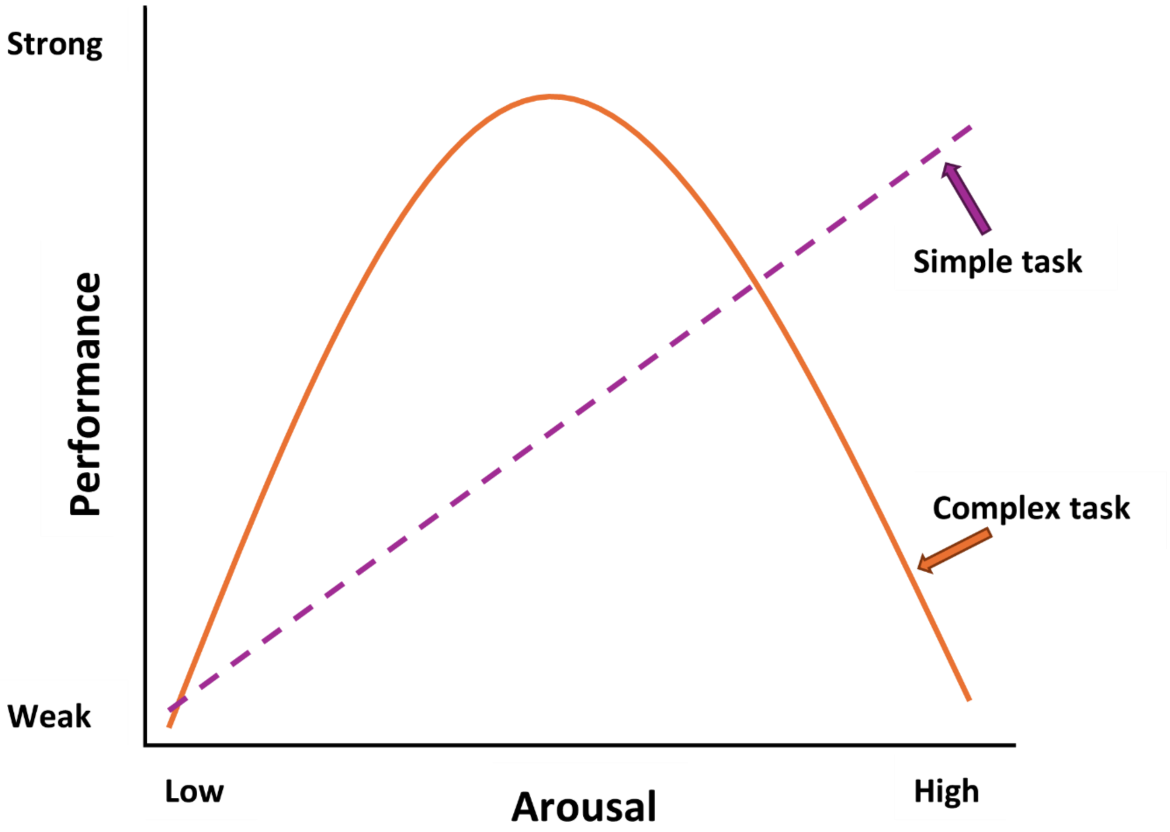

Figure 1.1. Graph showing the inverted-U-shaped relationship between arousal and performance (Yerkes and Dodson, 1908). Under high arousal situations, performance in simple tasks (purple dashed line) continues to improve, whereas performance declines in more complex tasks (orange curved line). (Yerkes and Dodson, 1908). Adapted from Diamond (2005).

The Yerkes-Dodson Law has been applied to equine cognition and training in a handful of studies (Starling et al., 2013;2016; Valenchon et al., 2013; Olczak et al., 2016). The focus of the work to date has been on the effects of arousal during a task on performance. For example, Valenchon et al. (2013) studied the effects of a stress on performance in a working memory task in horses, by comparing the performance of horses in a stressful experimental condition against the performance of horses in a control condition. Stress was induced during the experimental condition by the addition of aversive events such as novel sounds, objects falling and water spraying (Valenchon et al., 2013). This was designed to test how well horses would learn under stressful and unpredictable conditions, which may be relevant to specific training scenarios such as police horse training (Christensen et al., 2010).

However, studies that induce stress using novel stimuli such as those used in Valenchon et al. (2013) lack ecological validity in the context of most equine training practices. It would be useful to know how training-related arousal (i.e. arousal caused by the training itself), whether positive (food seeking, reward) or negative (frustration, punishment), influence learning performance. Additionally, the effects of baseline arousal (possibly indicating welfare state) on learning abilities is not commonly included in analyses, and has not been explored in horses to date. Temperament and learning performance has been studied (Valenchon et al., 2013), but this has been based on behavioural temperament tests, rather than baseline physiological arousal. Baseline arousal may provide a useful predictor of learning performance which could influence equine welfare and training efficacy.

4. The horse as a model

In their working roles, horses demonstrate cognitive flexibility, to flexibly adapt to cues from different riders and trainers, and novelty in their environments. On the other hand, horses are also required to give reliable responses to cues such as ‘stop’ or ‘slow’, for safety purposes. In doing so, ridden horses make effective use of cognitive control. This delicate balance between cognitive flexibility and control makes the horse a unique and valuable partner in horse-human activities. Understanding more about how horses learn to be both flexible and reliable is undoubtedly important and relevant to horse owners, trainers and riders. The horse's working role therefore requires reliable habit formation for rider safety and elite performance (McGreevy and McLean, 2007). Further, horses are regularly expected to learn, reverse and extinguish behavioural responses, as they are re-trained, re-homed, ridden and handled by various people (Innes and McBride, 2008). Individual horses show differences in their predisposition to form habits (Lansade *et al*., 2017) and yet are often expected to learn complex tasks and then reverse their training (Brubaker and Udell, 2016). Therefore, investigating equine learning and cognition may have applied practical and welfare benefits. Dopamine and arousal may be useful predictors of adaptive learning in horses, allowing horse trainers to adopt more effective training methods, which safeguards the horse's welfare and improves the safety of equestrian activities.

From a scientific perspective, domesticated horses provide a suitable model species for exploring the link between striatal dopamine, arousal and adaptive learning for several reasons. They are adept at operant conditioning and readily learn novel tasks when reinforced correctly (McGreevy & McLean, 2007), including visual discrimination tasks (Hall et al., 2003). Further, they often change trainers or working roles and may then be required to reverse or extinguish previously reinforced behaviour (Innes & McBride, 2008). Individual horses also show individual differences in their predisposition to form inflexible habits (Lansade et al., 2017) and in dopamine activity and sensitivity (Momozawa et al., 2005). Spontenous eye blink rate (SEBR) has also been validated in this species relating to stress

(Merkies et al., 2019; Mott et al., 2020), attention (Mott et al., 2020) and impulsivity (McBride et al., 2022).

Further, individual differences in DA activity and sensitivity have been evidenced in horses (Momozawa *et al*., 2005), and SEBR as an indicator of DA activity has been validated in this species (Roberts *et al.,* 2016). In fact, horses have been used to investigate stress-induced alterations to striatal dopamine physiology (McBride & Hemmings, 2005) and the relationship between SEBR at rest and reduced behavioural extinction in an operant task (Kirsty et al., 2015). The equine striatum is almost identical to that of the human (Roberts *et al.,* 2017). Additionally, horses have large, anterolaterally placed eyes, which make blink rate analysis much easier than smaller animals with small eyes. Horses are habitual animals, suited to structured routines, which means that they can be studied in their home environments but with an element of control over extraneous variables. Finally, multiple measures of arousal are well validated in this species (Garnett & Merkies, 2019; Hall et al., 2011; Ijichi et al., 2020; Merkies et al., 2019; Rietmann et al., 2004; Stucke et al., 2015).

5. Methods of measuring striatal dopamine and arousal in horses

5.1 Spontaneous eye blink rate

An eye blink is the brief, full closure of both eyelids (Cruz et al., 2011). There are three main kinds of eye blink: voluntary, reflexive and spontaneous (Cruz et al., 2011). The function of a reflexive eye blink can usually be deduced by observation of the context in which it occurs (VanderWerf *et al*., 2003; Bacher and Smotherman, 2004). For example, a reflexive blink is likely to occur as a protective function when a foreign object approaches the eye. A voluntary eye blink occurs on command, prompted by an exogenous cue preceding the blink.

Voluntary eye blinks can be distinguished from reflexive and spontaneous blinks by their increased amplitude (Kaneko and Sakamoto, 1999). Spontaneous eye blinks, however, do not appear to be caused by any known external stimulus in the environment and do not have any protective functions (Bacher and Smotherman, 2004). Spontaneous blinking appears to be mediated endogenously (Cruz et al, 2011), therefore the specific triggers are not always as obvious as with the other kinds of blinking (VanderWerf et al., 2003). Importantly, there appears to be a lack of consensus within the scientific community about what SEBR relates to. Broadly speaking, there are two “camps”: those suggesting that SEBR reflects purely attention; and those who argue that SEBR is a marker of tonic dopamine and therefore reflects not only attention but also various aspects of cognitive processing.

5.1.1 SEBR as a measure of stress and attention

Spontaneous eye blink rate (SEBR) has been studied in relation to a number of cognitive and psychological processes, such as stress (Shibutani *et al*. 1984; Pruessner *et al*., 2004; Belujon and Grace, 2015), attention (Orchard and Stern, 1991), memory (Holland and Tarlow, 1975; DeJong and Merckelbach, 1990; Bentivoglio *et al*.,1997; Aalto *et al*., 2005; McNab *et al*., 2009) and high cognitive load (Telford and Thompson, 1933; Hall, 1945; Poulton and Gregory, 1952; Kinsbourne and Warrington, 1963; Holland and Tarlow, 1972; 1975; Karson *et al*., 1981). It has been observed that SEBR increases in response to stress and arousal (Mott *et al*., 2020) and decreases in response to attention and high cognitive load (Maffei and Angrilli, 2018).

Oh *et al*. (2012) observed increased SEBR during a cognitive task, however the difficulty of the task and the stimulus type had no influence, suggesting that SEBR reflects attention shifts rather than learning processes. Similarly, Fogarty and Stern (1989) found a significant link between SEBR and eye movements associated with identifying stimuli, suggesting that SEBR reflects attention and information processing, but only at the level of identifying stimuli (Fogarty and Stern, 1989). Interestingly, their results also suggest that blinking behaviour is influenced by the location of the stimuli, with peripherally located stimuli eliciting longer blink latencies than centrally located stimuli (Fogarty and Stern, 1989). This may indicate that blinking behaviour is more dependent on the distance the eye has to travel in order to identify and process stimuli, than on the actual stimulus information processing itself.

5.1.2 SEBR as a measure of striatal dopamine

Since it was first proposed by Stevens (1978), the relationship between SEBR and central dopamine activity has been well-documented (Jongkees and Colzato, 2016 for review). At rest, SEBR is positively correlated with dopamine activity (Jongkees and Colzato, 2016), particularly the D2 receptor system (Groman *et al.,* 2014; Jongkees and Colzato, 2016). The role of the spinal trigeminal complex in the spontaneous blink generator circuit has been proposed as an explanation for the link between SEBR and striatal dopamine (Kaminer *et al*., 2011; 2015). The trigeminal nerve (CN V) is responsible for sensory and motor functions in the face, including reflexive blinking (Pellegrini and Evinger, 1995). Dopamine inhibits the trigeminal complex (Kaminer *et al*., 2011), which increases SEBR (Doughty *et al*., 2009;

Kaminer *et al*., 2011; Jongkees and Colzato, 2016). Therefore, increased dopamine activity may cause increased SEBR.

Early animal studies into the effect of dopamine on SEBR manipulated dopamine action using DA agonist and antagonist drugs and measured the resulting effects on SEBR. Dopamine antagonists inhibit dopamine by destroying the cells in the substantia nigra which synthesise dopamine and have a lowering effect on SEBR in African green monkeys (Lawrence and Redmond, 1991) and squirrel monkeys (Mavridis *et al*., 1991). One study has reported the effects of dopamine antagonists on SEBR lasting up to 90 minutes after administration of the drug (Baker *et al*., 2002), suggesting a long-lasting and potentially biphasic impact on DA activity, which is indicated by the altered SEBR (Jongkees and Colzato, 2016). However, effects lasting this long after antagonist administration have not been replicated in other studies (Jongkees and Colzato, 2016), so this must be interpreted with caution. Dopamine agonists appear to increase SEBR in animal studies, with effects lasting up to an hour (Casey *et al*., 1980; Karson *et al*., 1981b, 1981c; Lawrence and Redmond, 1991; Kleven and Koek, 1996; Kaminer *et al.,* 2011; Kotani *et al.,* 2016). Taken together this suggests a positive association between dopamine activity and SEBR

5.1.3 SEBR as a predictor of learning

Whether through associations with attention and stress or striatal dopamine, SEBR may predict learning performance. In the last two decades, research into SEBR as a potential indicator of learning and other cognitive processes has increased, and questions about the reliability of such measures have been identified. Reward learning has been the focus of such research, due to the role that dopamine plays in reward signalling systems. Individuals with lower SEBR appear to have greater success in learning from negative outcomes, compared to individuals with higher SEBR (Slagter *et al*., 2015). However, high SEBR individuals do not necessarily have greater learning success from positive outcomes, indicating that the relationship may not be linear, and that SEBR may only be a reliable indicator of learning when negative training outcomes are used, such as positive or negative punishment commonly seen in operant conditioning.

Further, there is some suggestion that SEBR may only indicate D2 receptor activity (Slagter *et al*., 2015), and since D2 receptors are involved in avoidance learning, this reinforces the notion that SEBR reflects learning from negative outcomes (Slagter *et al*., 2015). However, these findings were not replicated by Van Slooten *et al*. (2019), in which no significant relationship between SEBR and learning from negative outcomes was found. This challenges the proposal that SEBR indicates learning from negative outcomes, and instead suggests that SEBR is an index of reward sensitivity (Van Slooten *et al*., 2019). Similarly, Byrne *et al.* (2016) found that individuals with high SEBR, plus high incidence of depressive symptomology, appear to show elevated reward sensitivity. Such individuals were more likely to avoid options which led to net losses and persevere in selecting options which led to net gains (Byrne *et al*., 2016). This suggests that mood, personality, genetics and predisposing factors for mental illness may influence dopamine function during cognitive challenges, and that SEBR may reflect this.

Zhang *et al*. (2015) observed significant correlations between SEBR and various learning abilities and argue that SEBR is strongly related to the central dopamine system. SEBR was positively correlated with successful task-switching (cognitive flexibility) and with success in a Go/No-Go task (response inhibition) (Zhang *et al*., 2015). This is consistent with Pajkossy *et al*. (2018), observing increased SEBR and pupil dilation following task rule switches. Pupil dilation has been positively correlated with cognitive load (Siegle et al., 2008; Sibley et al., 2011; Gavas et al., 2017); spontaneous blinks appear to cluster around the peak pupillary response, which coincides with peak cognitive load. Therefore, it has been suggested that spontaneous blinking clusters signal changes in cognitive load (Siegle et al., 2008), Interestingly, in Paikossy et al. (2018), baseline SEBR was related to task performance immediately prior to the most difficult task-switching trials, and baseline pupil size was associated with performance during these most difficult tasks. SEBR was negatively correlated with the ability to replace irrelevant information with new, relevant information (reversal), and the ability to completely suppress information that is no longer relevant according to the altered demands of the task (Zhang *et al*., 2015). These findings suggest that SEBR is closely related to dopamine function during learning in tasks relating to cognitive flexibility, inhibition and impulse control.

SEBR fluctuates in stability diurnally, with the most stable blinking patterns occurring during the morning, afternoon and at mid-day (Barbato *et al*., 2000). By contrast, SEBR is significantly less stable during the evening and night-time, corresponding with tiredness and drowsy times (Barbato *et al*., 2000). As such, any studies which attempt to investigate SEBR as a potential indicator of learning should consider the time of day and the overall drowsiness of subjects, to avoid confounding the results. SEBR also appears to be seasonally influenced, with higher SEBR occurring during the spring and summer months, compared with the autumn and winter (Barbato *et al*., 2018). Interestingly, Barbato *et al.* (2018) found that this also correlates with beta activity on electroencephalogram (EEG), which is highest during spring and summer. Beta activity is relevant to learning and blink rate because it indicates an activation of arousal, maintained in the spring and summer months by dopamine systems. EEG beta activity is thought to reflect cognitive processing and the emotional valence of a task (Ray and Cole, 1985). The beta activity during a cognitive task is seen in the parietal cortex area of the brain (Ray and Cole, 1985), the same area of the brain where dopamine activity is thought to influence spontaneous blinking (Mott *et al*., 2020). Additionally, EEG beta activity has been linked to attention during performance of visual tasks in elderly humans (Gola *et al*., 2013). Taken together, these findings indicate a link between cognitive processes and spontaneous blinking.

SEBR may also be influenced by genetics. In a study by Dreisbach *et al*. (2005), individuals with high SEBR had increased cognitive flexibility, but decreased cognitive stability, and these results were exaggerated in individuals carrying the ‘DRD4 exon III 4/7’ genotype. This suggests that SEBR is reflective of the inverted-U-shaped curve of dopamine influence on cognitive performance, which has since been identified (Cools and D’Esposito, 2011; Hidalgo-Lopez and Pletzer, 2017). The genetic link suggests that this influence is exaggerated by certain genotypes, which may explain why there is so much individual variance in baseline SEBR and the resulting effects of cognitive strain on SEBR. Colzato *et al*. (2009) found that SEBR could indicate inhibitory control, as a reliable predictor of performance in a Go/No-Go task. Taken together with the results above, these findings suggest that it may be reductionist to consider SEBR purely as an indicator of learning as a broad concept. SEBR may indicate specific, complex cognitive processes, interacting with aspects of personality and genetic predisposition.

The mechanisms underlying the effect of dopamine on blinking behaviour are unclear, partly due to the wide-ranging actions of dopamine in the brain and nervous system (Mott et al., 2020). This is why it is so difficult to attribute specific processes to dopamine since dopamine has so many functions in the body which all interact with one another. Additionally, due to its great span of activity across all areas, dopamine levels in the brain cannot be reliably measured (Hartley *et al*., 2019). As such, scientists cannot correlate exact, measured increases in dopamine with SEBR, which means that such correlations are implied and demonstrated through behaviour and pharmacological knowledge, but they are not measured exactly. Despite this, it is accepted that dopamine is the neurotransmitter involved in marking reward and has been implicated in reward learning processes.

Furthermore, there is strong evidence that manipulating dopamine action by administering dopamine agonists and antagonists alters blink rate markedly (Karson, 1988). Therefore, a relationship between blink rate and learning can be predicted with some confidence. Several studies have used SEBR to indicate learning in cognitive neuroscience research, since it is a relatively easy, cheap and non-invasive method of measuring striatal DA-mediated learning processes (Dreisbach *et al*., 2005; Colzato *et al*., 2009; Oh *et al.,* 2012; Slagter *et al*., 2015; Zhang *et al*., 2015; Byrne *et al.,* 2016; Eckstein *et al*., 2017; Van Slooten et al., 2017; Pajkossy *et al*., 2018; Hartley *et al*., 2019; Tummeltshammer *et al*., 2019; Van Slooten *et al*., 2019).

Given how costly and inaccessible neurochemical imaging is, there is a need for reliable behavioural indicators of learning, which can be used on a variety of species non-invasively and without altering the animals' environment. However, as the research described here indicates, there is a degree of uncertainty regarding the validity of SEBR as an indicator of learning specifically, so further investigation is warranted. If SEBR is a reliable proxy indicator of striatal dopamine, blink rate should increase during reward learning, indicating increased dopamine activity. However, if SEBR is a measure of attention blink rate should decrease during periods of high cognitive load, such as during learning.

5.1.4 Investigating spontaneous eye blink rate in an equine model

Traditionally, rodent species have been used in blink rate research (Evinger et al., 1993; Desai et al., 2007; Kaminer et al., 2011). Rodents are selected due to their small size and apparent low-maintenance requirements, which mean that they can easily be housed in an artificial laboratory setting (Kaminer et al., 2011). This kind of setting allows for high control of extraneous variables, which can be valuable for obtaining valid and replicable results.

However, there are limitations of studying animals in artificial settings, as by their nature, laboratory studies lack ecological validity. As such, there is a benefit to studying an animal in a more realistic setting.

To date, only a handful of studies have investigated SEBR in horses. The existing literature has focused almost exclusively on the following areas: personality (Roberts et al., 2016), stress and attention (Merkies *et al*., 2019; Cherry *et al*., 2020; Mott *et al*., 2020). This means that very little is known about the effect of learning and complex cognitive processes on SEBR in horses. Stress is thought to increase SEBR, as demonstrated in traditional human studies (Meyer *et al*., 1953; Giannakakis *et al*., 2017). However, Merkies *et al.* (2019) found that SEBR decreased, and eyelid twitch frequency increased when horses were placed in stressful scenarios.

Mott *et al*. (2020) suggest that this discrepancy in the research between humans and horses could be due to the attentional effort required by the horse as a prey species to evaluate the presence of any potential threats during what is perceived to be a stressful scenario. Attentional focus is negatively correlated with SEBR (Cherry *et al*., 2020), so this may explain why horses appear to experience a decrease in SEBR when under environmental stress. This also highlights the importance of ensuring that there are no environmental stressors when conducting blink rate analysis in attentional focus studies. Blink rate may decrease as a result of increased attention, but the attention may be directed at a potential stressor in the environment, rather than the experimental stimuli. Conducting experiments on horses in companion pairs is one way to reduce the risk of environmental stress, by reducing social separation anxiety (Ijichi *et al*., 2020).

Attention, including attentional focus and choices about the direction of attention, is crucial for effective acquisition of learning (Kruschke, 2003). All the above demonstrate that there is a complex interplay between aspects of equine personality, stress, attention, and learning ability, all of which appear to modulate changes in SEBR. Understanding the link between SEBR and learning, going beyond attention, is valuable for equine training, welfare, and human safety. SEBR in horses is a relatively new tool for assessing cognition, and as such there is no validated protocol for measuring SEBR in an equine model. Therefore, further research is necessary to establish a standardised protocol which may be used in future studies to better understand the possible relationship between blink rate and learning.

5.2 Measuring arousal

Arousal is a key indicator of an animal's psychological response to its environment (Broom and Johnson, 1993; Feldman, 1995). One of the challenges of finding appropriate methods of measuring arousal in horses is that they are a prey species that is adapted to be reactive to stimuli in their environment (Frid and Dill, 2002). As such, methods of measuring arousal must at the very least be non-invasive and non-aversive to horses. Methods of measuring arousal often involve taking invasive samples, such as blood (Becker *et al*., 1985). Even when less invasive samples such as urine, hair and saliva are taken, the horse needs to be restrained, which can itself increase arousal (Borstel *et al*., 2017; Mott *et al*., 2021). Therefore, non-invasive methods which allow the horse to move relatively freely are preferred. While faecal samples for cortisol concentration analysis can be collected non-invasively, the samples must be stored under strict conditions and analysed in a laboratory using specialist equipment (Yamanashi *et al*., 2016), which involves a lot of time and money.

The current project uses methods of measuring arousal that are non-invasive, non-aversive, rapid, and validated for use specifically in equines.

5.2.1 Heart rate variability

Heart rate variability is a well-established, validated measure of arousal (von Borrell et al., 2007). The parasympathetic and sympathetic branches of the autonomic nervous system have functions which affect heart rate variability (HRV) (Saul, 1990). Heart rate is increased in response to increased sympathetic activity, and there is typically a decrease in HRV (Ottaviani, 2018), which reflects a shift away from parasympathetic activity of the autonomic nervous system during sympathetic activation (Saul, 1990). HRV therefore reflects not only cardiovascular health (Malpas and Maling, 1990; Lombardi and Mortara, 1998; Watanabe *et al*., 2017), but also emotional regulation (Applehans *et al*., 2006; Williams *et al*., 2015) and physiological response to stress (Taelman *et al*., 2009; Kim *et al*., 2018; Ottaviani, 2018).

Heart rate monitors (HRMs) non-invasively detect electrical activity from the heart and these are represented as waveforms. Heart rate variability refers to the inter-beat interval (IBI), the time elapsed between beats. High variability reflects a high level of inconsistency in the IBI, whereas low variability is characterised by regularly timed beats (Mott *et al*., 2021). HRV is used as an indicator of welfare in a wide range of species, including horses (von Borell et al., 2007; Squibb et al., 2018; Ijichi *et al*., 2020), but also pigs (Marchant-Forde *et al*., 2004), dogs (Katayama *et al*., 2016; Bowman *et al*., 2017) and cows (Erdmann *et al*., 2018).

HRV can be measured and analysed using either time-domain, frequency-domain or non-linear parameters (Mott *et al*., 2021). Time-domain parameters include the Root Mean Square of Successive Differences (RMSSD) and the Standard Deviation of N-N intervals (SDNN) (Hejjel and Roth, 2004). Time-domain parameters quantify the amount of variance measured over a specified time period of heart rate monitoring (Pitzalis *et al*., 1996). Frequency-domain parameters calculate the quantity of signal energy which lies within specific frequency bands, and this includes low (LF) and high (HF) frequency bands (Pitzalis *et al.,* 1996; Kuss *et al*., 2008).

Non-linear methods of analysing HRV are complex algorithms which account for the non-linear fashion in which complex dynamics of the cardiovascular system interact (De Godoy, 2016). Time and frequency domain analyses are most frequently used for the assessment of HRV in dogs (Jonckheer *et al*., 2012), production animals (Von Borell *et al*., 2007), cats (Khor *et al*., 2014) and horses (Physick-Sheard *et al*., 2000; Von Borell *et al*., 2007; Stucke *et al*., 2015). The use of frequency domain for stress/arousal analysis has been cautioned against, following evidence suggesting that LF/HF bands do not reflect sympathetic nervous system activity (Thomas et al., 2019). Further, the HF band may be confounded by respiratory effects on heart rate (von Borell et al., 2007), and may lack validity when used on horses moving at certain gaits (Lenoir et al., 2017).

Two-lead HRMs, such as the Polar V800 (Polar Electro Oy, Kempele, Finland), have been widely used for measuring equine heart rate (Squibb *et al*., 2018; McDuffee *et al*., 2019; Rosselot *et al*., 2019; Ijichi *et al*., 2020; Mott *et al*., 2020; 2021). The 2-lead HRM has been validated against electrocardiogram (ECG) in humans (Gamelin *et al*., 2006) and horses when stationary (Mott *et al*., 2021). However, ECG is recognised as the most valid and reliable method for heart rate variability monitoring (von Borrell et al., 2007), and there are several factors which make it the more optimal choice for the collection of heart rate data in horses (Mott *et al*., 2021). Firstly, horses have a high vagal tone (Reef, 1985; Verheyen *et al.,* 2010; Van Loon, 2011; Mott *et al*., 2021). This can cause arrythmia, which occurs in a large proportion of clinically healthy equines (Eggenssperger and Schwarzwald, 2017; Mott *et al*., 2021). The arrythmia occurs because the sinoatrial node impulses are blocked from reaching the atrium (atrioventricular block), as a function of the autonomic nervous system. This means that there is no R wave on the ECG, and therefore the data is misrepresented as the IBI appears to be double where there is a missing beat (Mott *et al*., 2021).

Additionally, there appear to be a large proportion of errors known as artefacts in 2-lead HRM- generated HRV data from animal studies (Marchant-Forde *et al*., 2004; Von Borell et al., 2007; Mott *et al*., 2021). This may be due to the marked differences in the waveform profile between humans and other species (Kaese *et al*., 2013). For example, the equine ECG profile has a more pronounced T-wave than the human ECG profile (Kaese *et al*., 2013; Mott *et al*., 2021; Figure 1.2), and this may be counted in error as an R-wave in HRMs which only collect R-R intervals (Mott *et al*., 2021).

Figure 1.2. Diagram comparing normal human ECG (top; Zhao et al., 2013) and equine ECG waveform (bottom; Verheyen et al., 2010) The T-wave and R-wave are labelled.

ECG monitors, such as the Actiheart 5 (CamnTech Ltd, Fenstanton, England) monitors, measure the full wave, and therefore may be more appropriate for use in horses as they mitigate some of the above issues of missing or mis-interpreted R-waves. Mott *et al*. (2021) validated the use of the Polar V800 heart rate monitor and the Actiheart 5 ECG device for heart rate variability data collection and analysis. Although data from both the Polar V800 and the Actiheart 5 correlated strongly with the Televet 100, a gold-standard veterinary diagnostic and monitoring tool, the Polar device required an artefact correction threshold of 0.4s (Mott *et al*., 2021). This indicates that the Actiheart 5 is a valid alternative to the clinical ECG for HRV analysis in horses. The high incidence of artefacts may be attributed to the aforementioned waveform differences. Unlike the Polar device, it is possible to manually correct artefacts with the Actiheart device, due to the Full Waveform and Beat Editor functions.

5.2.1.1 Artefact correction

Heart rate analysis software allow users to automatically correct artefacts, using various filters which remove outlying peaks in the data. The level of artefact correction used is important because without adequate correction results become skewed and interpretations of the data lose validity. However, over correction can lead to valuable data loss if genuine peaks are removed. Artefact correction is a particularly delicate artform when working with horses and other animals with hair (Butler *et al*., 1992; Brugarolas *et al*., 2015; Virtanen *et al*., 2018). Horses' hair can cause issues when collecting HR data, as the fur creates a barrier between the electrode and the skin and the movement of the fur can cause false peaks (Cotur *et al*., 2020). Therefore, careful artefact correction is particularly important in this research. Studies using ECG devices on horses have identified that while errors are generally low when the horse is stationary, movement causes a large number of artefacts, likely due to the above-mentioned factors of hair and skin displacement (Parker et al., 2010; Lenoir et al., 2013; Ille et al., 2014).

The method of artefact correction by Mott *et al*. (2021) in their analysis of the Actiheart 5 ECG monitors was to apply correction thresholds of zero, 0.9s, 0.6s and 0.3s and compare results. A correction threshold of 0.3s was initially selected as it had been the threshold used in previous equine studies (Squibb *et al*., 2018). For horses with a lower resting heart rate, 0.6s correction was deemed appropriate as it would give a 30% correction for horses with a resting HR of 30bpm. A 0.9s threshold was used to cover those horses with atrioventricular (AV) block, which doubles the average R-R interval (Mott *et al*., 2021). Results indicated that most artefacts were due to AV block, and this meant that the 0.9s threshold was the most effective, with data corrected to 0.9s correlating most closely to the Televet data (Mott *et al*., 2021). In a healthy population, artefact correction of between 0.3 and 0.6 is therefore careful enough to adequately correct for discrepancies in stationary horses, rising to 0.9 in cases where AV block is present and/or horses are not stationary.

To summarise, with appropriate ECG equipment and informed artefact correction, HRV is a valid and appropriate method of robust arousal monitoring in horses. However, it should be noted that HRV as a stand-alone measure reliably indicates level of arousal, but not the valence of arousal (von Borell et al., 2007 for review).

5.2.2 Infrared thermography

Infrared thermography (IRT) transforms reflected energy from the infrared spectral band into quantifiable temperature readings (Chekmenev *et al*., 2005), using a thermal imaging camera. Thermal images (thermograms) can be viewed and analysed using specific image processing software to identify the emitted temperature values at different locations within the images (Ijichi *et al*., 2020). IRT has clinical applications in human medicine (Markel and Vaĭner, 2005; Lahiri *et al*., 2012) including sports injury medicine (Hildebrandt *et al*., 2010). More recently, it has been identified as a useful tool for identifying emotional responses by measuring facial temperature associated with muscle activation and blood flow (Clay-Warner and Robinson, 2015; Esposito *et al*., 2015).

Eye temperature, as measured using infrared thermography, is a valid indicator of emotional stress in humans (Vinkers *et al*., 2013) and animals (Stewart *et al.,* 2007; Travain *et al*., 2015; Weimer *et al.,* 2020; Elias *et al*., 2021), including horses (Valera *et al*., 2012;

Fenner *et al*., 2016; Ijichi *et al*., 2020; Kim and Cho, 2021). When an animal is under psychological stress or arousal, eye temperature increases, to reflect increased blood flow to the eyes caused by vasodilation as a function of the autonomic nervous system. The eyes are thought to be the target of vasodilation due to their role in identifying threats such as predation.

IRT is non-invasive, rapid and accurate when conducted using standardised methodology, producing data which correlates strongly with HRV as an indicator of arousal in horses (Ijichi *et al*., 2020). A handful of studies have suggested that eye temperature can reveal more about the valence of emotion in animals than HRV alone can (Blessing, 2003; Stewart *et al*., 2008a; 2008b; Ijichi *et al*., 2020). For example, reduced eye temperature has been attributed specifically to fear in humans (Blessing, 2003) and cattle (Stewart *et al*., 2008b). Similarly, reduced nasal temperature in some primates has been associated with fear (Nakayama *et al.,* 2005). Therefore, though heart rate variability is considered a valid stand-alone method of measuring arousal in animals, it is beneficial to use additional methods of measuring emotional state, such as IRT, which may go further than HRV in indicating emotional valence. For example, Travain et al. (2016) found IRT changes in dogs anticipating desirable food, but no effect on HRV was found. Therefore, they suggest that IRT may reveal more about valence than HRV is able to detect. The simultaneous use of both HRV and eye temperature, as measured by IRT gives a more accurate and detailed analysis of subjects' responses to cognitive challenge than HRV in isolation.

5.2.2.1 IRT as a proxy indicator of hemispheric lateralisation

Previous research identified differences in left and right eye temperatures that may indicate hemispheric lateralisation (Lush and Ijichi, 2018). Therefore, measuring individual eye temperatures during cognitive tasks that utilise different hemispheres may have value. There are three main theories of hemispheric lateralisation: the right hemisphere hypothesis, the valence hypothesis and the Behavioural Inhibition System/Behavioural Activation System (BIS/BAS) theory.

The right hemisphere hypothesis suggests that the right hemisphere is dominant in processing emotional stimuli, regardless of valence (Smith and Bulman-Fleming, 2005). This is based on evidence that in humans, damage to the right hemisphere significantly impairs emotional perception, whereas damage to the left hemisphere has little or no effect on an individual's ability to perceive and respond to emotional stimuli (Borod, 1992; Smith and Bulman-Fleming, 2005). According to this theory, the left hemisphere is responsible for logical reasoning and problem-solving. Current knowledge of hemispheric lateralisation in animals appears to support the right hemisphere hypothesis (Rogers, 2021 for review). For example, it is suggested that, in mammals, vigilance to threat is more effectively processed in the right hemisphere, whereas sustained response to targets and foraging behaviour are left-lateralised (Rogers, 2021).

Alternatively, the valence hypothesis argues that emotional processing is divided by valence. According to the valence hypothesis, the left hemisphere is dominant in processing positive emotions and the right hemisphere processes negative emotions (Schwartz et al., 1979; Killgore and Yurgelun-Todd, 2007). People with right hemisphere damage demonstrate an impaired ability to discriminate negative facial expressions from neutral facial expressions

(Adolphs et al., 2001). There is also evidence suggesting that discriminating negative expressions is easier when presented on the subjects' left (Adolphs et al., 2001). In horses, this has been supported by visual lateralisation research, such as Larose et al. (2006), in which more reactive horses viewed novel objects with the left eye. It is thought that visual information is processed by the contralateral hemisphere (Schiffer et al., 2004), so this is consistent with a contralateral relationship between the right hemisphere and the left visual field.

Similarly, Austin and Rogers (2007) found increased fear response when stimuli was presented on horses' left side. Further, right-footed horses demonstrate more optimistic responses to judgement bias tests than left-footed horses (Marr et al., 2018). These findings support the valence hypothesis as they demonstrate that motor laterality, thought to be associated with hemispheric lateralisation (McGreevy and Thomson, 2006; Mutha et al., 2012), indicates emotional valence. Lateralisation research in domestic horses must be treated with caution, due to a dominance of left-side handling in traditional equitation (Waran et al., 2007; Farmer et al., 2011). However, remote observational research in feral horse populations has also identified left side bias in vigilance, reactive behaviour and agonistic interactions (Austin and Rogers, 2012), which cannot be explained by human handling.

Prete et al. (2015) found evidence to support both the right hemisphere and valence hypotheses. They cite the modified valence hypothesis (MVH; Davidson, 1984), which suggests that the right hemisphere and valence hypotheses are not mutually exclusive. According to MVH, emotional processing generally involves posterior structures dominant in the right hemisphere, but valence-specific processing is specialised in the prefrontal cortex (PFC). The left PFC specialises in positive emotional processing and the right hemisphere specialises in negative emotional processing. However, whether this can be applied to the horse, who has an under-developed PFC, has not yet been investigated. It has also been suggested that the right hemisphere is dominant in processing some negative emotions such as fear, but other negative emotions such as disgust are not right-lateralised (Najt et al., 2013).

The Behavioural Inhibition System/ Behavioural Activation System theory (BIS/BAS; Gray, 1981) suggests that approach and avoidance behaviour are processed in opposite hemispheres (Figure 1.3; Goursot et al., 2021). According to BIS/BAS, behavioural activation (approach behaviour) is left lateralised and behavioural inhibition (withdrawal or avoidance behaviour) is right-lateralised (Carver and White, 1994). Goursot et al. (2021) present a strong argument for an association between laterality and stable individual traits (affective style). These traits include emotional reactivity (broadly positive or negative), motivational tendency (approach or withdrawal), personality (extroverted or introverted) and coping style (proactive or reactive), all of which can be associated with left or right laterality according to the BIS/BAS theory (Figure 1.3; Goursot et al., 2021).

The BIS/BAS framework described by Goursot et al. (2021) aligns with the valence hypothesis, because positive affect is associated with approach behaviour (Harmon-Jones et al., 2012), and negative affect is associated with withdrawal behaviour (Jones and Boissy, 2011; Goursot et al., 2021). However, this framework goes further than the valence hypothesis, because it includes not only emotional lateralisation, but also coping style, behavioural outcomes and motivation (Figure 1.3).

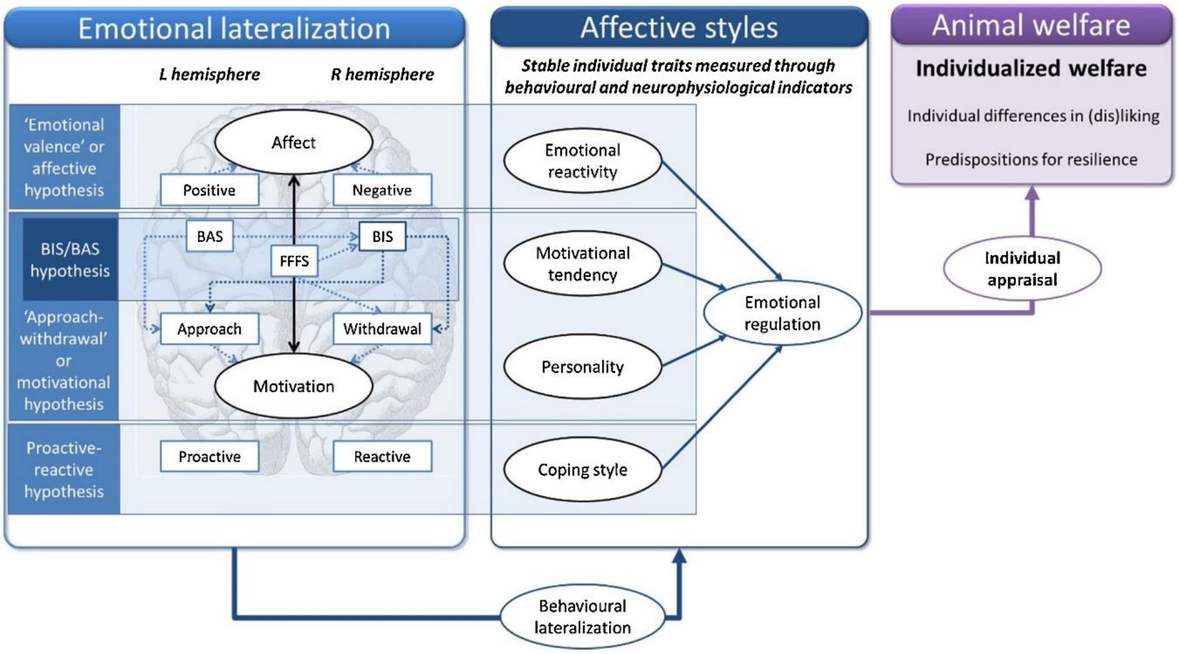

Figure 1.3. Diagram from Goursot et al. (2021) showing the BIS/BAS theory of emotional lateralisation, how it relates to affective style and animal welfare.

Direct measurement of hemispheric lateralisation in large, free-moving animals is challenging (Ye et al., 2008; Pereira et al., 2020), and there are currently no validated, non-invasive proxy measures available for use in horses. In this thesis, IRT is investigated as a potential proxy measure of hemispheric lateralisation. If successful, IRT of eye temperature may offer a rapid, non-invasive indicator of hemispheric lateralisation during cognitive tasks which, according to the three theories of hemispheric lateralisation (right hemisphere, valence and BIS/BAS), should be processed in either the left or right hemisphere.

6. Aims and Objectives of the thesis

The first aim of this thesis was to investigate spontaneous eye blink rate and arousal as novel predictors of cognitive performance in an equine model. This involved testing multiple theories relating to spontaneous eye blink rate, arousal and hemispheric lateralisation. Specifically, this thesis tested the theory that spontaneous eye blink rate is a valid proxy indicator of striatal dopamine activity. Also investigated was the theory that spontaneous eye blink rate is an indicator of attention and cognitive load. Additionally, this thesis tested the Right Hemisphere, Valence and Behavioural Inhibition System/Behavioural Activation System (BIS/BAS) hypotheses of hemispheric lateralisation.

A series of cognitive tasks, systematically increasing in complexity, were used as a tool to investigate cognitive performance. The broad cognitive skills tested in this thesis were cognitive flexibility, prospective model-based learning, behavioural inhibition and reward sensitivity. Some of these tasks were novel to this species, so a secondary aim was to determine whether horses had the cognitive capacity to complete such tasks.

The chapters of this thesis will address each of these aims in the following manner. Chapter 2 (General Methods) will provide details of the materials and methods used throughout this thesis. This will include information about the subjects, their housing and management, and the experimental environment. Chapter 2 will also provide detailed explanation of the equipment and methods used to collect physiological data from subjects throughout the experiments. These methods were used throughout all experiments in this thesis, except

Chapter 4. Where methods deviate from the general methods, this will be explained in full in the relevant chapter methods section.

Chapter 3 (Two-Choice Discrimination-Reversal Learning) will investigate spontaneous eye blink rate and arousal as possible predictors of performance in a task specifically designed to test cognitive flexibility. Chapter 4 (Model-Based Learning) was opportunistic in nature; during Chapter 5 (Go/No-Go) data analysis, a further research question arose, relating to the potential strategy that subjects may have been using to complete the task. As such, Chapter 4 investigated the use of model-based strategy in subjects during a Go/No-Go challenge, which investigates behavioural inhibition. As this chapter focused on the learning approach adopted by subjects, rather than physiological predictors of performance, no physiological data will be analysed in this chapter.

Chapter 5 investigated physiological responses to the Go/No-Go task and used statistical modelling to predict performance in this task. In particular, the BIS/BAS theory was investigated here as this task relied on effective use of the behavioural inhibition system.

Chapter 6 investigated reward sensitivity in horses, in two distinct phases. First, this chapter investigated reward value discrimination in horses, which is the ability to discriminate between rewards of differing values. Second, this chapter investigated responses to reward loss in subjects where actual reward value did not match expected reward value. This chapter focused on the valence hypothesis of hemispheric lateralisation in particular.

Each experimental data chapter will focus primarily on the theoretical underpinnings of each cognitive task and the physiological response, with limited comment on applications to the equine industry. These real-world applications will be discussed in Chapter 7 (General

Discussion). Chapter 7 will include a holistic review of the data across all experiments to evaluate the reliability of the novel methods and to derive general conclusions from the results. This chapter will also provide applications for the training, management and welfare of horses, plus recommendations for future work.

CHAPTER 2

GENERAL METHODS

1. Introduction

This chapter explains the general methods used across this project, including details about the sample, ethical considerations, equipment and the learning trials which form the main chapters of this thesis. The reduces unnecessary replication of methods within each chapter where only variations to the method are reported. As this project used horses as the model species, it was important to ensure that the methods used were both accurate and appropriate for use in horses. Further, it was vital that equine welfare was considered at every stage in this project, to ensure that ethical research which considered the three R's of animal research (Replacement, Reduction and Refinement) was carried out. This chapter forms the basis of the methodology sections of future empirical chapters and will serve as a point of reference throughout.

2. Sample

2.1. Selection

25 horses from Nottingham Trent University (NTU) were selected by yard manager Anna Gregory (AG) and equine research co-ordinator Catherine Hake (CH). There were no formal selection criteria, horses were selected based on their suitability to take part in research projects generally. Additionally, horses' availability to take part in a long-term project was considered, since the current project was a longitudinal study to be conducted over a period of three years. As such, horses nearing retirement and those not expected to remain at NTU

for the duration of the project were excluded from learning trials. However, these horses were included in pilot work such as habituation and preference testing. Table 2.1 provides details of all horses included in the wider sample.

Table 2.1. Subject details. Includes horse ID, sex (G=Gelding (castrated male), M=Mare (female)), age (years) at the start of the project, height (hh=hands), weight (kg), colour, and background. Participation in each study is indicated with Y =yes or N=no under the relevant study. Habituation study (column 9) refers to the habituation period study described in chapter 2 (General Methods).

Horse ID	Sex (G/M)	Age (years)	Height (hh)	Weight (kg)	Breed	Colour	Background	Habituation study (Ch 2)	Reversal (Ch 3)	Model-based learning (Ch 4)	Go/No-Go (Ch 5)	Reward sensitivity (Ch 6)
H001	G	14	16.2	638	ISH	Grey	Competition	Y	Y	Y	Y	Y
H002	M	14	17	628	Trakehner x WB	Bay	Riding School	Y	Y	Y	Y	Y
H003	G	15	15.2	640	Cob	Piebald	Leisure	N	N	Y	Y	N
H004	G	15	16.3	596	TB X WB	Dark Bay	Competition	Y	Y	N	N	N
H005	G	18	15.1	489	Connemara	Grey	Leisure	Y	Y	Y	Y	Y

H006	G	8	16.2	600	TBX	Chestnut	Competition	N	N	Y	Y	N
H007	G	17	16.2	546	ISH	Dark Bay	Competition	Y	Y	Y	Y	Y
H008	M	16	14.2	514	Haflinger	Palomino	Leisure	Y	Y	Y	Y	Y
H009	G	13	14.2	520	Cob	Black	Leisure	N	N	Y	Y	Y
H010	G	18	16.2	668	TB X SH	Bay	Competition	Y	Y	N	N	N
H011	G	15	16	642	ISH	Skewbald	Competition	Y	Y	Y	Y	Y
H012	M	14	15.1	548	Cob X Pony	Tricoloured	Leisure	Y	Y	Y	Y	Y
H013	M	10	15.1	550	Anglo-Arab	Chestnut	Leisure	Y	Y	Y	Y	Y
H014	G	22	15.2	478	Appaloosa	Grey	Charity	Y	Y	Y	Y	Y
H015	M	12	15	556	Cob	Piebald	Charity	Y	Y	Y	Y	Y
H016	G	18	16.2	626	ID X TB	Bay	Competition	Y	Y	Y	Y	N

H017	G	19	15.2	510	Connemara	Grey	Leisure	Y	Y	Y	Y	Y
H018	G	21	17.2	678	ISH	Liver Chestnut	Leisure	N	N	Y	Y	Y
H019	G	14	16.1	588	ISH	Dark Bay	Competition	N	N	Y	Y	N
H020	M	12	14.3	488	Welsh	Piebald	Charity	Y	Y	Y	Y	Y
H021	M	18	16.2	626	ISH	Bay	Competition	Y	Y	Y	Y	Y
H022	M	8	15.2	798	Suffolk Punch	Chestnut	Riding School	N	Y	N	N	N
H023	G	17	15.1	504	Connemara	Dun	Competition	Y	Y	Y	Y	Y
H024	G	17	16.3	670	TB X WB	Bay	Competition	Y	Y	Y	Y	Y
H025	G	18	16.2	614	BWB	Bay	Competition	Y	Y	Y	Y	Y

2.2. Age and sex

The age of horses ranged between 8 and 22 years (Table 1). The average age of subjects was 15.76 years. The sample included 8 mares and 17 geldings. Oestrogen is thought to influence striatal dopamine release (McDermott *et al.,* 1994) and, consequently, sex differences in SEBR have been identified (Chen *et al*., 2003; Hidalgo-Lopez *et al.,* 2020). However, this project had a within-subjects design, so the study was not compromised by an imbalance of mares and geldings in the sample.

2.3. Background and experience

The subjects came to NTU from a variety of backgrounds, including equestrian competition homes, equine welfare charities and family homes where they were ridden for leisure (Table 1). This was a unique sample of horses in that their role as university horses meant that they were regularly subject to research conditions, frequent handling and husbandry practice. Additionally, the sample were all NTU riding school horses, regularly used for Further Education (FE) and Higher Education (HE) riding lessons at NTU, except for an older horse (H012) who had retired from ridden work and two small ponies (H019 & H020), who were not suitable for ridden work but were used for horse handling modules.

All the horses were regularly handled by a variety of people, with a broad range of experience and for various purposes. As such, the effects of novelty were expected to be very minimal. This made the sample an ideal group for investigating adaptive learning. In particular, this sample of horses was regularly expected to reverse and extinguish previously learned behaviour, due to the volume of different riders and handlers they encountered.

Riding school horses often develop learned habits that are undesirable, so they are expected to reverse or extinguish these behaviours often.

One of the sample horses (H014) was a crib-biter. Since horses acted as their own controls it was appropriate to include the stereotypic horse. Interactions between oral stereotypies, learning, dopamine and spontaneous eye blink rate have been studied (McBride and Hemmings, 2005; 2009; Roberts *et al*., 2017), so the inclusion of this horse may provide an interesting individual case study.

2.4. Management and routine

Horses were housed and managed at NTU, in their usual stables. Horses' general routines were unchanged; their regular exercise and turnout times were unaffected and their usual night-time sleeping and resting hours were uninterrupted. On data collection days a training session lasting between 15 and 60 minutes was added to their schedule. Two of the horses in the sample shared a large combi barn away from the rest of the stables. These horses were loosely tethered at opposite ends of the combi barn during training sessions and learning trials. The decision not to separate the horses was due to welfare and validity concerns regarding separation anxiety. It was decided during the ethical review process that all horses should have at least one companion in close proximity (i.e. in adjacent or opposite stables). Since horses acted as their own controls for this study, it did not compromise the validity of the methodology that two of the horses had a different stabling design. Further, this was their usual stable, so these horses experienced the same experimental conditions as the rest of the sample, who all remained in their usual environments.

3. Ethical consideration

The proposal for this project was approved by the NTU Ethical Review Committee (approval code: ARE202145) for the use of animals in research. The committee were satisfied that adequate and appropriate measures were taken to design an ethical study which considered the welfare needs of the equine participants. At every stage, horse welfare was safeguarded. Only healthy individuals were selected for use in this study. None of the horses had any known injuries likely to impact their ability to take part in the study, nor were there any physically demanding aspects of the study likely to cause or exacerbate existing unknown injury.

There were no great adjustments made to the horses' usual routines. Since the NTU horses were regularly used for student practical sessions and research projects, being handled by students was familiar to them and a usual part of their routine. Where possible, training sessions and learning trials took place during the university term breaks, when there were no student riding lessons, practical workshops or demonstrations. Therefore, the addition of 15 – 60 minutes training did not put excessive physical or mental strain on horses' workloads.

The only change to horses' feeding routine was additional food used for reward. The food reward (Ulsakind cubes) was part of horses' usual diets, minimising any adverse nutritional effects. Even the addition of the food reward was not considered a great change to horses' diets, since they are regularly rewarded in this way using positive reinforcement as part of their daily management. Ulsakind cubes were carefully selected by AG and CH because they are highly palatable, but low-calorie, low-sugar and high-fibre and were not likely to have any negative impact on the health, weight or temperament of the horses.

For one horse (H021; Table 1), Ulsakind was substituted for Bailey's minty treats due to individual taste preference, which meant that the Ulsakind cubes did not provide adequate motivation for this horse's training. The decision to give this alternative food reward was approved by AG and CH, on the basis that this horse had previously shown an appetite for this feed. Providing one subject with a different food reward may have affected motivation towards the task, and therefore performance. However, it was necessary to ensure that all horses were adequately motivated by the reward.

All training and learning trials took place in the horses' usual stables at NTU. Stabling at NTU was designed to accommodate individual housing preferences. Two of the stables had a crew yard design and two were combi-barns, which incorporated indoor and outdoor housing. All stables had chat hatches to facilitate social interaction between horses. Companions were always visible to the horses due to this stabling design. This reduced separation anxiety (Waran, 2001) and promoted a more relaxed environment for learning (Park *et al*., 2001). Horses were stabled around their preferred companions, and this was determined by AG and CH on the basis of pre-established pair bonds.

Training was carried out using positive reinforcement only. Positive punishment was never used for incorrect responses, nor was negative reinforcement used as a training method. Negative punishment was used in three sessions of the Go/No-Go task (Chapters 2&3), as a means of testing whether horses were using model-based strategy. This consisted of a brief time-out period in which horses could not access the experimental stimuli. Full details, including justification for the use of negative punishment are given in Chapter 2.

The training sessions were entirely free choice by design, as horses were able to refuse to take part in training at any stage, including being able to turn 180° away from targets. Where horses omitted a response, they were allowed one further opportunity to continue training. If they refused a second time their training session was terminated. The decision to give horses a second chance to take part accounted for other factors such as environmental noise which may have distracted the horse. Interesting to note is that across the entire project, only one horse refused to take part in a training session. This was horse H021, who willingly participated once an alternative food reward was presented. It was therefore determined that this horse's initial refusal to take part was due to taste preference. At all times, a sympathetic and considered approach to horse handling was taken.

4. Measuring physiology in an equine model

This study investigated the relationship between learning, spontaneous eye blink rate (SEBR) and arousal. The methods of measuring SEBR and arousal were strictly non-invasive, safe and appropriate for use with horses. This section details the equipment and methods used to measure physiology throughout all learning trials.

4.1. Measuring spontaneous eye blink rate

To meet the primary aim of this thesis, a valid, accurate and non-invasive method of measuring spontaneous eye blink rate in an equine model must be found. There is currently no standardised method of measuring SEBR in horses, and there are very few studies which have investigated SEBR in horses. Some methods which have been used to measure blinking behaviour include a facial action coding system to classify half blinks as a facial indicator of pain (Rashid *et al*., 2020) and a mechanical counter to manually count blinks in real time (Roberts *et al*., 2015). Video analysis has also been used (Merkies *et al*., 2019; Cherry *et al*., 2020; Mott *et al*., 2020). Typically, this has been carried out using a static video camera and tri-pod positioned towards one eye. Since horses have anterolaterally placed eyes (Ijichi *et al*., 2020), it is not possible to capture both eyes simultaneously from a video camera positioned to the side of the horse's head. This makes it difficult to ascertain whether a blink was a true blink or a single eye twitch and does not account for blinking asymmetry which is observed in horses (Kassem and Evinger, 2006). Cherry *et al*. (2020) used a GoPro Hero 5 camera attached to the noseband of a horse's headcollar and aimed at both eyes to capture blinking behaviour in both eyes simultaneously. This method allows the horses to move freely without moving out of camera shot at any time, allowing for more accurate measures of blink frequency. Additionally, this means that horses do not have to be restrained for the duration of the cognitive challenges, which allows for more valid assessment of their physiological response to the learning tasks and limits the external influences on their responses.

A GoPro HERO 7 camera (GoPro, California, United States), fixed to a GoPro leg strap, was attached above the noseband of a headcollar, which was fitted to the horse as usual (Figure 2.1). The GoPro camera, with fisheye lens, was aimed towards the horse's eyes to capture incidence of blinking in both eyes simultaneously (Figure 2.2). An appropriate habituation period was determined using a pilot study and outlined in full below (see *section 6*). Video recordings from the GoPro camera were downloaded onto a computer, where video analysis was carried out to determine blink rate.

All videos were analysed by the same researcher (LE). Blinks were counted and divided by the length (min) of the counting period, to give blink rate. Blinks were defined as the full and momentary closure of one or both eyes (Merkies *et al*., 2019), for less than 1 second. The <1 second duration of a full blink was important because horses in a dozing state may close their eyes fully but this does not constitute spontaneous blinking. Half blinks, defined as any movement of the upper eyelid towards the lower eyelid which did not fully cover the eye (Merkies *et al.,* 2019), and eyelid twitches were not counted.

Figure 2.1. Photograph showing the position of the GoPro on the horse's noseband.

Figure 2.2. Photograph showing an example of the view of the horses' eyes from the GoPro camera.

4.2 Blink rate variability

Informed by the results of SEBR analysis in Chapter 3, an alternative method of analysing blinking patterns during learning was proposed and subsequently implemented. Full justification for this addition is provided in Chapters 3 and 5. Blink rate variability (BRV) is a measure of the temporal variability of inter-blink intervals (Paprocki et al., 2016; *General Introduction and Literature Review*). From Chapter 3 onwards, BRV was used in addition to SEBR. In these chapters, BRV was analysed using root mean square of successive differences (RMSSD; Sollers et al., 2007). Video footage taken from the GoPro HERO 7 cameras were analysed in BORIS (Friard and Gamba, 2016). BORIS (Behavioural Observation Research

Interactive Software) is an online software programme for behavioural observations (Friard and Gamba, 2016). In BORIS, every blink incidence was time-stamped using the behavioural ethogram feature. The time stamps were converted to text files which were analysed for RMSSD in R. The formula for RMSSD is as follows (sqrt = Square Root, mean = Mean, RR*i* = a given interval between R peaks, RR*i*+1 = the subsequent interval following RR*i*; Schaffer, 2017):

$$\mathrm{sqrt}\left\{\mathrm{mean}\left((\mathrm{RR}_{i+1}-\mathrm{RR}_{i})^{2}\right)\right\}$$

4.3. Measuring heart rate variability

Heart rate variability (HRV) was measured using an ECG device (Actiheart 5 ECG: CamNTech, Fenstanton, United Kingdom). Since horses have a large thorax, a surcingle designed specifically for equine use (Polar Electro Oy, Kempele, Finland) is required to collect heart rate data. The ECG was worn on a Polar Equine belt (Polar Electro Oy, Kempele, Finland), fitted around the thorax (Figure 2.3). The belt was fitted as per Mott *et al*. (2021) using warm water applied to the coat directly beneath the belt as a conductivity aid. Horses may have thick coats depending on breed and season which poses issues with heart rate data collection due to the interaction between hair and skin during movement, causing artefacts (Parker et al., 2010; Lenoir et al., 2013; Ille et al., 2014; Cotur *et al*., 2020). Where data were collected during colder months (Chapter 6, for example), horses were clipped to remove excess fur.

Figure 2.3. Photograph showing the position of the ECG device fitted to the horse via a Polar Equine belt.

The Full Waveform feature of the Actiheart software was used to live stream the data recording as it occurred via Bluetooth connection. This allowed for the position of the Polar belt and conductive elements to be adjusted according to the appearance of the wave output. The full waveform view was checked prior to the start of each training session. In

Chapter 3 (the first experiment), the IBI Monitoring mode was used to collect data. The IBI mode records the inter-beat interval and is a valid method of measuring HRV. Heart rate monitors commonly used in equine research to date use IBI measurement for HRV analysis (Frippiat et al., 2021; Mott et al., 2021). In subsequent chapters it was discovered that the Full Waveform mode would be more optimal for collecting HRV data due to the manual correction feature (Mott et al., 2021), which would allow for artefacts (including type 1-5 errors; Ille et al., 2014) to be removed (Marchant-Forde et al., 2004). It was not possible to re-collect the data from Chapter 3 using Full Waveform mode, nor was it necessary given the validity of the IBI data. However, it must be acknowledged that the software mode used to collect the HRV data was not the same for every chapter. Once recording was finished, the ECG device was immediately removed from the horse and connected to the laptop using the USB cable to download the data. Once the data were downloaded, the device was ready to be set up for the next subject.

ECG files were analysed in Kubios HRV Premium (Kubios Oy, University of Eastern Finland). Files were uploaded to the viewer pane and the test period was selected for analysis. The test period was standardised to 3 minutes from the beginning of the training session for all recordings to ensure that variability was not affected by the length of the recording. To account for the less optimal mode of collecting HRV data used in Chapter 3, artefact correction was set at 0.4s as per previously validated methods for correcting IBI data (Mott et al., 2021). In subsequent chapters, artefacts and type 1-5 errors were manually corrected as per Marchant-Forde et al. (2004).Heart rate (HR) and heart rate variability (HRV) were taken from the auto-generated table of results produced by Kubios.

4.3. Measuring eye temperature

Eye temperature was measured using a FLIR e60 bx camera (Teledyne FLIR LLC, Oregon, United States), a portable thermal imaging device which uses infrared thermography (IRT) technology to measure emissive temperature. The device is referred to throughout this and future chapters as the IRT camera. Thermal images of both the left and right eye of the horse were taken immediately before and immediately after each training session. Images were taken from a 1 metre distance and 90-degree angle in relation to the sagittal plane, using the standardised method as described in Ijichi *et al.* (2020). This method has been validated as a non-invasive indicator of welfare in horses (Ijichi *et al*., 2020). Thermal images were downloaded onto a personal computer and analysed using FLIR Thermal Studio (Teledyne FLIR LLC, Oregon, United States) software. In FLIR Thermal Studio, the hottest point of each image could be found and marked using the in-built spot meter function in the software. Absolute temperature was recorded for each eye, and the change in temperature from pre-training to post-training was calculated and recorded.

4.4. Baseline measures

Baseline physiological measures pose a challenge because physiological processes are affected by many external and internal factors, particularly when using a reactive prey species such as the horse as a model species. With this in mind, it was determined that baseline for the purpose of this project was to mean the horse's resting state, in their usual stable. Some external factors such as olfactory cues in the environment, temperature and light conditions could not be controlled for. However, these factors represented a 'normal' setting for this sample of horses. Horses are not laboratory animals and cannot be managed as such, but they did provide a useful model species for this project.

Baseline heart rate and heart rate variability were measured using the ECG device as described above. A habituation period of 5 minutes was given for horses to acclimatise to the polar belt. This was an appropriate habituation period based on the familiarity of the equipment to this sample of horses. Subjects were regularly fitted with heart rate monitors for various research and university teaching purposes. Further, the Polar belt was fitted in the same way as a girth or surcingle, both of which were familiar to the horses. On the Kubios computer software, the baseline recording period could be selected manually.

Eye temperature was measured immediately before and after each learning trial. Since eye temperature is sensitive to environmental factors such as direct sunlight, humidity and wind conditions (Church *et al*., 2014), it would be inappropriate to use a measure of eye temperature from a previous date to compare future eye temperature measures against.

Monitoring blink rate in horses using the GoPro HERO 7 and GoPro leg strap was a novel method. In Mott *et al*. (2020), horses were left undisturbed for 2 hours wearing the GoPro camera to habituate. Since the current study was a longitudinal, time-sensitive project, a pilot study was conducted to find the habituation period of the sample to the GoPro so that this could be implemented for the duration of the project. Section 5 of this Chapter describes this preliminary study and reports its findings.

5. Pilot study: Finding the habituation period for a video analysis method of measuring blink rate in horses.

5.1 Rationale

To ensure the most valid and reliable measures of blink rate, the habitation period for the GoPro was investigated. It was important that experimental effects were accurately measured against a baseline which was not influenced by horses' reactions to the equipment. To this end, a small-scale preliminary study was conducted to find the maximum habituation period of the current sample of horses. This habituation period was to be implemented in the blink rate method throughout this project.

5.2. Method

Twenty horses from the main sample (see *Sample* and Table 1 for details) were used for this preliminary study. Horses in their usual stables at NTU were fitted with an Actiheart 5 ECG device and a GoPro HERO 7, as described previously (*Sections 4.3* and *4.1*, respectively). Horses were left at rest in their stables with both the GoPro camera and the ECG device recording for up to 45 minutes. The exact recording period depended on horses' behavioural reactions to the GoPro camera, which was relatively novel to them. Horses were closely monitored to ensure they did not have any adverse behavioural response to the novel equipment, and to ensure that equipment was not removed or damaged by the horse. Recording was immediately terminated if horses performed any of the following behaviours: excessive and continuous head shaking, rearing, excessive vocalisation, rubbing the face against the stable walls or door and removal of the device. The GoPro footage and Actiheart ECG recordings were managed as previously described.

Blinks were counted in three-minute intervals every three minutes, starting at the beginning of the GoPro video recording. Each three-minute period was numbered (Table 2.2). R statistical software (R Foundation for Statistical Computing) was used to analyse the data from each count period. Wilcoxon Signed Ranks tests of difference were performed between each count period systematically, starting with count periods 1 and 2. Habituation was defined as the point at which there was no longer a significant difference in blink rate for 2 or more consecutive count periods. Heart rate (HR) and heart rate variability (HRV) were analysed in the same way, to monitor horses' sympathetic nervous system response to the cameras over time.

Table 2.2. Time stamps for each count period in the habituation pilot study.

Time	Count period
00:00 – 03:00	1
06:00 – 09:00	2
12:00 – 15:00	3
18:00 – 21:00	4
24:00 – 27:00	5
30:00 – 33:00	6
36:00 – 39:00	7
42:00 – 45:00	8

5.3. Results

Recording periods varied in length between individual horses, such that of the twenty horses in the sample, only four had the GoPro on for 45 minutes. Fifteen horses had the GoPro on for 30 minutes, three horses had it on for 25 minutes and one horse for only 5 minutes. This was due to individual differences in tolerance to the equipment, as explained above (*5.2 Method*). Blink rates during count periods 1-5 only (Table 2.2) were compared in this study, since most horses tolerated the equipment for thirty minutes. Blink rate decreased from count period (CP)1 to CP2 and increased in CP5 (Table 2.3). Heart rate increased significantly in CP4 (Table 2.4). Heart rate variability did not significantly change across any of the count periods (Table 2.5).

Table 2.3. Blink rate results across count periods. Bold indicates significant difference.

Count period	Wilcoxon Signed Ranks test statistic (V)	Direction	p-value	Power
1 – 2	**115.5**	**Decrease**	**0.01**	**0.34**
2 - 3	89	No change	0.82	0.06
3 - 4	115.5	No change	0.19	0.54
4 - 5	**17.5**	**Increase**	**0.01**	**0.27**

Table 2.4. Heart rate results across count periods.

Count period	Wilcoxon Signed Ranks test statistic (V)	Direction	p-value	Power
1 – 2	62	No change	0.78	0.9
2 – 3	98	No change	0.13	0.4
3 – 4	**106.5**	**Increase**	**0.005**	**0.05**
4 – 5	51	No change	0.73	0.88

Table 2.5. Heart rate variability (RMSSD) results across count periods.

Count period	Wilcoxon Signed Ranks test statistic (V)	Direction	p-value	Power
1 – 2	74	No change	0.78	0.9

2 – 3	78	No change	0.63	0.83
3 – 4	36	No change	0.1	0.35
4 – 5	54	No change	0.5	0.76

5.4. Discussion & conclusions

Results indicated that the habituation period was between 0 – 6 minutes, followed by a period of 15 minutes in which blink rate remained stable. The increase in blink rate at CP5 combined with the increased heart rate at CP4 indicated arousal, which may explain the behaviours leading to the GoPro being removed after 30 minutes in most cases. Around the 30-minute mark, 15 of the horses began headshaking and rubbing their faces against the stable walls and door which caused the GoPro to become loose and/or fall off. This suggests that there was a tolerance threshold of around 30 minutes and a window of stable blink rate between 6 to 24 minutes. For all future chapters, baseline blink rate measures were taken within this window.

Horses were closely monitored throughout this preliminary study. While this may have caused observer effects, it was necessary to monitor the horses to ensure their welfare was safeguarded. Since this was a novel protocol for this sample of horses, it was important to monitor their behaviour for any signs of discomfort or emotional distress. This study revealed not only the habituation period of the horses sampled, but also a tolerance threshold. Taken together, the behavioural observations, blink rate data and HR data revealed that horses generally became intolerant of the GoPro at around the 30-minute threshold. Not only was this finding important for welfare purposes in this study, but it also was useful from a health and safety perspective and for protecting the equipment. This highlights a possible limitation of the horse as a research model, because they are large animals which can pose a danger to themselves, researchers and equipment. These risks can be mitigated by careful monitoring of the equine subjects, though this may have unintended observer effects due to the presence of a human researcher. Horses cannot be managed in the same ways as lab rodents, and the health, safety and welfare of the animals must be prioritised over complete research validity in scenarios such as this. It is important to note that this sample of horses were unique in that they were accustomed to taking part in research projects regularly and are therefore generally very tolerant of novelty. In a more representative sample of horses, this habituation period would have to be revisited. This study used horses from the main sample of the wider project. As such, these results were inherently useful for the current project, though they may not be applicable to the general equine population and should be generalised with caution.

6. Adaptive learning

This thesis comprises three learning trails designed to test adaptive learning in horses, and act as experimental conditions in which to measure blink rate and arousal as predictors of learning performance. The learning trials, Two-Choice Discrimination-Reversal, Go/No-Go and Reward Sensitivity are outlined below (*Section 6.2*); further details are given in individual data chapters. Pre-trial training was necessary to condition the secondary reinforcer and shape a nose-touch response to the experimental stimuli. Details of this pre-training are given below (*Section 6.1*).

6.1. Pre-trial training

6.1.1 Initial whistle training

The touch response required for all chapters (outlined in section 6.1.2) was achieved through whistle training, which involved using a whistle sound as a secondary reinforcer and a food reward (Ulsakind cubes) as a primary reinforcer. The benefits of using the whistle were (1) This sample of horses had never been whistle trained previously (but had inconsistent clicker training experience), so the sound of the whistle was neutral to them prior to training; (2) The whistle could be worn around the trainer’s neck on a lanyard and operated with the mouth for instant delivery of the secondary reinforcement, timed accurately to reward the horses’ behavioural responses. (3) The whistle sound was consistent, which was important for operant conditioning so that the horse was able to quickly learn what the whistle sound meant.

Each horse completed two 15-minute whistle training sessions. Training sessions took place in the horses’ usual stables. Horses were loosely tethered in their usual stables. The reasons for loosely tethering horses were as follows: 1) The primary aim was to measure physiological responses to learning, so it was important that the physiological measures were not confounded by horses moving around. For example, the validity of the EGC relies on horses being stationary, eye blink rate is affected by movement and air displacement, and eye temperature is affected by exercise. 2) To comply with the health and safety regulations at the study site. 3) horses at this particular study site were not all housed in identical stabling: at least four of the subjects in each study were pair housed. Therefore, in order to test every horse in their usual home environment, it was necessary to loosely tether all subjects. The same trainer (LE) trained all horses using the same method and training schedule, as follows: First, positive reinforcement was used to condition the whistle sound. Every time the whistle sounded, the horse was given a small handful of Ulsakind (approximately 5-6 10x3x2mm pellets). This whistle-sound-food-reward training was repeated continuously for 3 minutes, followed by a 2-minute break, and then repeated for a further 3 minutes. This training schedule was repeated for a total of 15 minutes. Each horse had two 15-minute training sessions, conducted over consecutive days.

The food reward was kept in a closed treat bag behind the trainer's back, while the targets were presented in front of the horse. The reasons for this relatively close proximity between targets and food were 1) to ensure timely delivery of food for effective conditioning, and 2) to reduce movement of the horse during training which would have invalidated the physiological data.

Next, horses' understanding of the whistle training was consolidated with a basic behaviour training schedule. The same trainer stood at the shoulder of the horse, facing forwards, with a small handful of Ulsakind cubes in one hand and the whistle ready. The trainer kept the treat-containing hand closed and did not allow the horse to take the food. As soon as the horse stopped investigating and turned away from the trainer to face forwards, the whistle was sounded, and the horse was immediately rewarded with the food. This was repeated several times, from both the left and right shoulders equally, until the horse learned to stand still and wait for the food to come to them.

The purpose of this training was threefold: (1) The horse's knowledge that the whistle sound indicated reward was consolidated; (2) Further training using the whistle would be more effective as a result of this training, due to the association of the whistle sound as an indicator that a correct behavioural response had been offered. (3) The horse learned not to perform foraging behaviours directed at the trainer, which would distract from the training objective and lead to the formation of an undesirable habit.

6.1.2 Shaping the cue card touch response

Once subjects had successfully learned to associate the whistle sound with reward, the whistle was used to train a nose touch response. Each horse was trained to touch one of two A3 size, laminated cue cards (1 x black, 1 x white) with their nose. Cue card colour was pseudo-randomised and counterbalanced across the sample. The cue cards were black and white because these are two shades which are clearly visible to horses (Macuda and Timney, 1999). The cue card was introduced to the horses by the same trainer (LE), who held it in front of the horse, at the level of the horse's chest. During this shaping phase, any movement towards the cue card was rewarded with a whistle sound and food reward. As the horse began to give the nose touch response more readily, only complete touches were rewarded. The cue card response was considered successful when active and immediate nose touch responses were offered upon presentation of the cue card.

Each horse completed a minimum of two shaping sessions. Each session lasted one hour, with regular breaks throughout. Horses were given 5 minutes to rest for every 15 minutes of training completed. The criteria for completing the touch response training was at least 80% correct behavioural responses to the cue card in two consecutive training sessions (Neave et al., 2013). The number of correct responses given and any notes on the training sessions were recorded in a training logbook. As the horse's touch response improved, the cue card was held in different positions and at different angles, encouraging the horse to actively seek the cue card for reward. As the cards were laminated, light would reflect in different ways so moving the cue card into different positions meant that the horses learned to generalise their response to the cue card even when it looked slightly different depending on position and lighting.

6.2 Learning trials

Once all pre-training and pilot testing of methods was complete, the individual learning experiments began. Each experiment is described in full in the relevant data chapters, however the below gives a general overview of each experiment. Physiological responses (SEBR, blink rate variability (BRV), HRV and eye temperature (IRT)) to each task were recorded and analysed as described previously (*Section 4*).

6.2.1 Two-Choice Discrimination-Reversal (Chapter 3)

In the two-choice discrimination-reversal task, horses were trained to select one rewarded stimulus against an unrewarded stimulus. One black and one white cue card were presented to the horses; one card was rewarded and the other unrewarded in a counterbalanced design. The laterality of the cue card presentation was pseudo-randomised and counterbalanced such that each colour was presented on the left the same number of times that it was presented on the right. Correct nose-touch responses to the rewarded stimulus resulted in whistle activation and food reward. Incorrect responses and errors of omission (no response) were not rewarded. No punishment was used in the case of incorrect responses or errors of omission. Once learning criteria of 80% correct responses in two consecutive sessions was reached (Neave et al., 2013), the rewarded and unrewarded stimuli were reversed. For example, horses who were previously rewarded for selecting the white card were now rewarded for selecting the black card, and vice-versa. The reversal task tested horses' cognitive flexibility, the ability to reverse a behavioural response that has previously been rewarded in favour of a newly rewarded behaviour. Baseline physiology and physiological responses to the task were investigated as possible predictors of cognitive flexibility.

6.2.2 Go/No-Go (Chapters 4 and 5)

In the Go/No-Go task, a light cue was used as a "No-Go" signal, meaning that whenever the light was on, subjects were not rewarded for touching the previously rewarded stimulus. When the light was off, subjects could "Go" and their touch responses to the stimulus were rewarded. This challenged inhibitory control, a skill that horses are expected to perform regularly. For example, horses are routinely expected to inhibit their learned locomotory behaviour until the rider offers a "Go" signal. Learning criteria was 80% correct in two consecutive sessions as previously.

Both Chapters 4 and 5 investigated responses to this task, but with different aims. Chapter 4 examined horses' behavioural responses to the experiment, exploring whether they used model-based or model-free learning to complete the task. Chapter 4 was added to this project following an interesting observation about horses' behaviour towards the Go/No-Go task. Performance data during the Go/No-Go training sessions revealed that horses appeared to be responding indiscriminately to the stimulus, and it was decided that an additional experiment would be carried out to investigate this further, using a combination of positive reinforcement and negative punishment. Full details and justification are given in Chapter 4. In Chapter 5, physiological responses to the Go/No-Go task were investigated. Baseline physiology and physiological responses to the task were investigated as possible predictors of inhibitory control performance.

6.2.3 Reward Sensitivity (Chapter 6)

Reward sensitivity is the extent to which an individual can recognise the relative reward value of multiple reinforced behaviours (Martin and Potts, 2004). The Reward Sensitivity task was completed in two distinct phases. The first phase was a reward value discrimination task. In this phase, a nose-touch response directed at either of two stimuli was rewarded, but one stimulus was rewarded significantly more than the other. One stimulus elicited a whistle sound and one pellet of food; the other stimulus elicited the whistle sound and three pellets of food. Food preference testing was conducted prior to this experiment to determine if food quality could be used to signal high and low reward, but no significant preference was established. In addition, recent work suggests that horses respond better to differences in reward quantity rather than differences in reward quality (Brucks et al., 2022). Therefore, the stimulus worth three pellets was assigned as the high value target (HVT) and the stimulus worth one pellet was the low value target (LVT).

Targets were white, A3 sized, laminated cue cards with distinct symbols printed in black. In this experiment, targets were presented on poster stands directly outside the horses' stables. This allowed horses to move freely between the two targets. The reason for this approach here but not in previous experiments was that the targets did not need to be switched between attempts, so could remain in a fixed, consistent position throughout the session. Unlike previous experiments, the horse was this time allowed twenty opportunities to select the target of their choice. These choices were analysed for preference above chance, to determine whether horses would prefer the high value target over the low value target. Target position was pseudo-randomised and counterbalanced across the sample, such that half of the subjects (n=10) were presented with the HVT to their left and the other half (n=10) had the HVT to their right.

In Reward Loss (Chapter 6), both stimuli were presented to horses again for a further twenty opportunities to select the preferred target. However, this time both targets elicited one pellet of food. This meant that the previous high value target was now reduced in value (RVT), while the previous low value target remained consistent (CVT). The position of the targets switched in this phase, such that the target previously presented to the horse's left was now on the horse's right, to avoid side bias. Preference for the RVT or CVT, plus physiological responses to reward loss were examined in this study.

6.3 Measuring learning performance

A canon legria video camera (Canon Inc., Tokyo, Japan) and tripod were used to record all training sessions so that retrospective analysis of learning performance could be carried out. The number of attempts, number of correct responses, and (where applicable) the position of the target (e.g. in the reversal trial, whether the rewarded target was presented on the horse's left or right) was noted.

7. Conclusions

The methods detailed in this chapter have been carefully considered, refined and validated. As this project uses horses as a model to investigate physiological predictors of learning, it is important that measures of arousal are appropriately validated for use in horses. A preliminary study was carried out as part of this careful consideration of the methods used, and those results and conclusions have been presented here. All methods discussed in this chapter were selected with horse welfare in mind. As such, methods used were non-invasive and species appropriate, with relevant safeguards in place.

CHAPTER 3

EYE UNDERSTAND: PHYSIOLOGICAL MEASURES AS NOVEL PREDICTORS OF REVERSAL LEARNING PERFORMANCE

1. Introduction

Domestic horses are regularly expected to discriminate and reverse behavioural responses to stimuli, and this function of cognitive control is crucial for rider safety (Starling et al., 2016) and effective training (McGreevy and McLean, 2007). In equitation, reversal occurs when a horse is ridden or handled by different people and when horses are rehomed or retrained in a new discipline, which is common in the equestrian industry (Innes and McBride, 2008; Stowe and Kibler, 2016; Sigler, 2019; Evans and Williams, 2022). What the horse has associated as a cue (stimulus) for a particular behavioural response with one handler may be the cue for a different behavioural response with another handler. This requires the horse to have the cognitive flexibility (Scott, 1962) to adapt their response to different cues and stimuli. When horses are unable to adapt, this can lead to inappropriate behavioural responses which, when dealing with a large animal, can be dangerous (Hawson et al., 2010).

Reversal learning occurs when a previously rewarded behavioural response is no longer rewarded, but an alternative behavioural response is. Therefore, the animal must reject the previously rewarded response in favour of the newly rewarded response. The speed and accuracy with which an animal is able to do this is an indicator of cognitive flexibility. Therefore, it is in the interests of horse and rider safety, as well as training efficacy, to be able to reliably predict individual reversal learning performance. In this chapter, physiological predictors of performance are investigated in a two-choice discrimination-reversal learning paradigm, designed to test cognitive flexibility.

Animal behaviour is often explained in terms of reward learning, which refers to the idea that behaviour is more or less likely to occur based on the positive or negative consequences that follow it. The striatum controls value-based decision making which is the basis of reward learning (Filla et al., 2018; Graybiel, 2016; Kwak & Jung, 2019; Verharen et al., 2019). Striatal dopamine is important in learning (El-Ghundi M. et al., 2007; Izquierdo et al., 2006; Morita et al., 2016), as it is the neurotransmitter that marks reward (Mirenowicz & Schultz, 1996; Schultz, 2002).

There are two ways that striatal dopamine mediates reward learning: first, through tonic release of midbrain striatal dopamine and second, through phasic striatal dopamine release. Tonic dopamine affects individual sensitivity to reward and motivation to seek reward (Beeler et al., 2010; Berridge, 2007; Maia & Conceição, 2017), thus increasing an individual's tendency to perform a behavioural response when there is opportunity for reward (Berridge, 2007; Maia & Conceição, 2017). Phasic dopamine release is associated with the initiation of an operant response, predicting the reward that follows (Berridge, 2007; Pan et al., 2021). After the operant response has been acquired, phasic dopamine is released initially on presentation of reward, then it moves temporally backwards to cues that either indicate imminent arrival of reward or a window of opportunity to perform an operant response that will attain a reward (McBride et al., 2017).

Changes to dopamine activity are determined by the reward prediction error (RPE), which evaluates the actual level of reward elicited from a behavioural response, versus the predicted level of reward (Glimcher, 2011; Schultz, 2013, 2016). Where there is a discrepancy between actual and predicted reward, RPE (mediated by phasic dopamine activity in the midbrain) prompts the individual to alter their response in order to seek the expected reward (Schultz, 2013, 2016). Therefore, phasic dopamine, through the RPE function, plays an integral role in reversal learning.

Early work by Karson (1983) identified a relationship between striatal dopamine systems, including both tonic and phasic dopamine, and spontaneous eye blink rate (SEBR). SEBR can be manipulated using dopamine agonist drugs and variations in mental load (Karson, 1983). Indeed, a positive linear relationship between striatal dopamine activity and spontaneous eye blink rate (SEBR) has been observed in a variety of species (Jongkees & Colzato, 2016). Blink rate has also been linked to reward learning (Gregory, 2008; Slagter et al., 2015; van Slooten et al., 2017, 2019). Higher blink rate during learning indicates phasic dopamine release in the striatum during reward processing (van Slooten et al., 2017, 2019). Therefore, average blink rate may offer a proxy measure of tonic striatal dopamine activity during reward learning (van Slooten et al., 2017).

In addition to striatal dopamine, arousal may also be important for learning, though evidence for the direction of this relationship is inconsistent. Arousal is a state of physiological activation, regulated by the hypothalamus and brainstem through multiple neurotransmitter pathways (Marrocco et al., 1994). Physiological effects of increased arousal include increased heart rate (Azarbarzin et al., 2014; Davies et al., 2014; Graham & Jackson, 1970; Wascher, 2021) and lower heart rate variability (Applehans & Luecken, 2007; Scherz et al., 2020). In addition, increases in eye temperature caused by increased blood flow to the eyes as a sympathetic nervous system response have been observed (Jansson et al., 2021; Stewart et al., 2007, 2010; Travain et al., 2021), though decreases in temperature can be seen where this arousal is specific to fear, pain, or frustration (Nakayama et al., 2005; Stewart et al., 2008).

Increased arousal during learning has been associated with positive effects on information storage (Eysenck, 1976), retention (Levonian, 1972) and retrieval (Eysenck, 1976). Ferrari (2014) found that tadpoles at higher risk of predation, which are presumably chronically more aroused, performed better on a predator recognition and memory task than tadpoles at lower risk. This suggests that improved cognitive performance can be an adaptive response to arousal, as it increases chances of survival in threatening situations. However, increased arousal does not always aid learning. Sage & Bennett (1973) increased participants' arousal by administering small electric shocks at random intervals during a motor skill task and found that learning was not enhanced. Therefore, the effects of arousal on learning performance may be context dependent.

Further, it has been suggested that over-arousal can have detrimental effects on learning performance (Fisk & Warr, 1996). Maloney et al. (2014) suggest that when students experience high levels of exam anxiety, their academic performance is impaired, due to an overstimulation of the sympathetic nervous system. These contradictions may be explained by an arousal “sweet spot”, in which performance is optimal with moderate arousal which has been well documented particularly in human studies (Yerkes & Dodson, 1908). The Yerkes-Dodson law of arousal has been applied to attention and learning in an ungulate species, whereby performance on a visual attention task was hindered by both high and low levels of vigilance or fearfulness (McBride and Morton, 2018).

Reversal learning requires the cognitive flexibility to reverse a previously conditioned discrimination response in favour of a newly conditioned response (Fiske & Potter, 1979). In reversal learning, reward parameters are altered such that a behavioural response that has been learned through operant conditioning is no longer rewarded and/or is punished (Izquierdo et al., 2012). This means that it is no longer advantageous to offer this behaviour, therefore it must be inhibited in favour of a newly rewarded response (Izquierdo et al., 2012). The nucleus accumbens has a significant role in processing reinforcement learning, reward and motivation (Day and Carelli, 2007). The caudate nucleus processes information relating to the outcomes of responses, which has functions for instrumental learning (Brovelli et al., 2011).

Dopamine has different roles depending on the cortico-striatal-thalamo-cortical (CSTC) loop (McBride and Parker, 2015). For example, the salience network features a CSTC loop which has functions for cognitive control, including response selection and inhibition (Peters et al., 2016). McBride and Parker (2015) describe in detail the various CSTC loops and their relation to dopamine action. Increased dopamine activation is beneficial during nucleus accumbens predominant initial acquisition, due to the role of dopamine in habit formation, however, it is detrimental to caudate predominant action-outcome learning required for reversal learning (Eckstein et al., 2017; Gregory, 2008; Slagter et al., 2015; van Slooten et al., 2017, 2019). Therefore, individuals with higher dopamine activity readily learn new reward-based tasks but lack the flexibility to adjust responses under changing conditions as they rely on stimulus-response, rather than response-outcome, learning (Wickens et al., 2007). Reversal learning requires a dip in tonic striatal dopamine activation in order to modify action in terms of expected outcomes (Schultz, 2013, 2016). As such, reversal learning is an appropriate experimental vehicle in which to investigate novel predictors of performance, using SEBR as a proxy measure of dopamine.

It has been generally accepted that SEBR is reflective of striatal dopamine activity (Jongkees & Colzato, 2016; Karson, 1983) and striatal dopamine is implicated in learning performance (El-Ghundi M. et al., 2007; Izquierdo et al., 2006; Morita et al., 2016). However, it has not yet been directly established whether blink rate may be used as an indirect measure of striatal dopamine, to predict learning performance. SEBR could offer a non-invasive, practically applied alternative to Positron Emission Tomography (PET) and functional Magnetic Resonance Imaging (fMRI), current in vivo methods of measuring striatal dopamine (Calabro et al., 2023). Therefore, the first aim of this study was to investigate whether SEBR may be used to predict learning performance using a two-choice discrimination reversal learning task (referred to as reversal learning throughout) as a cognitive challenge.

It was hypothesised that reversal learning performance would be predicted by baseline blink rate, indicative of dopamine receptor activation in the striatum (tonic dopamine) (Beeler et al., 2010; Berridge, 2007; Maia & Conceição, 2017). Further, it was hypothesised that blink rate during reversal learning would predict reversal performance. Over activation of the dopamine system is detrimental to action-outcome learning and instead produces habitual stimulus-response learning (Schultz, 2013, 2016). While arousal has been demonstrated to affect performance in a range of cognitive tasks, there is less evidence for the relationship between baseline arousal and learning. Individuals with high resting arousal may become over-aroused during challenges which could compromise performance. The second aim of the current study was to investigate whether physiological indicators of arousal (infrared thermography of eye temperature and heart rate variability), both at baseline and during a reversal learning challenge, could predict learning performance. It was hypothesised that performance would be predicted by arousal, both at baseline and during reversal learning, though the nature of the relationship was not pre-conceived.

2. Materials and Methods

Ethical notes and subject information are presented in the General Methods Chapter.

2.1 Learning Protocols

The protocol was a two-choice discrimination reversal learning task. Subjects first undertook pre-conditioning (reported in full in General Methods), then visual discrimination training (referred to as Discrimination), followed by a Reversal challenge (referred to as reversal learning). All data collection took place in the horses' usual stables. All sessions were completed between 10:00 and 15:00. Twenty-two horses from the original sample were used for this experiment, as detailed in Table 1 of Chapter 3.

The same trainer (LE) trained all 22 horses, using the same method and training schedule (Figure 3.1). Subjects were given three sessions a day, each comprising ten trials with a short break between each session to rest subjects and re-adjust equipment where needed. Each day was capped at three sessions which equates to 30 total trials and 30 small potential food rewards, as horses are a trickle-feeding species and feed allocation was corrected for in subsequent meals.

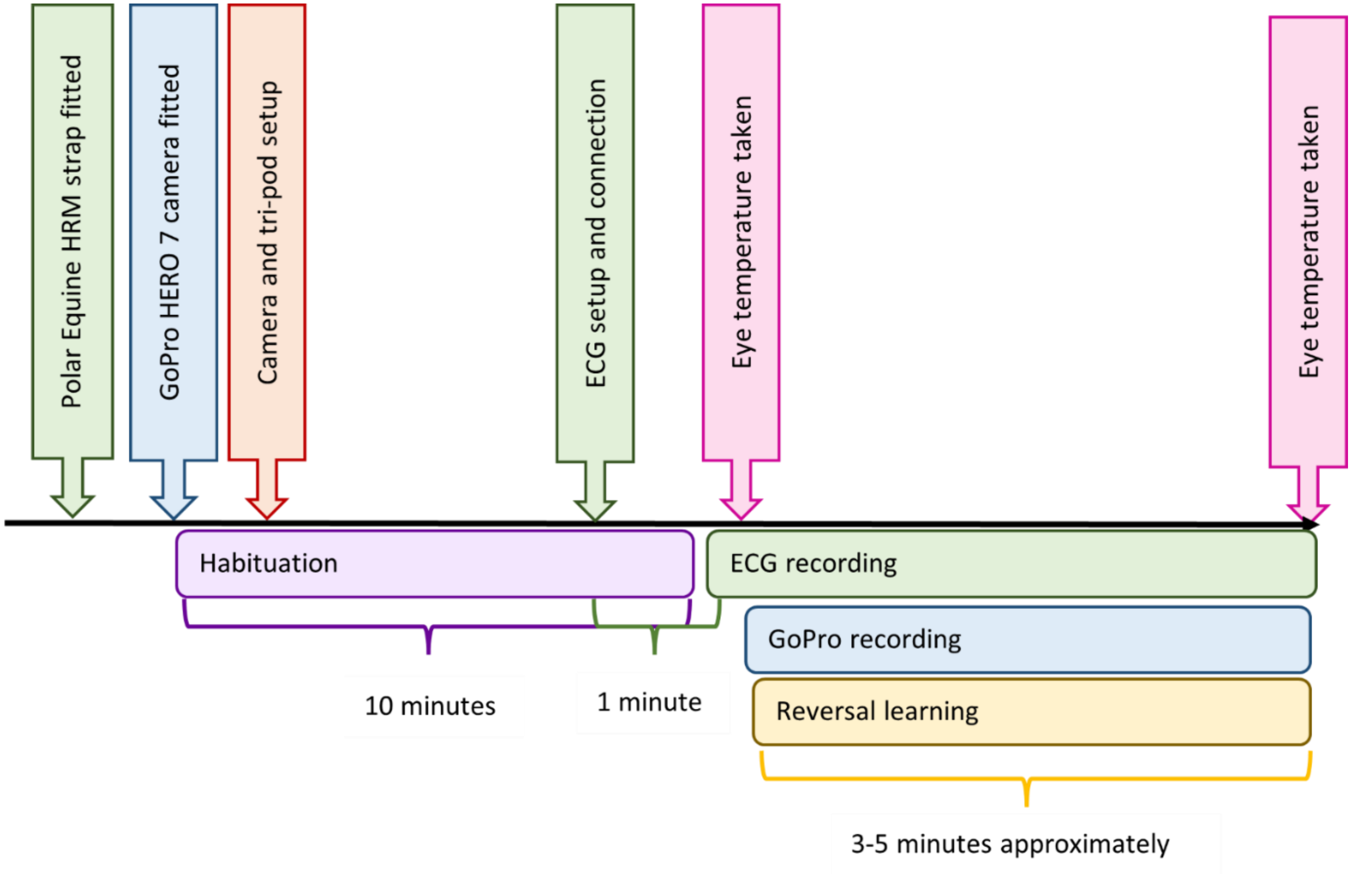

Figure 3.1. Timeline of the data collection protocol for the two-choice discrimination-reversal study.

2.2 Discrimination Training

Discrimination Training followed pre-conditioning (presented in General Methods). The aim of Discrimination was to train the subjects to select the conditioned target (S+) with a nose touch response. During Discrimination, subjects were trained to touch one of two A3 size, laminated cue cards (1 x black, 1 x white). The targeted cue card colour was pseudo-randomised using a random number generator for each horse and counterbalanced overall. The touch response was followed by the whistle sound as a secondary reinforcer and then followed by a food reward (Ulsakind cubes) as a primary reinforcer.

During Discrimination, subjects were presented with both the target (S+) and the distractor (S-) (the black and the white cards). Both targets were held by the trainer in front of the subject, side by side. Target position was pseudo-randomised and counterbalanced such that each target was presented on the subject's left the same number of times as it was presented on their right, to avoid the formation of a side bias (Austin & Rogers, 2014).

Subjects were loosely tethered, such that they were able to interact with or move away from the task, but that University health and safety regulations were complied with. Target presentation marked the start of a trial, and subjects were allowed up to 15 seconds to make their selection before targets were removed. This was considered one trial and if the subject failed to make a selection within the time allowed this was recorded as an error of omission. If the subject offered an incorrect response (error of emission) no reward was given and the next presentation was made. Between each trial the targets were removed from the subjects' sight and immediately re-presented to them for the next trial. Learning criteria was reached when subjects gave at least 80% correct responses in over 30 consecutive trials (Neave et al., 2013).

2.3 Reversal Learning

Reversal learning took place in the same setting, with the same subjects, except for three who were removed from the study at this stage due to being rehomed (sample: 7 females, 12 males. Mean age = 16.3 ± 2.57). Subjects did not proceed to reversal learning until they had reached learning criterion during Discrimination. Reversal learning saw the previously unrewarded target (S-) from Discrimination become the new rewarded target, and vice versa. Errors of omission (lack of response), emission (incorrect response) and number of attempts taken to reach criteria were recorded. Learning criteria was set as at least 80% correct over 30 consecutive trials (Neave et al., 2013). Subjects were trained until they successfully achieved reversal or until they reached the training limit of 150 attempts.

2.4 Behaviour & Physiology

2.4.1 Spontaneous Eye Blink Rate

SEBR was measured at rest (referred to as baseline throughout) and again during learning trials under the same environmental conditions as per General Methods. Resting SEBR was measured as a potential indicator of tonic dopamine and during learning as a potential indicator of phasic dopamine (Jongkees & Colzato, 2016; Kirsty et al., 2015; McBride et al., 2017).

2.4.2 Heart Rate Variability

Heart rate variability was measured at rest (referred to as baseline throughout) and again during learning trials under the same environmental conditions as per General Methods to provide evidence of both arousal day-to-day and in response to the cognitive challenge. Actiheart was set to record in the IBI (Inter-Beat Interval) Monitoring window on the Actiheart software (see *Section 4.3* of the *General Methods* chapter for justification). Files were exported from Actiheart as time stamped IBI files, then analysed in Kubios HRV Premium (Kubios Oy, University of Eastern Finland). Artefact correction was set at 0.4s as per previously validated methods for correcting IBI data (Mott et al., 2021).

2.4.3 Infrared Thermography

Eye temperature (IRT) was measured to indicate both arousal and potential lateralised responses to the cognitive task as per General Methods. Absolute temperature was recorded and the change in temperature from pre-training to post-training was calculated and recorded for both eyes.

2.4.4 Learning Performance

A Canon Legria video camera (Canon Inc., Tokyo, Japan) and tripod were used to record the training sessions so that retrospective analysis of learning performance could be carried out. The number of trials to reach learning criterion and number of errors was recorded. A Reversal Performance Index for each horse was calculated using the following formula: 1000/T/ME, where T = number of trials to reach reversal learning criteria and ME = mean number of errors across all reversal learning trials (Fiske & Potter, 1979). Higher indices indicate better learning performance.

2.5 Data analysis and modelling

Data were statistically analysed in R version 4.1.2 (2021 R Core Team, R Foundation for Statistical Computing, Vienna, Austria). Shapiro Wilks tests of normality were used to determine normality of the data. Subsequently, Wilcoxon signed-rank tests of difference were used to determine whether IRT, RMSSD and SEBR changed in response to reversal learning trials. A full R script for the analysis methods used throughout this thesis is provided in Appendix IV.

Data were modelled to determine whether SEBR or arousal variables could predict learning performance. The purpose of this was to satisfy the two aims of the study: (1) to investigate SEBR as a predictor of learning performance; (2) to investigate arousal both at baseline and during a reversal learning challenge as a predictor of learning performance. Both baseline SEBR and reversal learning SEBR were included in the statistical model to account for the possible effects of both tonic (baseline) and phasic (reversal learning) dopamine activity. Data were modelled in R using the MASS package (Venables & Ripley, 2002) to conduct a negative binomial generalised linear model (GLM). This model is suitable for over-dispersed data (high variability in the dataset; Zuur and Ieno, 2016).

The model was carried out according to the 10-step process recommended by Zuur and Leno (2016). Data were examined for missing values, outliers, zeros, collinearity using Variance Inflation Factor (VIF) and normal distribution using Shapiro-Wilks. The following covariates were tested in the GLM: blink rate, baseline blink rate, RMSSD, baseline RMSSD, left eye temperature change and right eye temperature change. The reasons for including each covariate (including the individual hypothesis for each variable) can be found in Table 3.1. The response variable was the Reversal Learning Performance Index. A stepwise regression using Akaike Information Criterion (AIC) was conducted to find the best model significance and fit. Effect sizes were calculated following Farrar et al. (2020).

Table 3.1. Predictor covariates included in the NB GLM, with justification for inclusion and individual hypotheses.

Covariate	**Reason for inclusion in the model**	**Hypothesis**
Blink rate	This study was interested in whether phasic blink rate may be a useful predictor of adaptive learning.	Higher blink rate, indicating increased phasic dopamine firing, would be associated with better performance (Schultz, 2013, 2016).
Resting blink rate	Baseline blink rate is reflective of tonic striatal dopamine (Jongkees and Colzato, 2016), which may predict individual sensitivity to reward learning.	Higher baseline blink rate, as an indicator of higher tonic dopamine, would predict better learning performance (Jongkees & Colzato, 2016).
Heart rate variability (RMSSD)	As a measure of physiological arousal during learning. Arousal may predict learning performance according to the Yerkes-Dodson Law.	Moderate arousal, as indicated by a moderate RMSSD would be associated with better performance (Yerkes & Dodson, 1908)
Resting RMSSD	As a measure of baseline stress sensitivity, which may predict learning performance/ trainability.	Moderate arousal, as indicated by a moderate baseline RMSSD would be associated with better performance (Yerkes & Dodson, 1908)

Left eye temperature change	As a marker of arousal in response to learning. May predict learning performance.	Moderate arousal, as indicated by a moderate change in eye temperature would be associated with better performance (Yerkes & Dodson, 1908)
Right eye temperature change	As a marker of arousal in response to learning. May predict learning performance.	Moderate arousal, as indicated by a moderate change in eye temperature would be associated with better performance (Yerkes & Dodson, 1908)

3. Results

3.1 Physiological responses to learning.

Heart rate variability (RMSSD) increased during reversal learning (median + IQR = 99.81 + 38.39), compared with Resting (median + IQR = 84.44 + 55.36; Figure 3.2). This pattern was not statistically significant (Wilcoxon: V = 48, p = 0.06) but did demonstrate a medium effect size (Cohen's d = 0.58).

There was no significant difference between Resting blink rate (median = 14.66, IQR = 4.84) and blink rate during reversal learning (median + IQR = 15.8 + 8.71; Wilcoxon: V = 69, p = 0.31; Cohen's d = 0.31).

Temperature (°C) of the left eye decreased significantly from pre-reversal learning (median + IQR = 35.7 + 0.75) to post-reversal learning (median + IQR = 35.1 + 0.9; Wilcoxon: V = 167.5, p = 0.002; Figure 3.3a), with a medium effect size (Cohen's d = -0.74).

Temperature (°C) of the right eye decreased significantly from pre-reversal learning (median + IQR = 35.8 + 0.62) to post-reversal learning (median + IQR = 35.3 + 0.67; Wilcoxon: V = 157.5, p = 0.05; Figure 3.3b), with a medium effect size (Cohen's d = -0.69).

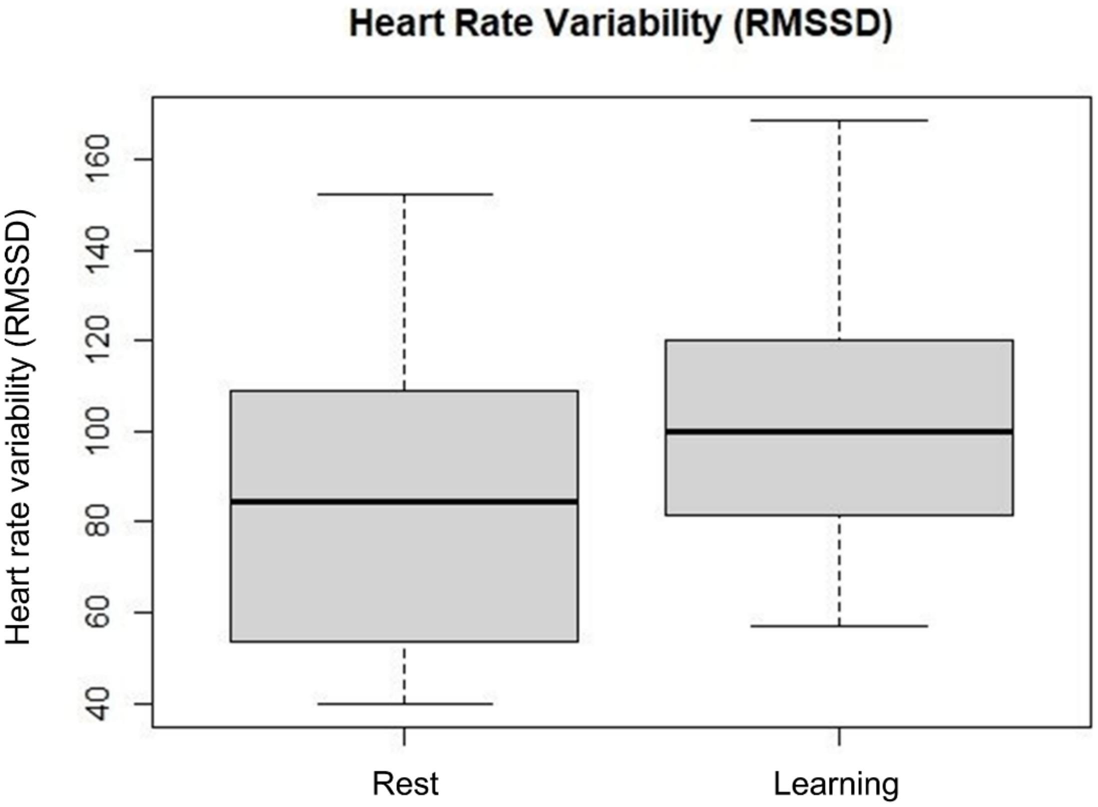

Figure 3.2. Boxplot of heart rate variability as calculated by root mean square of successive differences (RMSSD) in nineteen horses at rest (baseline) and during Reversal learning for visual inspection of a possible change (n=19, V = 48, p = 0.06). The boxes represent the first to the third quartile of data, with a bold line at the median value. The maximum and minimum values are indicated by the upper and lower whiskers, respectively.

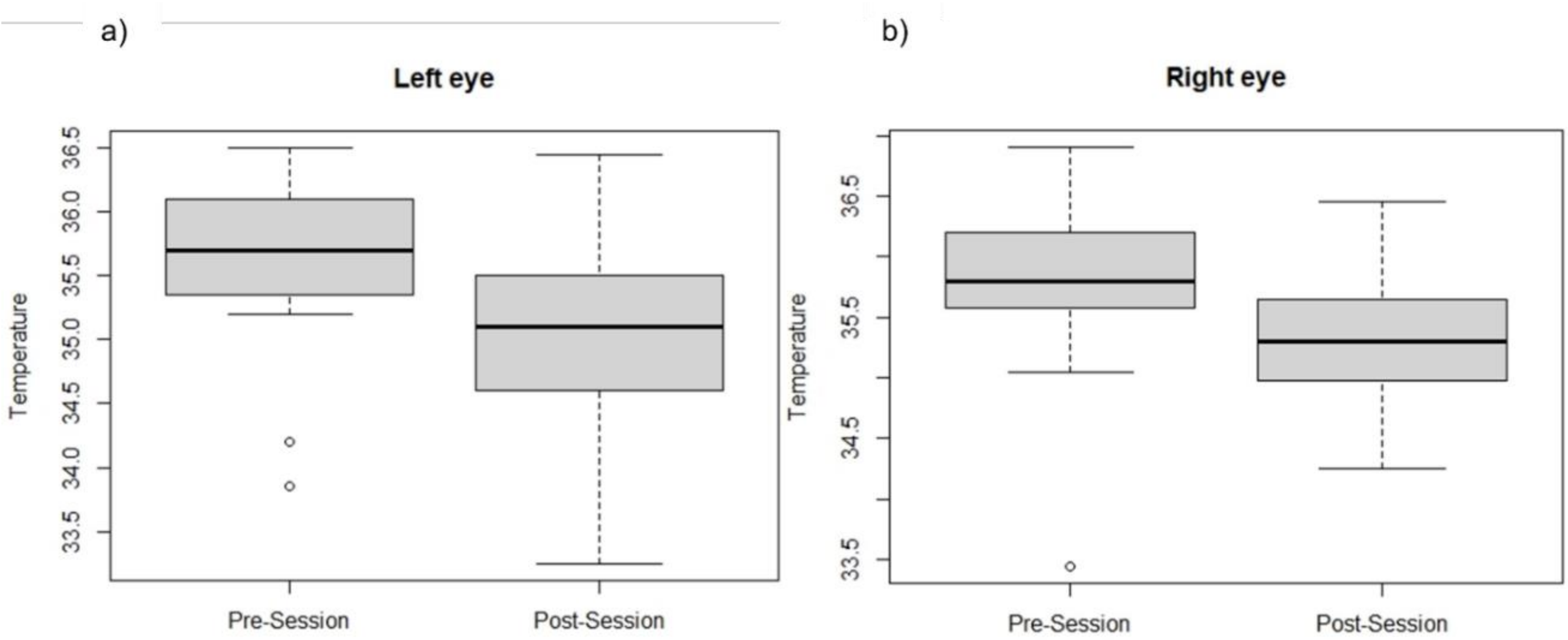

Figure 3.3 a) Boxplot showing the significant difference in left eye temperature of horses (n=19) from pre-session to post-session of the two-choice discrimination-reversal task. The boxes represent the first to the third quartile of data, with a bold line at the median value. The maximum and minimum values are indicated by the upper and lower whiskers, respectively. b) Boxplot showing the significant difference in right eye temperature of horses (n=19) from pre-session to post-session of the two-choice discrimination-reversal task. The boxes represent the first to the third quartile of data, with a bold line at the median value. The maximum and minimum values are indicated by the upper and lower whiskers, respectively.

3.2 Physiological predictors of learning performance

The results of the negative binomial GLM of best fit are given in Table 3.2. Performance indices ranged from 2.22 to 49.75 (mean + SD= 14.35 ± 15.28).

Left eye temperature change was a significant positive predictor of performance index (p <0.0001). Resting heart rate variability (RMSSD) was a significant predictor of performance index (p=0.009). RMSSD during learning was also a significant predictor of performance index (p=0.002).

Table 3.2. Table of results from the negative binomial generalised linear model. Significance level is coded as follows: p=0.05 = '·', p<0.05 = '*', p<0.01 = '**', p< 0.001 = '***'. Bold indicates significant predictors

Coefficient	**Estimate**	**Std. Error**	**Z value**	**p value**	**Significance**
Intercept	0.47	0.71	0.66	0.51	n/s
Left eye temperature change	**1.23**	**0.18**	**6.75**	**<0.001**	*******
Resting RMSSD	**0.01**	**0.004**	**2.59**	**0.009**	******
RMSSD	**0.02**	**0.005**	**3.15**	**0.002**	******

4. Discussion

Baseline blink rate did not predict performance in the reversal learning challenge. This was surprising, given the existing research linking tonic striatal dopamine levels and reward learning (humans: Berridge, 2007; rodents: Beeler et al., 2010; Flagel et al., 2011). However, there are some issues associated with measuring blink rate in horses, which could have confounded the results. Blink rate in horses is influenced by factors such as time of day (Barbato et al., 2000), exercise (Cherry et al., 2020), stress (Merkies et al., 2019; Mott et al., 2020) and attention (Cherry et al., 2020; Merkies et al., 2019). Therefore, true baseline blink rate is difficult to define, particularly in a reactive prey species that is susceptible to novelty effects (Krueger et al., 2014; Yngvesson et al., 2016). For these reasons, despite early research suggesting that SEBR during tasks may indicate phasic dopamine (Kirsty et al., 2015), baseline SEBR may not be a robust measure of tonic dopamine in this species under real-world conditions.

In addition, SEBR during reversal learning did not differ significantly from baseline, nor was blink rate during learning a significant predictor of reversal learning performance. This was a surprising result, given that in human studies blink rate is higher during reward learning due to phasic dopamine release (McGovern et al., 2020; Peckham & Johnson, 2016). This finding also disagrees with studies of attention and cognitive load, which have suggested that blink rate is significantly lower during a visual cognitive challenge due to increased attention (Chan & Chen, 2004; Chen & Epps, 2014; Jongkees & Colzato, 2016; Magliacano et al., 2020). Considering the similarities between the horse and human striatum (Hemmings et al., 2018), it was hypothesised that equine blinking behaviour would mirror that which is seen in human blink rate research (Jongkees & Colzato, 2016) but this was not observed.

As well as differences between testing under laboratory conditions for human research and applied settings in this case, part of the difference in findings may be related to methodological differences. The human literature observes blink rate in periods of between 12 and 60 seconds, far more concise than the three to five-minute period observed in this study. It may be that blink rate was generally reduced due to focus but had sudden increases due to phasic dopamine release which may be obscured by taking an average over the total session. With multiple presentations of the target in each minute, even minute-by-minute averages may not identify subtle changes in blinking. Additionally, the method of taking mean blink rate across training sessions may have blunted the resolution of blink rate as a potential proxy measure of striatal dopamine activity. It may be more appropriate to take measurements at specific points within the learning paradigm, relating to key and non-key periods of functional dopamine activation (McBride et al., 2017).

The average blink rate across a reversal learning task (as utilised here) may simply not be a sensitive enough measure to explain the interaction between blink rate and learning, yet it is the standard method for quantifying blink rate in animal studies. It may be more appropriate to calculate blink rate variability, i.e., a measure of variance in the successive temporal differences between each blink (Lenskiy & Paprocki, 2016). Fukuda & Matsunaga (1983) first suggested that the temporal distribution of blinks may be more salient than blinks per minute, following an observation that blink rate peaked immediately following a stimulus presentation and progressively decreased between stimuli in a discrimination task.

Taking an average, as done in the current chapter, would not detect such a change. Paprocki & Lenskiy (2017) argue for the use of blink rate variability in cognition research, as the temporal distribution of blinks distinguish spontaneous eye blinks from reflexive or voluntary eye blinks.

Blink rate variability has been successfully trialled as a measure of cognitive load and performance in a limited number of human studies (Gebrehiwot et al., 2016; Lenskiy & Paprocki, 2016; Paprocki & Lenskiy, 2017). Blink rate variability was higher during a memory recall task than at baseline in humans but lower during a reading task than at baseline (Lenskiy & Paprocki, 2016). This suggests that blink rate variability is a more sensitive measure of cognitive performance than blink rate average and that blink rate variability may be applied in scenarios where blink rate alone proves not to be a useful measure (Paprocki & Lenskiy, 2017). However, this is highly time-consuming to achieve using manual counts and the development of automated blink detection is warranted for this reason.

Increased eye temperature is recognised as a sympathetically mediated arousal response, caused by increased blood flow to the eyes (Bartolomé et al., 2013; Blessing, 2003). By redirecting blood flow away from the skin surface and towards the eyes, the sympathetic nervous system prepares the animal for a fight-or-flight response (Blessing, 2003). Increased eye temperature has been associated with emotional arousal in horses (Bartolomé et al., 2013; Butterfield et al., 2018; Fenner et al., 2016; Hall et al., 2014; Redaelli et al., 2019; Valera et al., 2012). Further, increased eye temperature as an indicator of arousal has been validated against heart rate variability (Ijichi et al., 2020). In the current study, subjects experienced a decrease in eye temperature in both the left and right eye over the course of reversal learning. A decrease in ET may suggest that blood flow is directed away from the eye (Blessing, 2003), indicating that subjects were possibly less aroused after the cognitive task compared to before and this is supported by reduced (non-significant) changes in HRV. Positive reinforcement was used for all training, the task was a free-choice paradigm and subjects were never punished for incorrect target selection and so this might be expected.

Left, but not right, eye temperature change during learning was a significant predictor of performance; an increase in temperature of the left eye was associated with a higher Performance Index in reversal learning. Since eye temperature changes are thought to be mediated by blood flow (Blessing, 2003), it is possible that there was increased blood flow to the left eye, but not to the right eye, in response to the reversal learning challenge. In the current study, stimuli were presented equally to the left and right of the horse, in a counterbalanced design. Therefore, it is unlikely that the effect on left eye temperature reflected the location of the target.

Instead, the association between left eye temperature changes and performance may be due to brain hemisphere lateralisation (Rogers, 2021). Hemispheric lateralisation is an asymmetry of the left and right brain functions and is advantageous as it can increase cognitive capacity by creating cognitive “space” (Rogers, 2021). There is a wealth of evidence across a variety of vertebrate and invertebrate species that this approach directly aids cognitive performance (Bisazza & Dadda, 2005; Dadda & Bisazza, 2006; Rogers, 2021). In particular, the right hemisphere is responsible for sustained background detection and assessment of potential threats in the environment (Robins & Rogers, 2006; Rogers, 2002;

Rogers & Andrew, 2002), while the left hemisphere deals with specific targeted responses to stimuli in the foreground (Robins & Rogers, 2006; Rogers, 2021). Therefore, the left hemisphere of the brain is responsible for selecting and directing appropriate responses towards specific stimuli (Andrew, 2009; Kimura, 1982; Rogers, 2021). This includes directing sustained responses to targets (Rogers, 2021), which is the basis of both discrimination and reversal learning. As such, it is proposed that during successful reversal learning, the left hemisphere of the brain was activated, resulting in increased blood flow to the left side of the horse's head, which indirectly increased the temperature of the left eye in horses with better performance.

Recent research suggests that the hypothalamus mediates motivation and cognition in feeding and associative learning (Burdakov & Peleg-Raibstein, 2020). The hypothalamus also shows lateralised responses and is positioned within the ventral diencephalon of the forebrain which is relatively close to the eye within the horse's brain (Beltran et al., 2022; De Lahunta et al., 2015). Left hypothalamus activation is consistent with an increase in left eye, but not right eye, temperature associated with performance in reversal learning. On the other hand, a key aspect of reversal learning in the current study was the food reward, which was only issued when subjects gave correct responses. Since the left hemisphere is also responsible for feeding behaviour (Robins & Rogers, 2006; Rogers, 2002, 2021), it would be expected that the left hemisphere would be activated by delivery of the food reward. Therefore, it may be that successful performance led to increased instances of food reward which increased left eye temperature, rather than being driven by cognitive processing of the targets. Further trials without food reward, or monitoring of eye temperature following food presentation, would be required to differentiate potential causes of left eye temperature increases. In addition, both hypotheses warrant further investigation since the proximity of the eye in horses to the brain is likely affected by the large nasal cavity and extensive network of sinuses (De Lahunta et al., 2015). Regardless of the cause of increased temperature, it is consistent with a lateralised hemispheric response associated with learning and observable using IRT.

Heart rate variability (HRV), as measured by RMSSD, was also a significant predictor of reversal learning performance. Subjects with higher Resting HRV (undisturbed in the home environment), indicative of lower baseline arousal, performed significantly better in the cognitive task than those with lower Resting HRV. Resting HRV has been associated with learning in the context of fear and safety conditioning in humans (Pappens et al., 2014; Wendt et al., 2015) and dogs (Bray et al., 2015). Taken with the findings of the current study, this suggests that resting arousal may be a useful indicator of potential learning performance.

The results of the current study also indicate that individuals with higher HRV during reversal learning performed better than those with low variability, which suggests that lower arousal has performance benefits. These results therefore are not in agreement with the conceptual framework offered by Starling et al. (2013) for use in horses and dogs, which suggests that the optimal condition for training to touch a target using positive reinforcement is moderate arousal, consistent with the Yerkes -Dodson Law (Yerkes & Dodson, 1908). It may be that in a flight species such as the horse, even moderate arousal inhibits attention to the task and cognitive processing due to demands from environmental noise overshadowing the required response (McLean, 2008). Alternatively, the welfare positive methods used to train in this study may mean that no subjects experienced high levels of arousal.

Taken together, the results of the current model suggest that Resting RMSSD, RMSSD during reversal learning and left eye temperature predict performance. A body of evidence has established that the autonomic nervous system is differentially affected by activity of the right and left hemispheres (Burtis et al., 2014; Hartikainen, 2021; Wittling et al., 1998). Evidence suggests that the right hemisphere controls sympathetic, while the left hemisphere controls parasympathetic cardiac activity (Rogers, 2010; Wittling et al., 1998). Additionally, the hypothalamus has a lateralised regulatory influence on the autonomic nervous system linking motivation, cognition and arousal, and potentially explaining the effects observed here (Burdakov & Peleg-Raibstein, 2020). As such, it is possible that activity of the left hemisphere influenced the apparent increase in RMSSD during reversal learning, observed in the current study.

Further, Resting RMSSD also predicted reversal learning performance, suggesting that increased parasympathetic tone at rest also interacts with hemispheric dominance during learning. This indicates that the relationship between cardiac activity and hemispheric lateralisation during learning may be bidirectional. As such, it is proposed that arousal, both at rest and during reversal learning, and left hemisphere activation interact together to predict learning performance. A possible explanation for this interaction is that parasympathetic activity works to down-regulate the neurotransmitter systems involved in vigilance (Oken et al., 2006), allowing for left hemisphere dominance. Individuals with increased parasympathetic tone (von Borell et al., 2007) have reduced demands for vigilance to potential threat (Dukas & Clark, 1995), therefore right hemisphere dominance becomes redundant (Hartikainen, 2021). This is adaptive for learning because it means that fewer resources are spent on arousal processing and vigilance. Indeed, Hartikainen (2021) reports that task performance is impaired when arousal processing demands are high. In the current study, physiological measures consistently evidenced that the cognitive task reduced arousal which is consistent with right hemisphere non-dominance. This may explain why the right eye temperature was not a predictor in the statistical model.

It would have been interesting to conduct multiple reversals to investigate these effects further, as has previously been done in horses (Fiske and Potter, 1979; Sappington et al., 1997; Martin et al., 2006) and other species such as rats (Mackintosh et al., 1968), passerine birds (Cauchoix et al., 2017) and fish (Lucon-Xiccato and Bisazza, 2014). However, it was unfortunately not practical to conduct multiple reversals within the investigative scope and time constraints of the current project, which was primarily interested in physiological responses as predictors of cognitive performance.

It is also important to note that physiological effects observed here may be caused by mental effort generally, rather than the specific learning paradigm used. The current study used a two-choice discrimination reversal paradigm as the experimental vehicle with which to investigate novel predictors of adaptive learning in horses. A study investigating physiological effects across different training paradigms would be required to determine whether reversal learning *per se* affects physiology in equine subjects.

5. Conclusions

SEBR did not predict learning performance, either at rest or during learning trials. I suggest that average SEBR is not sufficiently sensitive to have predictive validity in cognitive studies and that blink rate variability may offer a more appropriate measure. Therefore, SEBR variability will be incorporated into subsequent chapters. Performance was highest in subjects that showed lower arousal, measured by HRV, both at rest and during reversal learning. To the author's knowledge this is only the second study to provide data on how baseline arousal relates to learning performance in a non-human species (Bray et al. 2015).

Subjects who performed highest on the reversal learning task experienced an increase in left eye temperature and this was a significant predictor of performance. One speculative explanation is that the left eye temperature increased as a by-product of increased blood flow towards the left hemisphere of the brain. This is consistent with the left hemisphere being responsible for learning targeted responses to stimuli and feeding behaviour, both of which were integral aspects of the reversal learning task. This is the first observation of this phenomenon and, if a causal link can be demonstrated, provides a non-invasive, readily available proxy measure of hemispheric asymmetry in real-time. Subsequent chapters will be examined to determine if patterns predicted by hemispheric laterality are consistently observed and predictive of learning performance.

The association between resting arousal, arousal during learning and lateralisation of eye temperature observed here are intriguing. Horses in a low arousal state have fewer emotional processing demands, enabling left-hemisphere dominance, conducive to learning success. This hemispheric lateralisation may also mediate parasympathetic cardiac activity in a bidirectional relationship, allowing successful individuals to maintain lower arousal throughout training. Further, left hemisphere predominance is potentially observed here as higher left eye temperature, measured by IRT. Taken together, this Chapter provides a novel model of arousal and learning performance and tentatively demonstrates that arousal, both in the home and training environment, has critical importance for learning. Future work should seek to reveal more on equine cognition by conducting multiple reversals.

CHAPTER 4

WHOA, NO-GO: EVIDENCE CONSISTENT WITH MODEL-BASED LEARNING IN HORSES

1. Introduction

In Chapter 3, it was established that physiological measures of arousal can predict learning performance in horses completing a two-choice discrimination-reversal (reversal) task. Further, all horses completed the reversal task within a few short training sessions, indicating that this species has a reasonably strong ability for reversal learning. Good reversal performance indicates cognitive flexibility, which is important for equestrian training and activities. In the current chapter, it was originally intended that the same physiological measures would be investigated as possible predictors of cognitive control, another important skill for equestrian training (McLean and Christensen, 2017). However, an interesting observation during the Go/No-Go experiment prompted the current investigation.

It is thought horses lack the prospection and brain architecture which may be required for goal-directed, reflective model-based learning which considers future states. However, as will be examined in this chapter, it may be possible that horses use model-based strategies when faced with reward-based cognitive tasks. This chapter will begin by outlining the processes involved in model-based and model-free learning. Following this, the conceptualisation of the current investigation will be described and explained within the context of the thesis.

1.1 Model-free and model-based learning

Instrumental learning can be acquired through both model-free and model-based learning. Model-free learning is a relatively simple computation based on accrued trial-and-error learning that forms habits. As such, changes in contingencies and environmental conditions can only be responded to slowly, as a new “cache” of trials must be built (Dayan and Berridge, 2014). The accruing cache adjusts the reward prediction error which signals the value achieved by a given action to maximise future expected rewards (Sutton and Barto, 2018). This is achieved by comparing differences between rewards actually received and those expected, based on previous experience (Schultz, 2016). Simple tasks can be reliably learnt, given sufficient opportunities, by building associations between stimuli and their outcomes. For example, an animal may reflexively touch a target because they have been conditioned through repeated exposure to associate it with a positive outcome such as food. The target elicits an urge to make contact with it, and no more complex processing of what the target signifies. Therefore, there is no need for higher order executive function. By contrast, a model-based approach involves prospective cognition (Dayan and Berridge, 2014) which refers to the ability to, consciously or unconsciously weigh possible future states and the likely outcomes of various responses so that optimal responses can be selected. It therefore allows an individual to select the response most suitable to the current situation out of all potential options.

The model refers to this cognitive “map” of possible outcomes and is built using the state prediction error (SPE; Gläscher et al., 2010). SPE registers violated expectancies by comparing discrepancies between the current cognitive model (cognitive map) and the current observed state (reality; Gläscher et al., 2010). Whilst model-based is goal-directed and reflective, model-free cognition is habitual and reflexive. The former is considered more computationally demanding than the latter which is counterbalanced by its value in promoting flexible adaptive responses in dynamic situations (Huang et al., 2020).

1.2 Investigating model-based learning in a Go/No-Go paradigm

As outlined in the General Introduction (*Section 6. Aims and Objectives of the Thesis),* this thesis utilised several learning paradigms to test the relationship between dopamine, arousal and learning across a series of increasingly complex cognitive tasks. Two-Choice Discrimination-Reversal (Reversal) was tested in Chapter 3, and the next cognitive skill to be tested was inhibitory control, using a Go/No-Go task. It was originally intended that the inhibitory control investigation would therefore follow Reversal, however an interesting opportunity to conduct an additional experiment arose, and this will be explained here.

In Chapter 3, subjects were positively reinforced with food rewards to touch a target (Evans et al, 2024). In the subsequent Go/No-Go inhibitory control test subjects were rewarded for touching the target unless a new light cue was on, in which it was a No-Go contingency, and no reward was offered for touching. The aim of Chapter 5 was originally to investigate individual differences in inhibitory control and cue dependency, using physiological predictors. However, after three training sessions, all subjects still failed to reach criterion in this task which had previously been sufficient for the cohort to learn a two-choice discrimination reversal task (Evans et al., 2024). In addition, this species should be able to inhibit previously learned responses (Brucks et al., 2022). Instead, subjects responded indiscriminately and touched the target, regardless of the light cue and reward delivery. This is known as error of emission where a response is given but is incorrect (as opposed to error of omission, where no response is given).

Three explanations for this poor performance are presented in order of increasing cognitive complexity following Lloyd Morgan's Canon Dwyer and Burgess, 2011). First, horses lack the ability to complete Go/No-Go tasks due to poor behavioural inhibition or executive function limitations. However, horses have evidenced Go/No-Go ability, albeit in a spatial task (Hintze et al., 2018) and demonstrate inhibitory control in a delayed-gratification task (Murphy et al., 2009). Second, it may be that horses can learn Go/No-Go paradigms, but the stimulus used was not available to them. This was unlikely as the No-Go stimulus was a light presented within the right field of vision (Murphy et al., 2009) and was distinctly off and on. In addition, they may be able to learn this paradigm but needed longer to do so. Finally, subjects developed a model of the task and were using a cost-benefit analysis. Touching the target often results in reward and there is minimal energetic cost to indiscriminate responding, whilst contingent responding requires higher computational cost (Falkenstein et al., 1999). Therefore, it may be beneficial to touch the target regardless of the contingency. However, prospective cognition and executive function required for this approach are not thought to be well developed in horses due to their limited prefrontal cortex (de Lahunta et al., 2015).

To determine whether subjects might be using a cost-benefit analysis, an additional experiment was conceptualised and included as the current Chapter (4). The aim was to determine whether horses had developed a model of the possible outcomes, allowing them to use model-based learning to respond optimally, using the least effort to gain the most reward. A cost was introduced for errors of emission and these errors monitored and compared to predictions (Figure 4.1).

If subjects had no model of the task, the addition of the cost may have helped them understand the task and thus improve performance gradually, by using model-free operant conditioning. This is because model-free learning requires building a new cache of experiences of the cost, to modify behaviour. If so, a steady reduction in errors would be expected following introduction of the cost (Figure 4.1a; Dayan, 2009). If the Go/No-Go stimulus was not available or salient to the subjects, no reduction in errors would be expected (Figure 4.1b). This is because adding the cost would not help them perceive the light cue, thus behaviour would not be modified.

If horses cannot inhibit responses in a Go/No-Go test, again, no reduction in errors would occurs after introducing the cost (Figure 4.1b). If subjects developed a model of the task and were making errors of emission due to using model-based learning based on the low cost of the error, a sudden reduction in errors following the introduction of a cost would be observed (Figure 4.1c). This is because the cost chosen removed the opportunity to gain reward, therefore the most efficient approach to gain reward was to touch in Go contingencies and inhibit under No-Go. To achieve this flexible response to a dynamic situation, subjects would need to adaptively compute the ideal response by searching simulated outcomes (Huang et al., 2020). This would be expected to be relatively stable over the subsequent three sessions as a result of latent learning (Tolman, 1948). Therefore, errors would remain low across sessions 4, 5 and 6.

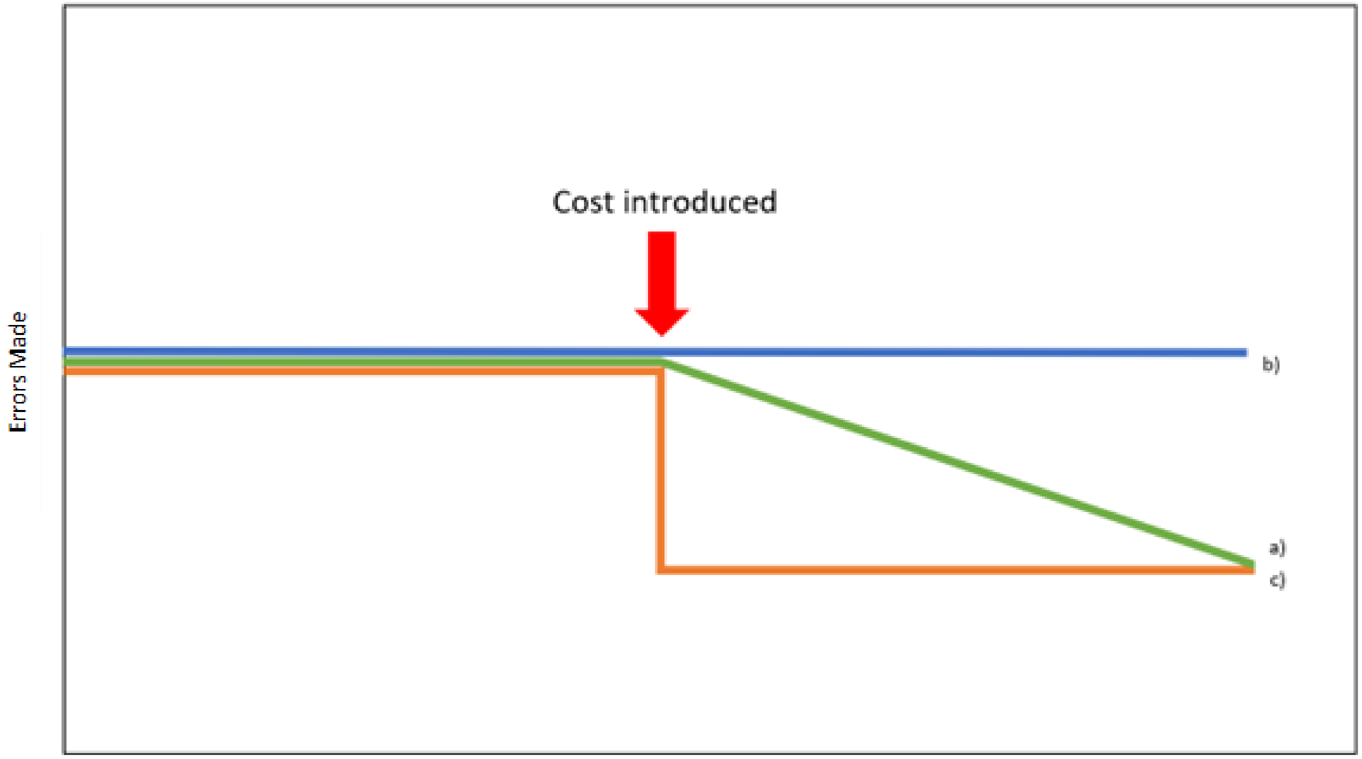

Figure 4.1. Expected patterns of responding if subjects: a) did not understand the task but the addition of negative punishment improved learning; b) did not understand the task, even after negative punishment was introduced *or* the no-go signal was not available; c) were using a cost/benefit approach and had successfully built a model of the task.

2. Methods

Ethics statement and Subject information is presented in General Methods

2.1 Pre-conditioning

Go/No-Go paradigms test inhibition of a previously conditioned responses, rather than the ability to learn a cue dependent response from the outset to determine when a target should be touched. To ensure that Go/No-Go tested inhibition of a conditioned response, subjects needed to be pre-conditioned through target training to touch a target. Pre-conditioning was applied to shape a nose touch response to the cue card target as per General Methods Chapter. The motor requirements of this touch response were to touch any part of the target with the nose or muzzle.

All subjects reached learning criteria of 80% correct responses in three consecutive sessions (1 session=10 trials; Neave et al., 2013), indicating that they were successfully conditioned to touch a particular target and not simply any item placed in front of them.

2.2 Go/No-Go Paradigm

Only the conditioned (Reversed) target for each subject from the previous study (Chapter 3) was presented in Go/No-Go testing as the intent was to measure inhibitory control of a conditioned response, rather than discrimination between two targets. Since only a single target was now being presented that target was now positioned centrally in front of the horse. Sessions lasted approximately five minutes, consisting of 28 Go contingencies and 12 No-Go contingencies (Neave et al., 2013). Within each contingency subjects could offer multiple responses, limited only by the length of time the contingency lasted. Therefore, although a total of 40 continencies were offered, error rates could be much higher than this if multiple errors of emission (touching the target when inappropriate to do so, such as during a No-Go trial) were made within the timeframe. All subjects completed the same number of sessions (see *section 2.5*).

No-Go was signalled by a light cue, consisting of a battery-powered LED cyclists' helmet torch light. The light was attached to an adjustable, elasticated helmet band, worn by the trainer in the central upper abdomen area. The trainer (LE) was 151 cm in height, so the position of the light was such that it was visible to the horses, but not shining directly into their eyes. The light was a bright white LED, as this is a shade known to be visible to horses (Roth et al., 2007). In Go, the light was switched off, as this was consistent with pre-conditioning of the touch response, where no light cue was present. The light was manually operated using an on/off switch on the top edge of the LED casing.

The No-Go load was 30% which is sufficient to maintain motivation (Neave et al., 2013). After two to three Go contingencies No-Go was initiated and maintained for between five to twenty seconds (*a priori* selected using a random number generator), preventing subjects predicting when the Go contingency would restart and ensured that it was the light itself that signalled the No-Go contingency and not predictable intervals. Touches of the target under "Go" resulted in a whistle sound and reward (+R). Responses under No-Go depended on the experimental condition (see section 2.5).

2.3 Experimental Design

The experiment was a within-individual design, with all subjects completing both treatments and acting as their own control. Subjects completed three sessions (one session per week) with only positive reinforcement (+R). Under No-Go, the target was presented and maintained until the condition ended. Should a subject touch the target in this time this was not rewarded and the target remained in position. Each touch was an error of emission. Following a fallow period of three weeks, in which the design for this additional chapter was conceived and approved by the NTU ethical committee, subjects completed three further sessions (one session per week) with positive reinforcement plus the introduction of negative punishment which represented a “cost” (+R/-P). Negative punishment is the removal (-) of a desirable stimulus to reduce the likelihood of the animal offering that response again (Punishment).

Errors of emission resulted in a 10-second “time-out” where no rewards could be earned in addition to the already scheduled five to twenty second No-Go contingency. The trainer stepped back out of the reach of subjects, lowered the target and looked down. At the end of the time-out period, they stepped back in range and re-presented the target to complete that current No-Go continency. Thus time-outs extended the time subjects must wait for the next opportunity to earn reward but did not limit how many errors could be made within the No-Go contingency. For example, upon completion of the 10-second time-out, the handler may present the target again only for the subject to immediately make another error of emission and trigger a further time out period. Time-outs extended total session length, dependent on the performance of the individual, but 28 Go and 12 No-Go contingencies were always available. Any reduction in errors observed during sessions 4-6 was therefore due to improved behavioural inhibition and not time-outs reducing the opportunity to make errors.

2.4 Learning Performance

A Canon Legria video camera (Canon Inc., Tokyo, Japan) and tripod were used to record sessions for retrospective analysis of errors. Touches of the target under No-Go contingencies were recorded as errors of emission, defined as a given response which is incorrect. The distinction between errors of emission (making an incorrect touch response when inappropriate, i.e. during a No-Go trial) and errors of omission (failing to give a response when required, i.e. during a Go trial), was important. A horse with poor inhibitory control would be expected to make errors of emission: touching the target when inappropriate to do so, such as during a No-Go trial. A horse omitting to give a response (error of omission), while also indicative of poor performance, would not necessarily suggest poor inhibitory control, but rather a lack of understanding of the task and/or unwillingness to participate. Performance was scored by the trainer (LE) and validated by a second hypothesis-blind rater with excellent agreement (weighted Cohen's kappa = 0.97).

2.5 Statistics

Data were statistically analysed in R version 4.2.1 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria). Data were assessed for normality using a Shapiro-Wilks test. Wilcoxon tests were used throughout to determine differences in Errors of Emission between consecutive sessions. P-values reported are adjusted using Bonferroni correction.

3. Results

No significant differences were seen between sessions 1-3 (+R) and sessions 4-6 (+R/-P) (Table 4.1). A significant reduction in errors of emission was observed between treatments (*sessions 3 and 4)* (Table 4.1; Figure 4.2).

Table 4.1. Tests of differences for errors of emission made between consecutive sessions (n=20). Treatments were positive reinforcement (+R) and positive reinforcement plus negative punishment (+R/-P). Significance level is coded as follows: p>0.05= ‘n/s’, p=0.05 = ‘·’, p<0.05 = ‘*’, p<0.01 = ‘**’, p< 0.001 = ‘***’. Bold indicates significant differences.

Sessions	Treatment	Median (IQR)	Wilcoxon V	p value	Significance
1	+R	12.5 (11.75)	72.5	0.86	n/s
2	+R	14.5 (20.5)			
2	+R	14.5 (20.5)	52.5	0.86	n/s
3	+R	16 (16)			
3	**+R**	**16 (16)**	**206.5**	**0.02**	*****
4	**+R/-P**	**5.5 (8.25)**			
4	+R/-P	5.5 (8.25)	95.5	1	n/s
5	+R/-P	7 (6.5)			
5	+R/-P	7 (6.5)	72	1	n/s
6	+R/-P	6.5 (7.5)			

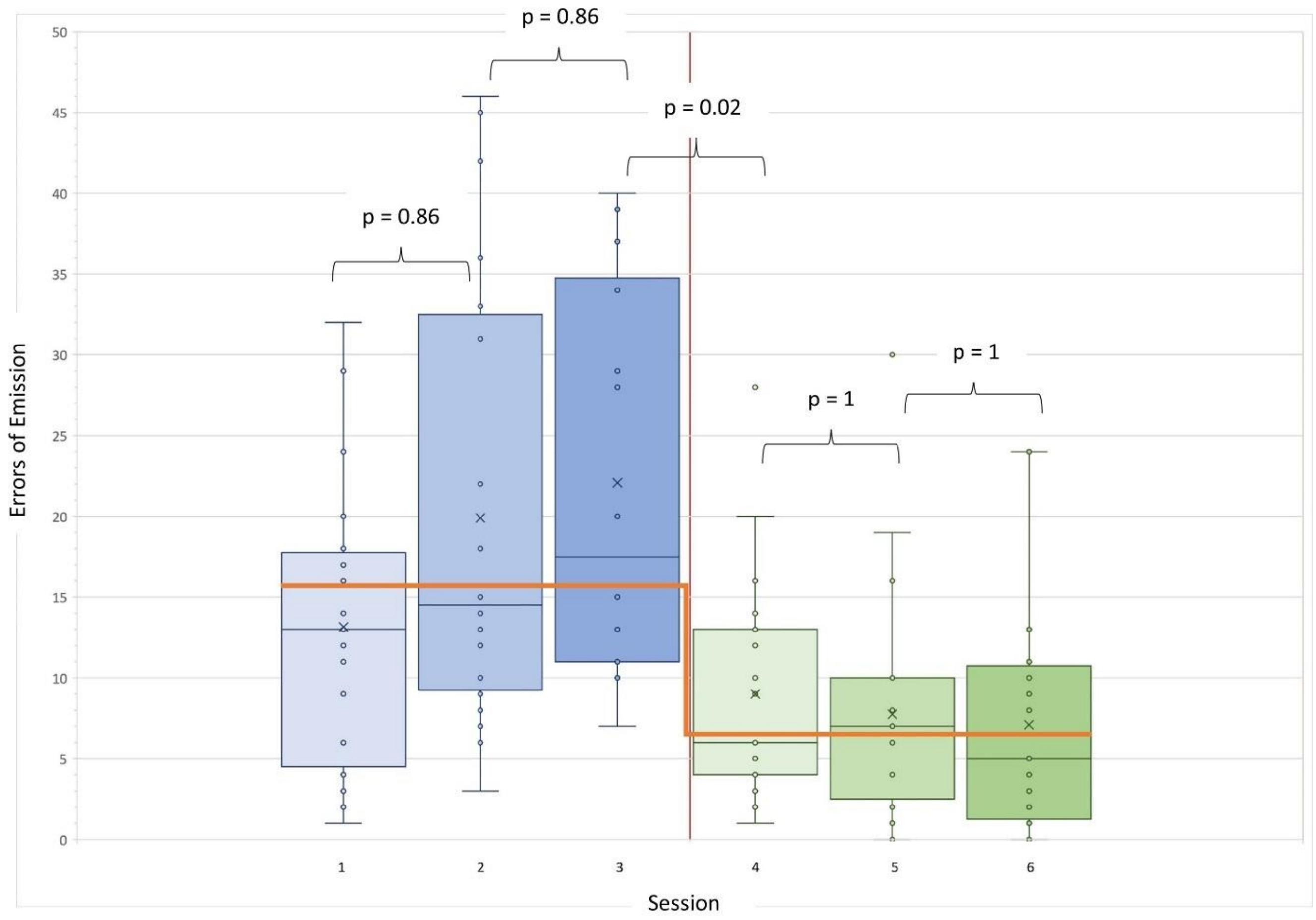

Figure 4.2. Errors of emission for each session. In sessions 1-3 (blue) only positive reinforcement (+R) is used. In sessions 4-6 (green) a "cost" for errors is suddenly introduced using negative punishment (+R/-P), resulting in an immediate, significant reduction in errors (p=0.02, n=20). The predicted pattern of errors for

model-based approach (Fig. 1) is superimposed for comparison. The boxes represent the first to the third quartile of data, with a bold line at the median value. The maximum and minimum values are indicated by the upper and lower whiskers, respectively.

4. Discussion

The results of this chapter are consistent with model-based learning. It appears that in sessions one to three, horses developed a model of the task based on a cost-benefit analysis of outcomes. Indiscriminate touch responses did not result in loss and sometimes resulted in reward. The level of reward was therefore the same regardless of whether horses followed the rules, requiring mental energy (Falkenstein et al., 1999), or responded indiscriminately to the target. The introduction of a time-out period for errors of emission at session four appears to outweigh the mental cost of following the rules, altering the horses' approach in favour of a cost-avoidant model-based approach. The evidence for this is that from session three (+R) to session four (+R/-P), there was a significant, immediate reduction in errors.

By contrast, model-free learning requires building a new cache of experiences of the cost to modify behaviour which would result in a steady reduction in errors. Therefore, the response observed here does not appear to be a subsequent learning effect, as the reduction in errors was immediate without the opportunity to cache new information (Dayan, 2009). According to Lloyd Morgan's Canon (Morgan, 1903), animal behaviour should only be considered evidence of higher cognitive processing if it cannot be explained by simpler mechanisms. If subjects had no model of the task, no reduction in errors would have been observed in session four but a slow reduction may have been observed over three sessions if the cost improved their understanding of the task. If horses cannot complete Go/No-Go tasks, or the light was not salient, no reduction would be seen in any session. Therefore, the explanation consistent with the observed reduction in errors was that horses used a model-based cost-benefit analysis.

There are other suggested reasons why performance improved at session four, and it is important to acknowledge these. First, seasonal effects might have an influence on motivation in animals (Zucker, 1983; Greggor et al., 2016). There was a three-week period between sessions three and four due to the logistical challenge of conceptualising, planning and data collection for the new treatment (+R/-P), which was not originally part of this thesis. As such, it is possible that within that three-week period, horses experienced seasonal changes. However, this is unlikely given that the data collection period still took place in summer and management was not changed during this time. Additionally, it would be expected that any changes would occur gradually, whereas there was an immediate, highly significant decrease in errors between sessions three and four (Figure 4.2). Seasonal changes in cognition and motivation have not been evidenced in horses specifically. In addition, there was a two-week gap between sessions one and three, and no improvement in performance occurred in that time, when there was ample opportunity to rest and consolidate learning.

A second, important point to acknowledge is the Clever Hans effect (Henschel, 2016). It is possible that the subjects used subtle, unintended cues from the trainer which influenced their responses. However, though it is never possible to rule out a Clever Hans effect when a human is present, such unintended cues would have been present in sessions one to three, where subjects did not demonstrate that they understood the task. Therefore, Clever Hans cannot explain the sudden improvement in performance between sessions three and four. The only cue that changed was intentional, in that the trainer stepped back and gave a time out. However, if horses found the task difficult, but the time-out cost aided their learning, a gradual improvement in performance over the course of sessions four, five and six would be expected, as this would reflect learning and caching responses.

Horses may have used Clever Hans in sessions one to three but chose a low-cost indiscriminate responding approach. When this approach was no longer effective due to cost introduced, they switched to cue-dependent discriminate responding. The same horses with the same targets and experimenter exhibited the ability to inhibit the initial learned discrimination in order to reverse in the previous chapter. They achieved this in three sessions, demonstrating they can do this. This is why I felt an alternative explanation for their failure to inhibit responses during the Go/No-Go task should be investigated in the current chapter.

An additional factor to acknowledge is that the sample of horses investigated here were unique. Subjects were University owned horses, who regularly experienced novel experimental settings and may experience confused signals from multiple riders, though the skill level of such riders is likely to be higher than in a general riding school, owing to the age and entry requirements of the university. These factors may contribute to the responses seen here; therefore these results should be generalised with caution. Further research is required, using different horse populations, to determine the repeatability of such results.

It would have been interesting to include a further three training sessions after the +R/-P phase, returning to +R only. This would have allowed for further analysis of responses to determine whether horses would return to indiscriminate responding, as per the model. However, this was an operant task, which meant that subjects would have to make an error of emission in order to discover that the negative punishment had been removed and there was no longer cost for errors.

After three sessions with cost and such a significant improvement in performance (Figure 4.2), it is possible that horses would not have trialled an incorrect response (error of emission). Therefore, many of the sample may not discover that the cost is removed. The best performers would be least likely to make an error in a final +R only phase, therefore these individuals would not have the opportunity to demonstrate strategic model-based responding.

Additionally, this chapter was not included within the scope of the original thesis plan, it was an additional investigation which came about opportunistically and makes an interesting contribution to the thesis. Therefore, it was not possible to digress from the original thesis plan for further +R sessions. Once it had been established that subjects could complete the Go/No-Go task, it was important to progress to Chapter 5 for the physiological analysis, as per the original scope.

It could be suggested that the negative punishment introduced at session four may have had the effect of 'one trial learning', owing to the negative experience of punishment. However, the seemingly mild nature of the negative punisher used (targets removed for ten seconds) makes this theory unlikely to apply here. Temporal cognition in horses is not fully understood; it is possible that ten seconds feels like a long time to a horse. However, one trial learning is traditionally seen in scenarios where a pain-inducing positive punishment is used, such as electric shock (Drew, 1938; Hudson, 1950). The idea is that if a punisher is powerful enough, animals can learn (usually avoidance) in only a single trial. This is evolutionarily adaptive; it is too costly to perform a behavioural response more than once if the outcome is so extremely punishing, particularly if pain is involved.

Though it is clearly inappropriate to conflate the negative punishment used here with such extreme forms of positive punishment, it would be relatively easy to test whether the one trial learning theory is at play here. Future work could repeat this experiment but omitting sessions 1-3 to prevent horses from building up an understanding of the light cue. A naïve group of horses could be tested only on sessions 4-6 (+R/-P). If the one trial learning theory applies here, it would be expected that naïve horses would follow the light cue immediately from the first time they are punished (ten second time out) for making an error of emission (touching during a No-Go contingency). This would indicate that the ten second removal of the targets is so punishing that horses immediately learn the task rules. I argue that this would be unlikely to occur, as the punisher is seemingly so mild and horses generally require repetitions to build a cache of responses and outcomes from which to learn.

Basic cost-benefit analysis has been observed in a range of species (Georgiev et al., 2013; Burtsev and Turchin, 2006), including snails (Gillette et al., 2000), bats (Wilkinson, 1992) and starlings (Wiebe, 2003). As a simple strategy, starlings use cost-benefit analysis to decide where to spend the most time and energy on foraging, based on predicted yield (Dall and Cuthill, 1997). More complex strategies, such as creating shortcuts, have been observed in dogs (Chapuis and Varlet, 1987). Macaque monkeys have demonstrated highly complex strategy use in a three-armed bandit task, where the reward values of three different behavioural options fluctuated (Walton et al., 2010).

It is unclear what selection pressures may be driving the behavioural response to the task in the current study. Free-ranging horses may experience water shortages and would therefore need to understand the environment they live in. Water is of higher value than the mental cost of developing a model of the environment, so it’s possible this kind of cognition is involved. The hippocampus is thought to be instrumental to building the horse’s spatial model of its environment, so it’s possible that a similar tool was utilised here to build a model of the task.

Model-based learning does not always require the development of a model (i.e. not the neural circuitry: the neural substrate circuitry is already there in the brains of animals in all species-specific behaviours across the taxa). The circuitry just needs to be populated with learning. For example, swallows have model-based learning neural circuits enabling the building of mud nests, and they populate this neural circuitry with learned experience. Model-based learning may not be associated with deliberate reasoning (Delius and Siemann, 1998), however it does rely on the learner’s mental model of the environment (Wunderlich et al., 2012; Huang et al., 2020; Huys et al., 2014). This may be a relatively complex cognitive skill requiring good executive function, particularly for an animal with an underdeveloped prefrontal cortex (PFC), such as the horse (Hausberger et al., 2019; Schmidt et al., 2019). The PFC is thought to be instrumental in model-based learning (Mcdannald et al., 2012; Tsujimoto et al., 2011) in humans and likely other primates and cetaceans, due to its role in processing cause-and-effect relations between choices and outcomes (Bunge et al., 2003; Tsujimoto et al., 2011). However, other brain structures more recently implicated in model-based learning are available to the horse, including the hippocampus (Schmidt et al., 2019; Vikblagh et al., 2019; Jacobs and Schenk, 2003).

The hippocampus creates a cognitive map (Jacobs and Schenk, 2003; O’Keefe and Nadel, 1979), which allows an animal to form a model of their environment. In model-based learning, a map of events and environmental stimuli is created, allowing the learner to prospectively evaluate the consequences of their actions (Dayan and Berridge, 2014). The horse has a particularly well-developed hippocampus (Schmidt et al., 2019), contributing to their adeptness for spatial learning (McLean, 2004; Baragli et al., 2011; Hanggi, 2010; Murphy, 2009). The horse's demonstrable ability to form spatial models (McLean, 2004; Baragli et al., 2011; Hanggi, 2010; Murphy, 2009) may have relevance to the apparent model-based approach observed in the current study. However, the mechanisms underlying the use of model-based learning seen here are unclear without the use of in vivo brain imaging, not yet developed in horses (Walton et al., 2010; Schmidt et al., 2019; Tsujimoto et al., 2011).

The results presented here indicate that horses may have higher-order cognitive capabilities than have been previously demonstrated (Hanggi, 2005). Higher order functions, also referred to as executive functions, allow animals to optimise their response to novel circumstances. The horse's ability for conditioned stimulus-response learning (Hanggi, 2005; Brubaker and Udell, 2016; Murphy and Arkins, 2007) and spatial memory (McLean, 2004; Baragli et al., 2011; Hanggi, 2010; Murphy, 2009) has been well evidenced. However, the results of the current chapter suggest that horses may be capable of certain higher-order processes.

Concept learning appears to have been observed in a small number of equine studies to date (Hanggi, 2003; Uller and Lewis, 2009; Gabor and Gerken, 2012). For example, horses were able to select novel, two-and-three-dimensional objects based on the concept of relative size difference (Hanggi, 2003). In a separate study, horses repeatedly demonstrated an ability to select the greater of two quantities of apples, indicating a basic understanding of relative quantity difference (Uller and Lewis, 2009). If horses can form concepts, it is possible that conceptualisation helps them to develop models during learning, as the results of the current study suggest.

A recent experiment found that horses were able to use self-control to wait for higher value rewards (Brucks et al., 2022). This suggests that horses may consider consequences, including costs and benefits of their behaviour. Recently, it has even been suggested that horses may have the ability to use tools to help them access food, aid social interactions and for comfort (Krueger et al., 2022). Though this requires further investigation, it indicates a possible sense of cause-and-effect, and even prospective planning (Krueger et al., 2022; Osman et al., 2014). The research to date has identified behaviours in horses which point towards some higher-order cognition (Hanggi, 2003; Uller and lewis, 2009; Brucks et al., 2022; Krueger et al., 2022). The current study has experimentally tested this proposed potential for horses to use prospective planning to achieve a goal (which is here referred to as strategy). It is worth noting that in the current study, considerable individual variation in errors was observed in all sessions indicating this approach was not consistently demonstrated by all subjects. However, though some individuals reduced their errors from session three to four more than others, all but one individual reduced errors in response to the introduction of the cost suggesting a remarkably prevalent ability within the group to do so. Variation in model-based learning use across individuals and the reasons for this are intriguing but beyond the scope of the current thesis. However, the data does indicate a species-level capability for model-based learning.

It is important that the results of this study are not used to excuse harsh punishment of horses on the basis that they are culpable, deceitful or capable of morality (knowing right from wrong). This would be an incorrect interpretation of these results; there is no data here to suggest that horses possess the sense of self required to behave in a way which could be considered deliberately ‘naughty’ or devious. This chapter is an observation of an interesting response worthy of further investigation but is not on its own enough to make any recommendations or to consider modifying current evidence-based methods of training based on sound learning theory. The results presented here are novel and interesting, but this was an opportunistic study based on an observation about horses’ responses to the following chapter. Further research is required to understand the underlying brain mechanisms and selection pressures which may be driving the behaviour observed here.

5. Conclusions

This chapter provides novel evidence consistent with model-based strategic decision making in horses. Subjects rapidly altered their responses in a Go/No-Go paradigm, shifting from indiscriminate responding with high rates of errors of emission to conservative responding with few errors when a cost was introduced. This is not consistent with the counter arguments that horses cannot complete Go/No-Go tasks, cannot see the light stimuli or needed further trials to cache and consolidate their learning. To the author’s knowledge, this is the first evidence indicating higher order, executive function in horses, a species thought to possess only moderate cognitive capabilities. In addition, subjects were successfully trained to correctly respond to the Go/No-Go stimuli required for Chapter 5.

CHAPTER 5

STANDING IN THE WAY OF CONTROL: PHYSIOLOGICAL PREDICTORS OF PERFORMANCE IN A GO/NO-GO PARADIGM

1. Introduction

In Chapter 3, it was demonstrated that novel physiological measures could predict performance on a two-choice discrimination-reversal task (Reversal). Reversal requires cognitive flexibility, the ability to alter behavioural responses when reward-related contingencies change (Izquierdo et al., 2017). Previously, it was believed that Reversal tasks measure response inhibition, as the subject is required to stop offering a learned response, in favour of a newly rewarded response (Jones & Mishkin, 1972). However, this theory has since been revised, based on evidence from a variety of species suggesting that Reversal and inhibitory control have different underlying brain mechanisms (Chudasama et al., 2006; Izquierdo et al., 2017; Izquierdo & Jentsch, 2012; Jentsch et al., 2014). As such, this Chapter investigated whether the same physiological markers that predicted performance in the Reversal task can be used to predict performance in a task specifically aimed at testing inhibitory control.

1.1 Inhibitory control

Inhibitory control is a function of cognitive control, defined as the action of ignoring distracting or emotion-inducing stimuli, to maintain task focus and increase the likelihood of successful outcomes (Neill et al., 1995). Inhibitory control has been studied using Go/No-Go learning paradigms (Gomez et al., 2007; Rubia et al., 2001). Go/No-Go is a learning paradigm suitable for a range of species which requires an individual to give a motor response to a specified ‘Go’ cue and inhibit this response in the presence of a specified ‘No-Go’ cue (Trommer et al., 1988).

Success in a Go/No-Go task relies on effective cognitive control to maintain task focus, even where there is competition between possible responses, in order to attain a specific goal or reward (Gunther & Pérez-Edgar, 2021). Unlike the Reversal task, the Go/No-Go task provides the additional challenge of inhibiting the correct response until it is the appropriate moment to offer it, contingent on an additional cue. Building on Chapter 3, the current study aimed to investigate whether physiological predictors of Reversal learning performance also predict performance in a Go/No-Go task.

Spontaneous eye blink rate (SEBR) may relate to performance in an inhibitory control task in two possible ways via associations with cognitive flexibility and attention. SEBR is thought to be a proxy indicator of striatal dopamine activity, a neurotransmitter involved in learning and response inhibition (Colzato et al., 2009; Gallo et al., 2022; Gunther & Pérez-Edgar, 2021). The association between blink rate and dopamine was observed in patients with Schizophrenia and Parkinson's disease, among other conditions characterised by dopamine disfunction (Chan & Chen, 2004; Grace, 1991; Sandyk, 1990). These observations were supported by pharmacological studies, which found that dopamine agonist drugs increase SEBR, while dopamine antagonist drugs decrease SEBR (Blin et al., 1990; Kleven & Koek, 1996).

Individuals with high SEBR have been shown to perform well in cognitive flexibility tasks, such as Reversal (Izquierdo et al., 2006; Jongkees & Colzato, 2016; Morita et al., 2016; Van Slooten et al., 2017), though this was not observed within this study population in Chapter 3. However, high SEBR predicts poor inhibitory control performance, due to high distractibility as a byproduct of flexibility (Dreisbach et al., 2005; Jongkees & Colzato, 2016). Alternatively, blink rate has also been associated with attention and cognitive load (Chan & Chen, 2004; Chen & Epps, 2014; Daza et al., 2020a; Ledger, 2013). It has been reported that blink rate decreases during periods of high attention, as an adaptive function to maximise information gathering potential (Daza et al., 2020b; Oh et al., 2012).

1.2 Blink rate variability

In Chapter 3, blink rate was not a significant predictor of Reversal performance, and it was proposed the blink rate variability may be a more valid measure of cognitive performance. This is because blink rate uses an average of the number of blinks per minute (Colzato et al., 2009; Fukuda & Matsunaga, 1983; Karson, 1983; Maffei & Angrilli, 2018; Merkies et al., 2019), which may not be sensitive enough to account for the observed pattern of blinking that occurs during high cognitive load. For example, it has been observed that in humans, blink rate decreases significantly during periods of focus (Lenskiy & Paprocki, 2016), followed by rapid serial blinks at the peak of cognitive load (Nomura & Maruno, 2019). Therefore, blink rate variability has been proposed as an alternative method of analysing blink patterns during learning (Gebrehiwot et al., 2017; Lenskiy & Paprocki, 2016; Paprocki & Lenskiy, 2017). Higher blink rate variability indicates fluctuation between periods of attention and concentration (characterised by reduced blinking; Lenskiy and Paprocki, 2016), and periods of reward anticipation and acquisition (characterised by increased blinking in clusters; Nomura and Maruno, 2019).

1.3 Heart rate variability and inhibitory control

Heart rate variability (HRV), indicative of lower arousal, has been positively associated with cognitive performance across a range of cognitive tasks (Forte et al., 2019). HRV has been associated specifically with inhibitory control performance (Gillie et al., 2013; Moretta et al., 2019; Ottaviani et al., 2021). Ottaviani et al. (2019) describe the Neurovisceral Integration Model

(NIM) of the relationship between HRV and inhibitory control. According to NIM, the vagus nerve mediates a heart-brain connection during inhibitory control. The vagus nerve is one of the most influential nerves in the autonomic nervous system, mostly responsible for parasympathetic responses (Kolman et al., 1975; 1976).

The vagus nerve also appears to mediate inhibitory control by linking structures, such as the prefrontal cortex, in the brain to the heart (Hilgarter et al., 2021). HRV reflects vagus nerve activity and has been associated with inhibitory control in the prefrontal cortex (Ottaviani et al., 2019). Higher resting HRV has been associated with more effective prefrontal inhibitory circuits (Thayer & Lane, 2000, 2009; Sakaki et al., 2016; Ottaviani et al., 2019). The horse does possess a prefrontal cortex (Frees et al., 2001; Ferrell et al., 2002; Schmidt et al., 2019), however it is thought to be under-developed (Kellerman et al., 1990), therefore comparisons to human data should be treated with caution. Despite this, the heart-brain connection is expected to apply to horses (Felici et al., 2023).

In human studies, individuals with higher HRV at rest perform better on inhibitory control tasks than those with low variability (Gillie et al., 2013). However, there is a lack of comparable data in non-human animals, and the horse makes a suitable model for investigating the relationship between inhibitory control performance and arousal. This is because horses are expected to have good inhibitory control in their working roles, for example inhibiting flight responses and waiting for cues from the rider. Additionally, horses are adept at operant conditioning. Chapter 3 demonstrated that horses can be trained to complete cognitive tasks within a few short training sessions. Further, heart rate variability is a validated measure of arousal in this species and the equipment required to do so is non-invasive and tolerated well.

1.4 IRT as an indicator of hemispheric lateralisation in inhibitory control

Sympathetically mediated blood flow towards the eyes (Blessing, 2003) has been validated as a measure of arousal in horses (Ijichi et al., 2020; Lesimple 2020; Kim and Cho, 2021; General Methods). Additionally, eye temperature was a significant predictor of performance in the two-choice discrimination-reversal task in Chapter 3. An increase in temperature of the left eye was associated with better performance. This novel finding is consistent with brain lateralisation during reversal learning, as the left hemisphere is thought to be responsible for sustained responses to targets and feeding behaviour (Rogers, 2010, 2021; Rogers & Andrew, 2002), which were both key elements of the reversal paradigm. Go/No-Go is thought to be right lateralised in humans, with response inhibition being one of the skills processed by the right hemisphere (Goursot et al., 2021). As such, if IRT technology can reveal hemispheric lateralisation through eye temperature changes, then it would be expected that individuals who perform well in the Go/No-Go task would experience an increase in the right eye temperature.

There are several theories of hemispheric lateralisation that support predictions within Go/No-Go paradigms. According to the right hemisphere hypothesis, all emotion is processed in the right hemisphere whereas reasoning and problem-solving are left-lateralised (Rogers, 2021). The left hemisphere should therefore be dominant during a complex reasoning task such as Go/No-Go, according to this theory. The BIS/BAS theory suggests that approach and avoidance behaviour are processed in opposite hemispheres (Goursot et al., 2021). According to BIS/BAS, behavioural activation (approach behaviour) is left lateralised and behavioural inhibition (withdrawal or avoidance behaviour) is right-lateralised (Goursot et al., 2021). Success on a Go/No-Go task relies on excellent inhibition, therefore according to BIS/BAS, the right hemisphere should be dominant during Go/No-Go. Predicting patterns expected by the valence hypothesis in a Go/No-Go paradigm is more difficult because it depends on individual experience. Inhibiting strong behavioural urges may be associated with frustration, which would mean right hemisphere dominance. However, the reward acquired in a Go contingency may counteract this, so depending on the individual's response this may be left-lateralised.

1.5 Physiological response to training methods

Positive reinforcement (+R) training is a principle of operant conditioning which uses the addition (+) of a pleasant or rewarding stimulus to reinforce (R) or encourage a pattern of desired behaviours (Ormrod, 1999). Positive reinforcement has been associated with increased arousal due to reward anticipation (Gieske and Sommer, 2023) and the combination of increased arousal and increased reward-related dopamine activity is thought to enhance memory performance in humans (Gieske and Sommer, 2023). However, this has not yet been observed in horses. Negative punishment (-P), by contrast, is the removal (-) of a rewarding stimulus as a means of punishment (P), to deter unwanted behavioural patterns from forming (Polling et al., 2002). Positive reinforcement and negative punishment may elicit different physiological responses due to their intended purposes as training methods. For example, while positive reinforcement may increase arousal due to reward anticipation (Gieske and Sommer, 2023), according to the valence hypothesis (Schwartz et al., 1979), it is hypothesised that this response would be left-lateralised.

In Chapter 3, it was proposed that lateralised eye temperature changes may reflect hemispheric lateralisation, therefore this would correspond to an increased left eye temperature during positive reinforcement training. Negative punishment may increase arousal due to frustration of having a desired stimulus removed (Amsel, 1990), therefore this would be right-lateralised according to the valence hypothesis, and would be reflected in an increased right eye temperature during training if eye temperature is a proxy measure for lateralised hemispheric activity.

1.6 Aims of the chapter and predictions

The aim of this chapter was: 1) to determine physiological responses to a Go/No-Go inhibitory task and potential differences between two training methods (+R and +R/-P); 2) investigate physiological indicators as possible predictors of inhibitory control performance. To this end, blink rate, blink rate variability, heart rate variability and eye temperature were measured while 20 horses completed Go/No-Go trials. Subjects completed three sessions with only positive reinforcement (+R) and a further three sessions with the addition of negative punishment (+R/-P). It was hypothesised that better performance on the Go/No-Go task would be predicted by lower average blink rate, but higher blink rate variability, in agreement with the human literature reviewed above (Dreisbach et al., 2005; Jongkees & Colzato, 2016). Similarly, it was hypothesised that higher baseline HRV would predict better performance, in agreement with human studies (T hayer & Lane, 2000, 2009; Sakaki et al., 2016; Ottaviani et al., 2019) and the results of Chapter 3.

Eye temperature was hypothesised to predict performance in one of three ways, according to the three theories described above. According to the right hemisphere hypothesis, left eye temperature increase should predict better performance because the left hemisphere is dominant during sustained responding, logical reasoning and feeding behaviour, whereas the right hemisphere processes emotions (Rogers, 2021). Right eye temperature decrease may predict better performance according to the valence hypothesis. This is because individuals who perform well would have fewer errors, and therefore fewer negative punishments, and the right hemisphere is thought to process negative emotions. Therefore, reduced activity in the right hemisphere would predict better performance. However, as discussed above, the valence hypothesis is not easily validated in a Go/No-Go task as the hemispheric dominance is dependent on the individual emotional response to the task. According to BIS/BAS, increased right eye temperature would predict better performance because inhibition is processed in the right hemisphere.

2. Methods

2.1 Go/No-Go paradigm and experimental design

Subjects completed the Go/No-Go paradigm as described in full in Chapter 4. As previously stated, subjects completed three sessions (one session per week) with only positive reinforcement (+R). Following a fallow period of three weeks, subjects completed three further sessions (one session per week) with positive reinforcement plus the introduction of negative punishment which represented a "cost" (+R/-P). In Go conditions, subjects were rewarded for touching a pre-conditioned target. In sessions 1-3 (+R), under No-Go conditions, target touches were not rewarded. This was an error of emission. In sessions 4-6 (+R/-P), errors of emission resulted in a 10-second "time-out" where no rewards could be earned. The trainer stepped back out of the reach of subjects, lowered the target and looked down. At the end of the time-out period, they stepped back in range and re-presented the target to complete that current No-Go continency. The experiment was a within-individual design, with all subjects completing both treatments (+R and +R/-P; Chapter 4).

2.2 Physiological measures

2.2.1 Spontaneous eye blink rate and blink rate variability

SEBR was measured at baseline (control) and again during learning trials as per the General Methods and Chapter 3.

To calculate blink rate variability, text files time-stamped with each blink incidence were processed in R. Root mean square of successive differences (RMSSD; see General Methods for formula) was calculated in R using the Varian package (Wiley & Elkhart Group Limited, 2016).

2.2.2 Heart rate variability

Heart rate variability was measured at baseline (control) and again during learning trials as per General Methods. The ECG was set to record in the Full Waveform mode so that artefacts could be manually corrected as per Marchant-Forde *et al*. (2004). This recording and correction method was adopted in the current and subsequent chapters following the discovery at Chapter 3 that IBI Monitoring, though valid, was not the most optimal method of analysing the ECG data (*Section 4.3* of the General Methods chapter for full details). Artefacts were manually corrected as per Marchant-Forde et al. (2004). Corrected waveform files were exported from Actiheart and analysed in Kubios HRV Premium (Kubios Oy, University of Eastern Finland).

2.2.3 Infrared Thermography

Eye temperature (IRT) was measured, as per detailed reporting in General Methods, to indicate both arousal and potential lateralised responses to the Go/No-Go cognitive task. Absolute temperature was recorded and the change in temperature from pre-learning trial to post-learning trial was calculated and recorded for both eyes.

2.3 Learning Performance

A Canon Legria video camera (Canon Inc., Tokyo, Japan) and tripod were used to record sessions for retrospective performance analysis. Performance was quantified for each subject by calculating the percentage of correct responses in session four only (Lhost et al., 2021). Session four was chosen based on the results of Chapter 4, which revealed that session four was likely the first demonstration of subjects' true learning performance. It appeared that performance in sessions 1-3 was not a true reflection of how well subjects understood the task, as it seemed that they were responding in a manner consistent with model-based learning (Chapter 4). As such, the current chapter refers to performance as the percentage score from session four of the Go/No-Go paradigm.

2.4 Statistics

Data were statistically analysed in R version 4.2.1 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria). Data were assessed for normality using a Shapiro-Wilks test (Field et al., 2012). Kruskal-Wallis tests were used to test for differences in physiological responses across training sessions and for all physiological measures (Field et al., 2012. Post-hoc pairwise Wilcoxon tests were used to calculate pairwise comparisons between training sessions. Significance values were corrected using Bonferroni adjustment to control the family-wise error rate (FWER) for multiple comparisons (Sedgwick, 2012). Wilcoxon signed ranks and paired t-tests were used to test physiological arousal responses to the addition of cost. Mean blink rate, blink rate variability (RMSSD), heart rate variability and eye temperature change were calculated across sessions 1-3, when there was no cost for errors. These were compared to the mean values across sessions 4-6, when cost was added, using Wilcoxon signed ranks tests.

A negative binomial GLM was performed following the ten-step process outlined by Zuur & Ieno (2016). This model is suitable for over-dispersed data (high variability in the dataset; Zuur and Ieno, 2016), which applied here. The model investigated whether baseline physiology and/or physiological measures in session four (first experience of cost added) predicted performance (see *2.6 Learning Performance* for performance parameters; Table 5.1). The GLM was carried out using the MASS package (Venables & Ripley, 2002). Stepwise backwards selection and Akaike Information Criterion (AIC) were used to determine best model fit and significance.

Table 5.1. Covariates included in the negative binomial GLM, with justification for inclusion and individual hypotheses.

Covariate	**Reason for inclusion in the model**	**Hypothesis**
Blink rate (blinks per minute)	This study was interested in whether phasic blink rate may be a useful predictor of inhibitory control performance (Colzato et al., 2009; Jongkees & Colzato, 2016)	Lower blink rate will predict better performance
Blink rate variability (RMSSD)	Blink rate variability may offer a more appropriate method for investigating the relationship between SEBR and learning performance (Paprocki & Lenskiy, 2017)	Higher blink rate variability will predict better performance
Baseline blink rate (blinks per minute)	Baseline blink rate is reflective of tonic striatal dopamine (Jongkees & Colzato, 2016) which may predict individual sensitivity to reward learning	Higher baseline blink rate, as an indicator of higher tonic dopamine, would predict better performance

Heart rate variability (RMSSD)	As a measure of physiological arousal during learning. Arousal may predict learning performance, (Eysenck, 1976; Yerkes & Dodson, 1908) as in Chapter 1.	Moderate arousal, as indicated by a moderate RMSSD will predict better performance
Baseline heart rate variability (RMSSD)	As a measure of baseline stress sensitivity, which may predict learning performance/ trainability (Eysenck, 1976; Yerkes & Dodson, 1908)	Moderate arousal, as indicated by a moderate baseline RMSSD will predict better performance
Left eye temperature change (°C)	As a marker of arousal in response to learning. May predict learning performance (Eysenck, 1976; Yerkes & Dodson, 1908)	Moderate arousal, as indicated by a moderate change in eye temperature will predict better performance. Left eye temperature will increase during +R sessions, according to the valence hypothesis

Right eye temperature change (°C)	As a marker of arousal in response to learning. May predict learning performance (Eysenck, 1976; Yerkes & Dodson, 1908)	Moderate arousal, as indicated by a moderate change in eye temperature will predict better performance. Right eye temperature will increase during +R/-P-sessions, according to the valence hypothesis

3. Results

3.1 Physiological response to the Go/No-Go task.

3.1.1 Average spontaneous eye blink rate and blink rate variability

There was no significant difference in average SEBR between baseline and all Go/No-Go sessions (Kruskal-Wallis chi-squared = 11.54, df = 6, p = 0.07; Table 5.2; Figure 5.1). There was no significant difference in blink rate variability between baseline and all sessions (Kruskal-Wallis chi-squared = 6.27, df = 5, p = 0.28; Table 5.3; Figure 5.2).

Table 5.2. Results of the pairwise Wilcoxon calculations for blink rate across baseline and all training sessions. Significance values given in the table are corrected using the Bonferroni adjustment method. Significance level is coded as follows: p=0.05 = ‘·’, p<0.05 = ‘*’, p<0.01 = ‘**’, p< 0.001 = ‘***’. Values in brackets are the corresponding effect sizes for each comparison. Effect size was calculated by dividing the test statistic by the square root of the sample size (Z/√N). Wilcoxon effect size has the following interpretation: <0.3=Small; 0.3-<0.5=Moderate; >0.5=Large (Fritz et al., 2012). Effect sizes in bold are moderate to large. Significance values in bold are statistically significant (threshold=p<0.05).

	Baseline	**Session 1**	**Session 2**	**Session 3**	**Session 4**	**Session 5**
Session 1	1.00 (0.16)	-	-	-	-	-
Session 2	1.00 (0.00)	1.00 (0.14)	-	-	-	-
Session 3	1.00 (0.05)	1.00 (0.09)	1.00 (0.07)	-	-	-
Session 4	0.06 **(0.68)**	0.39 **(0.59)**	0.30 **(0.56)**	0.13 **(0.58)**	-	-

Session 5	1.00 (0.25)	1.00 (0.17)	1.00 (0.18)	1.00 (0.13)	1.00 **(0.65)**	-
Session 6	1.00 (0.01)	1.00 (0.16)	1.00 **(0.30)**	1.00 (0.18)	0.80 **(0.71)**	1.00 **(0.41)**

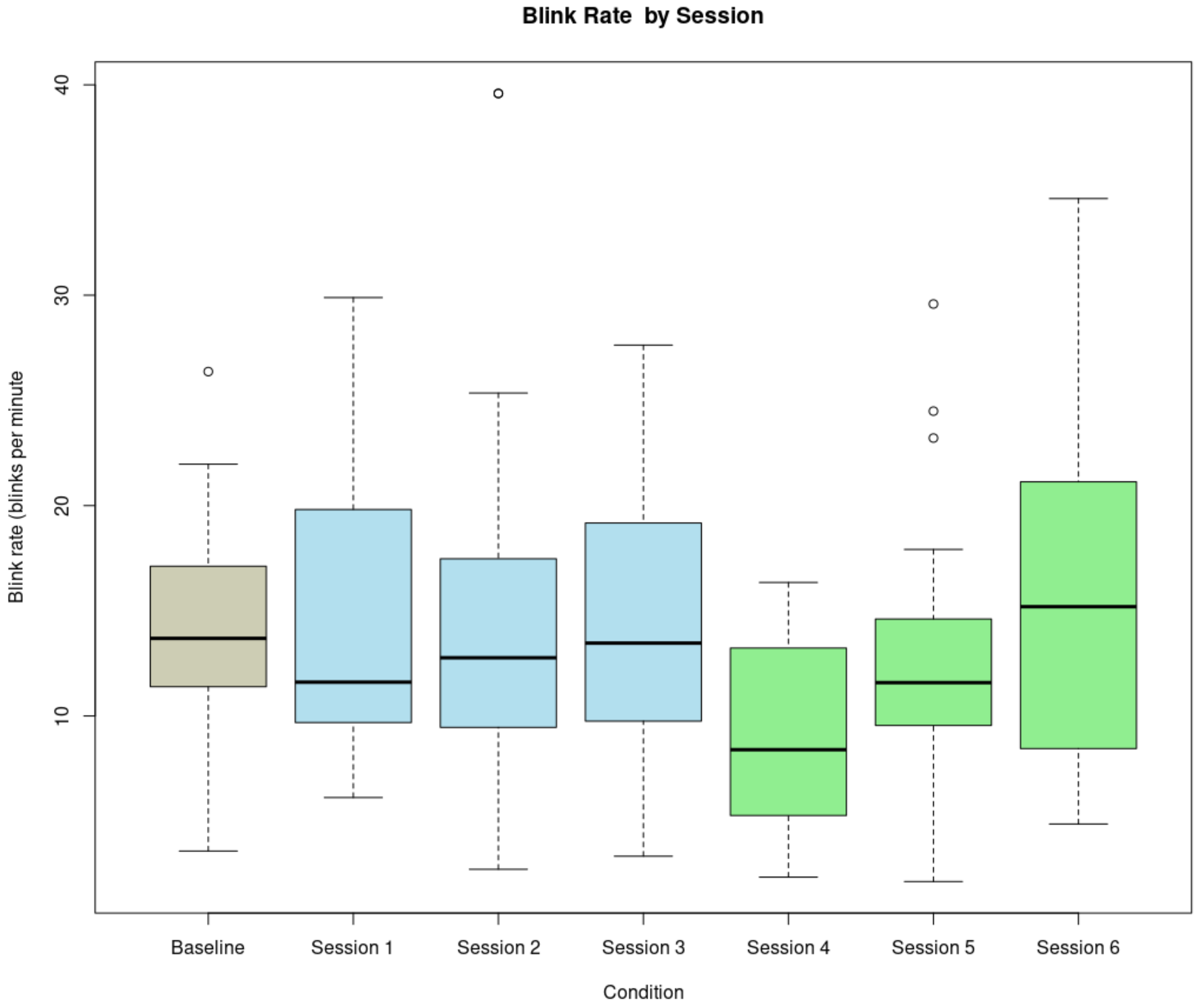

Figure 5.1. Boxplot showing blink rate (blinks per minute) across each session of the Go/No-Go task. The grey box represents the baseline (control) blink rate. Blue boxes represent sessions where there was no cost for errors (+R) and green boxes represent sessions where cost was added (+R/-P). The boxes represent the first to the third quartile of data, with a bold line at the median value. The maximum and minimum values are indicated by the upper and lower whiskers, respectively.

Table 5.3. Results of the pairwise Wilcoxon calculations for blink rate variability across baseline and all training sessions. Significance values given in the table are corrected using the Bonferroni adjustment method. Significance level is coded as follows: p=0.05 = ‘·’, p<0.05 = ‘*’, p<0.01 = ‘**’, p< 0.001 = ‘***’.

Values in brackets are the corresponding effect sizes for each comparison. Effect size was calculated by dividing the test statistic by the square root of the sample size (Z/√N). Wilcoxon effect size has the following interpretation: <0.3=Small; 0.3-<0.5=Moderate; >0.5=Large (Fritz et al., 2012). Effect sizes in bold are moderate to large. Significance values in bold are statistically significant (threshold=p<0.05).

	Baseline	**Session 1**	**Session 2**	**Session 3**	**Session 4**	**Session 5**
Session 1	1.00 (0.09)	-	-	-	-	-
Session 2	1.00 (0.08)	1.00 (0.00)	-	-	-	-
Session 3	1.00 (0.20)	1.00 (0.09)	1.00 (0.13)	-	-	-
Session 4	1.00 **(0.47)**	1.00 **(0.46)**	1.00 **(0.34)**	0.39 **(0.55)**	-	-
Session 5	1.00 (0.18)	1.00 (0.20)	1.00 (0.13)	1.00 (0.11)	1.00 **(0.45)**	-
Session 6	1.00 (0.22)	1.00 **(0.42)**	1.00 (0.01)	1.00 (0.19)	1.00 (0.26)	1.00 (0.13)

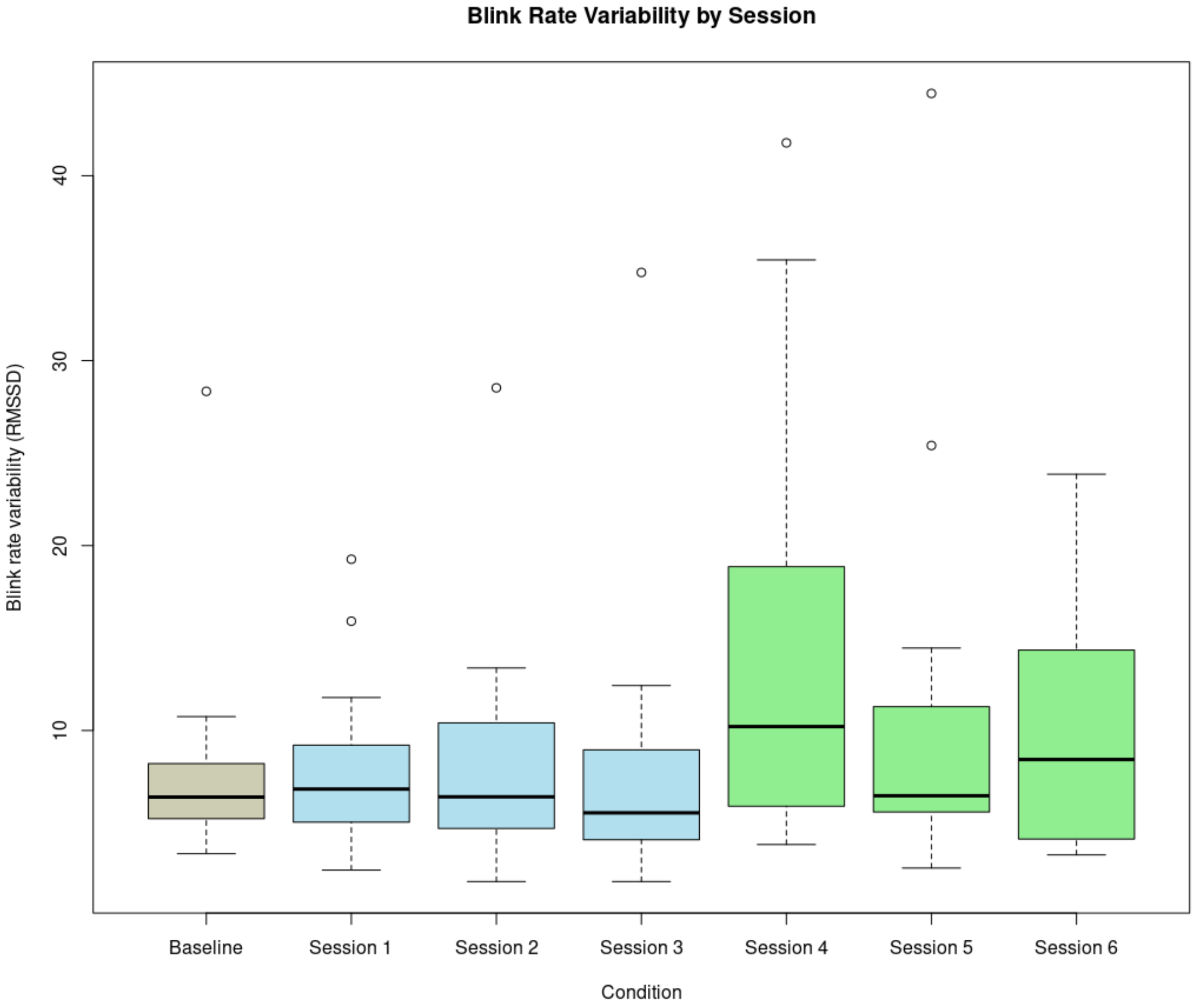

Figure 5.2. Boxplot showing blink rate variability (RMSSD) across each session of the Go/No-Go task. The grey box represents the baseline (control) blink rate variability. Blue boxes represent sessions where there was no cost for errors (+R) and green boxes represent sessions where cost was added (+R/-P). The boxes represent the first to the third quartile of data, with a bold line at the median value. The maximum and minimum values are indicated by the upper and lower whiskers, respectively.

3.1.2 Heart rate variability (HRV)

HRV was significantly different between baseline and Go/No-Go sessions (Kruskal-Wallis chi-squared = 15.72, df = 5, p = 0.008; Figure 5.3). Post-hoc pairwise Wilcoxon tests using Bonferroni (BH) p-value adjustment revealed that HRV was significantly lower in session 1 than in sessions 5 and 6 (p=0.005, p=0.009, respectively; Table 5.4).

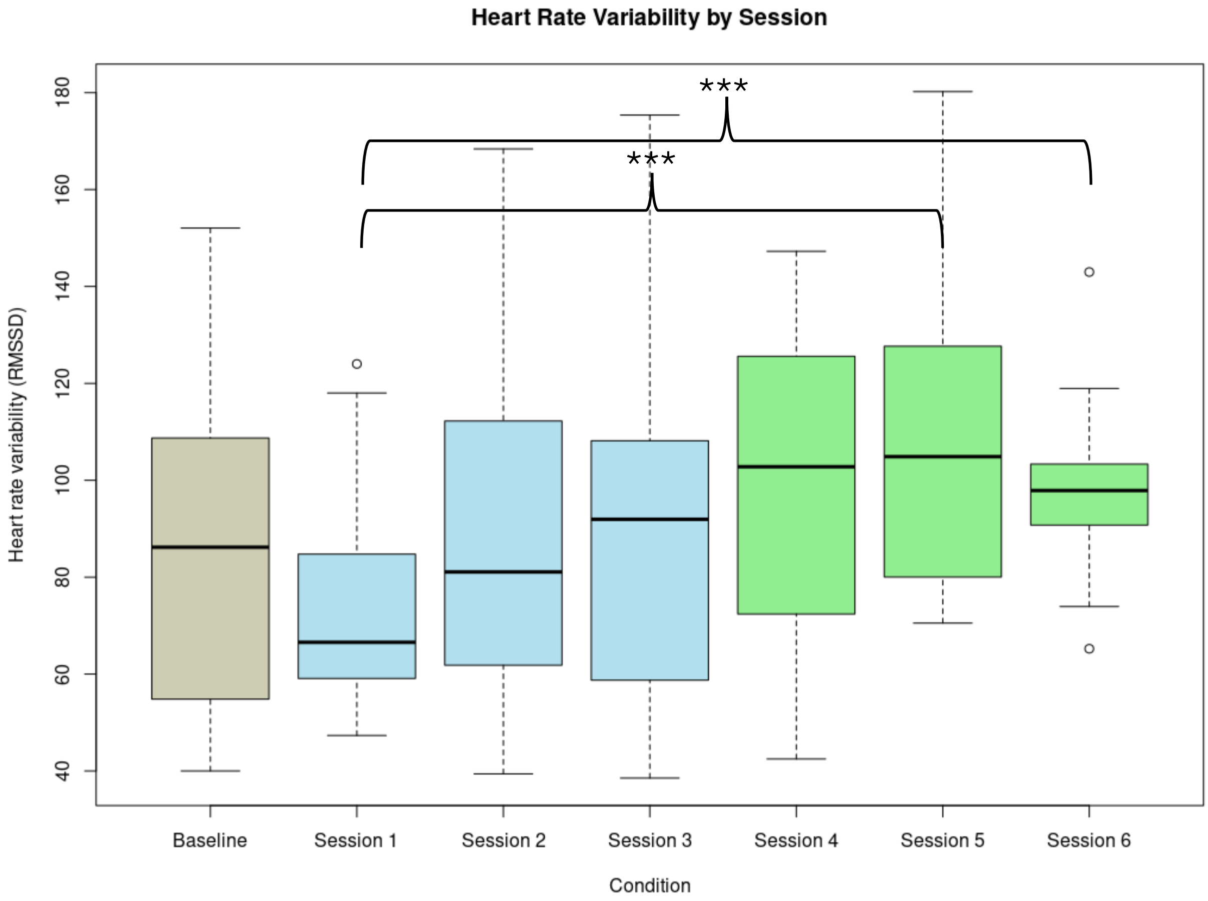

Figure 5.3. Boxplot showing heart rate variability across each session of the Go/No-Go task. The grey box represents the baseline (control) HRV values. Blue boxes represent HRV when there was no cost for errors (+R) and green boxes represent HRV when cost was added (+R/-P). The boxes represent the first

to the third quartile of data, with a bold line at the median value. The maximum and minimum values are indicated by the upper and lower whiskers, respectively.

Table 5.4. Results of Post-hoc pairwise Wilcoxon tests between HRV in each session and at baseline. Significance values given in the table are corrected using the Bonferroni adjustment method Significance level is coded as follows: p=0.05 = '·', p<0.05 = '*', p<0.01 = '**', p< 0.001 = '***'. Values in brackets are the corresponding effect sizes for each comparison. Effect size was calculated by dividing the test statistic by the square root of the sample size (Z/√N). Wilcoxon effect size has the following interpretation: <0.3=Small; 0.3-<0.5=Moderate; >0.5=Large (Fritz et al., 2012). Effect sizes in bold are moderate to large. Significance values in bold are statistically significant (threshold=p<0.05).

	Baseline	**Session 1**	**Session 2**	**Session 3**	**Session 4**	**Session 5**
Session 1	1.00 **(0.30)**	-	-	-	-	-
Session 2	1.00 (0.04)	1.00 (0.40)	-	-	-	-
Session 3	1.00 (0.12)	1.00 **(0.36)**	1.00 (0.17)	-	-	-
Session 4	1.00 (0.21)	0.16 **(0.69)**	1.00 **(0.36)**	1.00 (0.27)	-	-
Session5	1.00 **(0.46)**	**0.005** (0.76)**	1.00 **(0.49)**	1.00 **(0.43)**	1.00 (0.28)	-
Session 6	1.00 (0.28)	**0.009** (0.70)**	1.00 **(0.42)**	1.00 (0.18)	1.00 (0.01)	1.00 (0.19)

3.1.3 Eye temperature (IRT)

There was a significant decrease in temperature of the right eye during Go/No-Go session 2 ($p<0.001$; Table 5.5). Both the left and right eyes significantly decreased in temperature during session 5 ($p=0.03$ and $p=0.04$, respectively; Table 5.5).

There was a significant decrease in temperature of the right eye in session 6 ($p<0.001$; Table 5.5). Eye temperature did not significantly change during any other sessions. Left eye temperature changes were not significantly different between Go/No-Go sessions (Kruskal-Wallis chi-squared = 3.79, df = 5, p = 0.58; Figure 5.4; Table 5.6). Right eye temperature changes were not significantly different between Go/No-Go sessions (Kruskal-Wallis chi-squared = 5.86, df=5, p=0.32; Figure 5.5; Table 5.6).

Table 5.5. Results of the tests of difference between pre-session and post-session eye temperature. Effect size was calculated using Cohen's D for paired t-tests, with the following interpretation: <0.5=Small; 0.5-<0.8=Moderate; ≥0.8=Large (Cohen, 1988). For Wilcoxon tests, effect size was calculated by dividing the test statistic by the square root of the sample size ($Z/\sqrt{N}$). Wilcoxon effect size has the following interpretation: <0.3=Small; 0.3-<0.5=Moderate; >0.5=Large (Fritz et al., 2012). Effect sizes in bold are moderate to large. Significance values in bold are statistically significant (threshold=$p<0.05$.

Session	**Eye**	**Mean (±sd)/ Median (IQR) temperature (°C) pre-session**	**Mean (±sd)/ Median (IQR) temperature (°C) post-session**	**Test**	**Test statistic**	**Effect size**	**Significance value**
1	Left	36.53	36.37	Paired t-test	-0.79	0.18	0.44
	Right	36.54	36.40	Paired t-test	-1.18	0.26	0.25
2	Left	36.74	36.55	Paired t-test	-0.88	0.19	0.39
	Right	36.80	36.45	Paired t-test	-3.91	**0.87**	**<0.001**
3	Left	36.03	36.14	Paired t-test	0.54	0.12	0.59

	Right	36.15	35.94	Paired t-test	-1.48	0.33	0.15
4	Left	36.38	36.05	Paired t-test	-1.59	0.36	0.13
	Right	36.30	35.80	Wilcoxon	-155.5	**0.42**	0.06
5	Left	36.58	36.29	Paired t-test	-2.32	**0.52**	**0.03**
	Right	36.46	36.24	Paired t-test	-2.15	0.48	**0.04**
6	Left	36.47	36.36	Paired t-test	-0.95	0.22	0.35
	Right	36.5	36	Wilcoxon	-164.5	**0.81**	**<0.001**

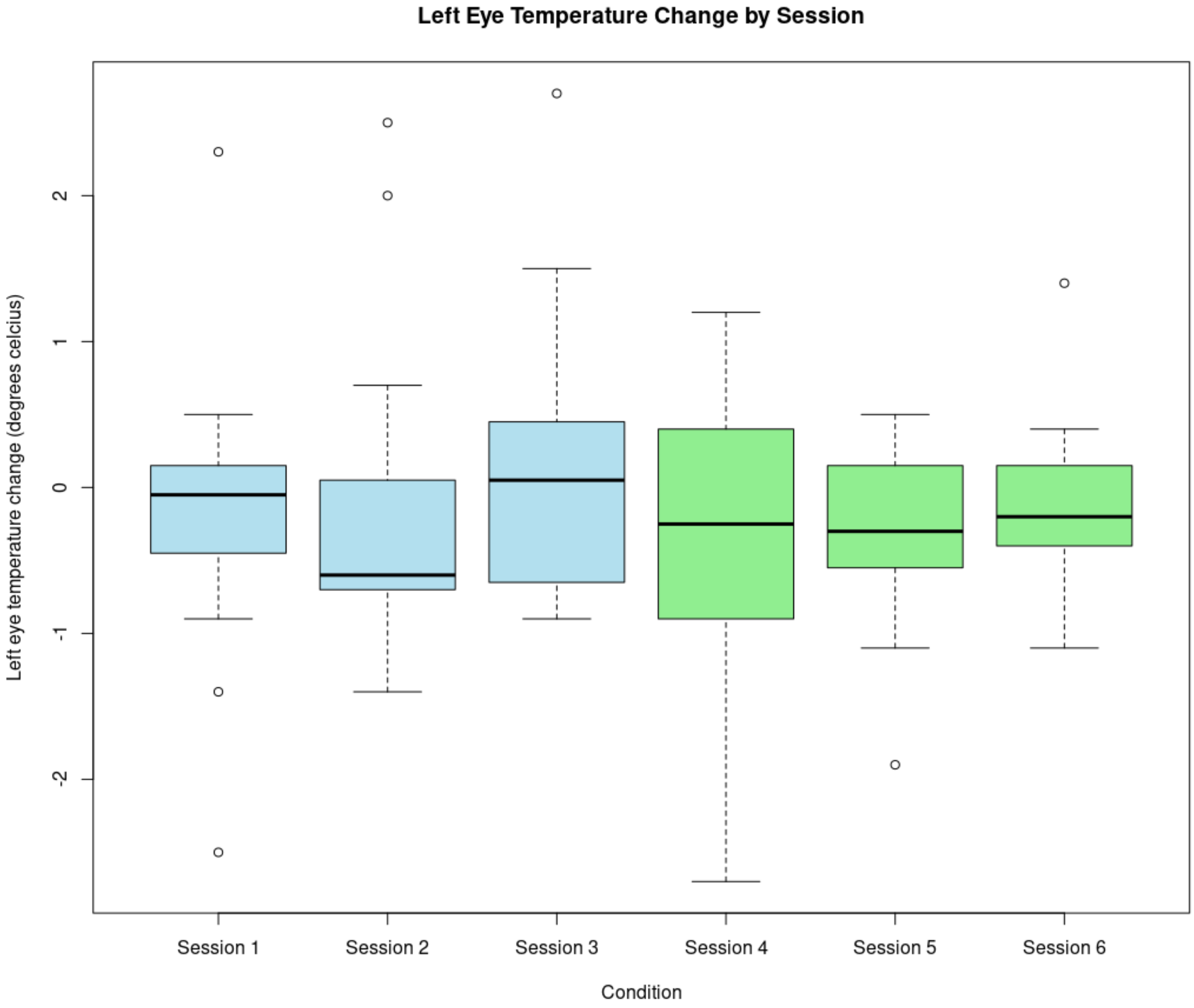

Figure 5.4. Boxplot showing left eye temperature change across each session of the Go/No-Go task. Blue boxes represent sessions where there was no cost for errors (+R) and green boxes represent sessions where cost was added (+R/-P). The boxes represent the first to the third quartile of data, with a bold line at the median value. The maximum and minimum values are indicated by the upper and lower whiskers, respectively.

Table 5.6. Results of Post-hoc pairwise Wilcoxon tests between left eye temperature change in each session. Significance values given in the table are corrected using the Bonferroni adjustment method Significance level is coded as follows: p=0.05 = ‘·’, p<0.05 = ‘*’, p<0.01 = ‘**’, p< 0.001 = ‘***’. Values in brackets are the corresponding effect sizes for each comparison. Effect size was calculated by dividing the test statistic by the square root of the sample size (Z/√N). Wilcoxon effect size has the following interpretation: <0.3=Small; 0.3-<0.5=Moderate; >0.5=Large (Fritz et al., 2012). Effect sizes in bold are moderate to large. Significance values in bold are statistically significant (threshold=p<0.05).

	Session 1	**Session 2**	**Session 3**	**Session 4**	**Session 5**
Session 1	-	-	-	-	-
Session 2	1.00 (0.12)	-	-	-	-
Session 3	1.00 (0.21)	1.00 (0.15)	-	-	-
Session 4	1.00 (0.23)	1.00 (0.04)	1.00 **(0.40)**	-	-
Session 5	1.00 (0.05)	1.00 (0.04)	1.00 (0.21)	1.00 (0.08)	-
Session 6	1.00 (0.02)	1.00 (0.23)	1.00 (0.12)	1.00 (0.10)	1.00 (0.29)

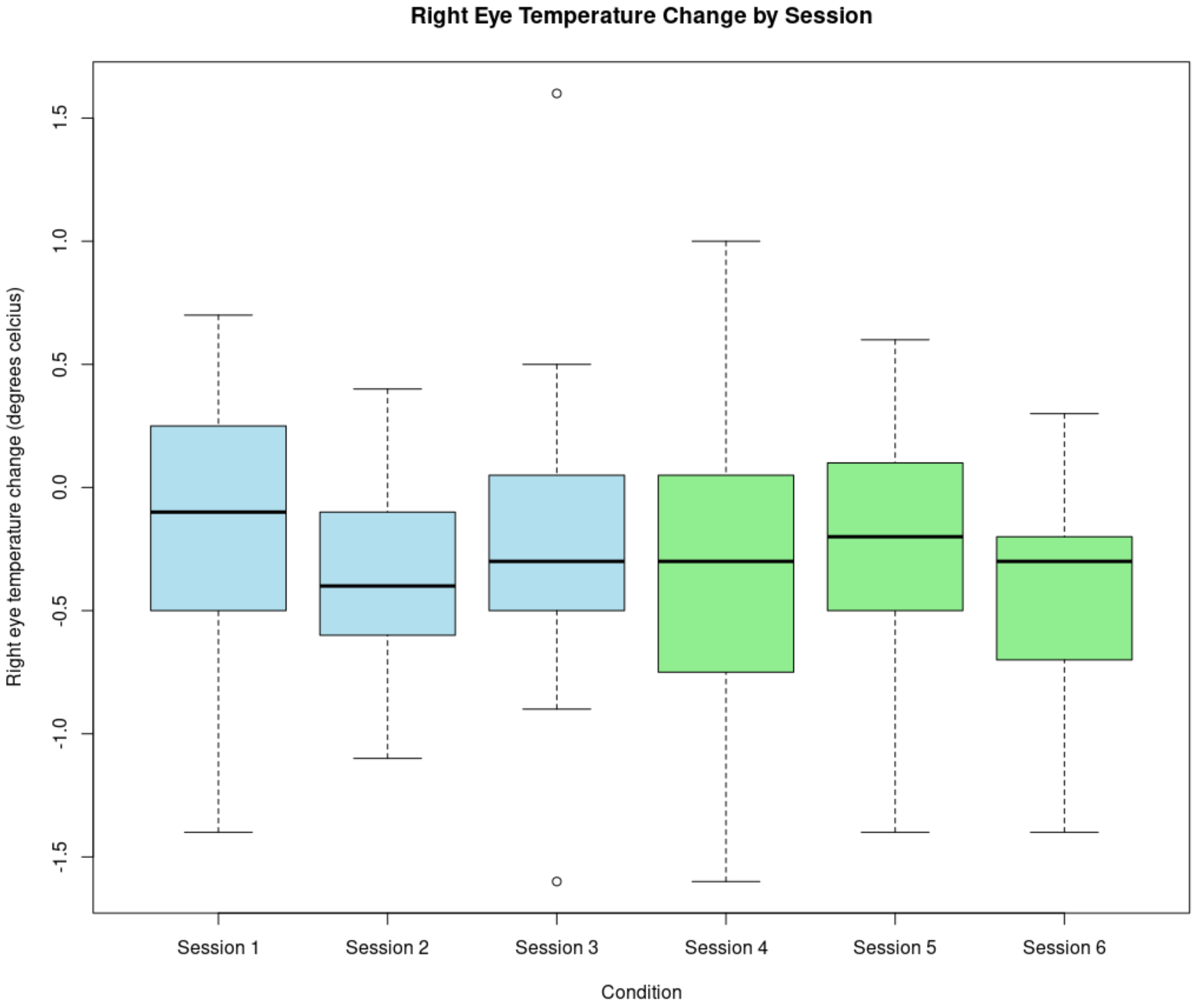

Figure 5.5. Boxplot showing right eye temperature change across each session of the Go/No-Go task. Blue boxes represent sessions where there was no cost for errors (+R) and green boxes represent sessions where cost was added (+R/-P). The boxes represent the first to the third quartile of data, with a bold line at the median value. The maximum and minimum values are indicated by the upper and lower whiskers, respectively.

Table 5.7. Results of Post-hoc pairwise Wilcoxon tests between right eye temperature change in each session. Significance values given in the table are corrected using the Bonferroni adjustment method Significance level is coded as follows: p=0.05 = '·', p<0.05 = '*', p<0.01 = '**', p< 0.001 = '***'. Values in brackets are the corresponding effect sizes for each comparison. Effect size was calculated by dividing the test statistic by the square root of the sample size (Z/√N). Wilcoxon effect size has the following interpretation: <0.3=Small; 0.3-<0.5=Moderate; >0.5=Large (Fritz et al., 2012). Effect sizes in bold are moderate to large. Significance values in bold are statistically significant (threshold=p<0.05).

	Session 1	**Session 2**	**Session 3**	**Session 4**	**Session 5**
Session 1	-	-	-	-	-
Session 2	1.00 **(0.40)**	-	-	-	-
Session 3	1.00 (0.16)	1.00 (0.11)	-	-	-
Session 4	1.00 (0.26)	1.00 (0.24)	1.00 (0.09)	-	-
Session 5	1.00 (0.11)	1.00 (0.28)	1.00 (0.04)	1.00 (0.05)	-
Session 6	0.43 **(0.49)**	1.00 **(0.30)**	1.00 **(0.41)**	1.00 (0.26)	1.00 **(0.45)**

3.1.4 Physiological response to cost added

Blink rate was not significantly different in sessions where cost was added (4-6) than in sessions where there was no cost for errors (1-3; Wilcoxon V=126, p=0.22). Blink rate variability was significantly higher during sessions where cost was added than in sessions where there was no cost for errors (Wilcoxon V = 42.5, p=0.04; Figure 5.6).

Heart rate variability was significantly higher in sessions where cost was added than in sessions where there was no cost for errors (t= 3.47, df=19, p=0.002; Figure 5.7). The change in eye temperature from pre-learning session to post-learning session was not significantly different between sessions with cost added and sessions with no cost for errors (Left eye: t=1.11, df=19, p=0.28; Right eye: t=1.15, df=19, p=0.26).

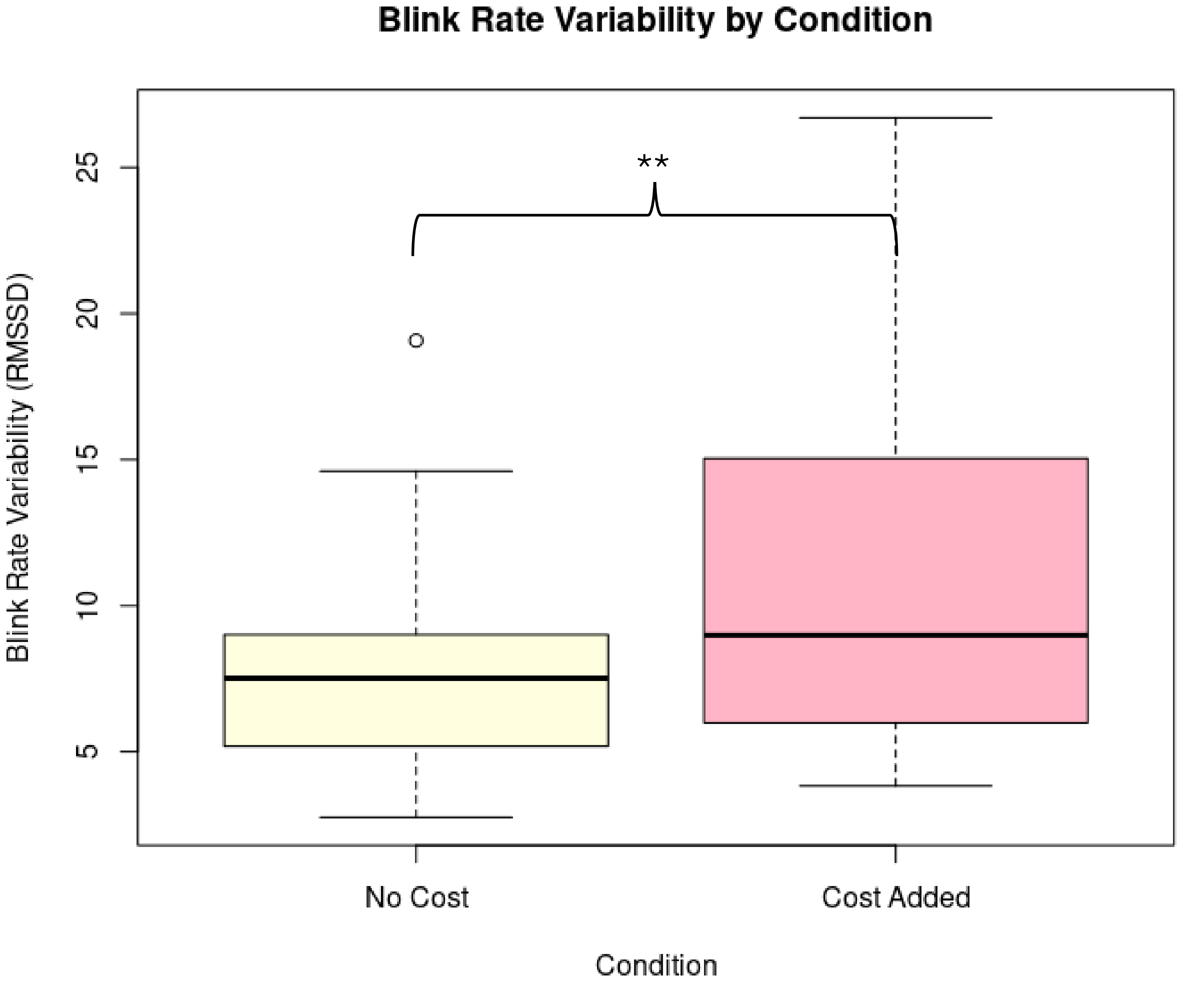

Figure 5.6. Boxplot of blink rate variability across training conditions. The yellow box (No Cost) represents the mean blink rate variability from sessions 1-3, where only positive reinforcement was used. The pink box (Cost Added) represents the mean blink rate variability from sessions 4-6, where positive reinforcement plus negative punishment was used. BRV was significantly higher when cost was added than when there was no cost (Wilcoxon V=42.5, p=0.04). The boxes represent the first to the third

quartile of data, with a bold line at the median value. The maximum and minimum values are indicated by the upper and lower whiskers, respectively.

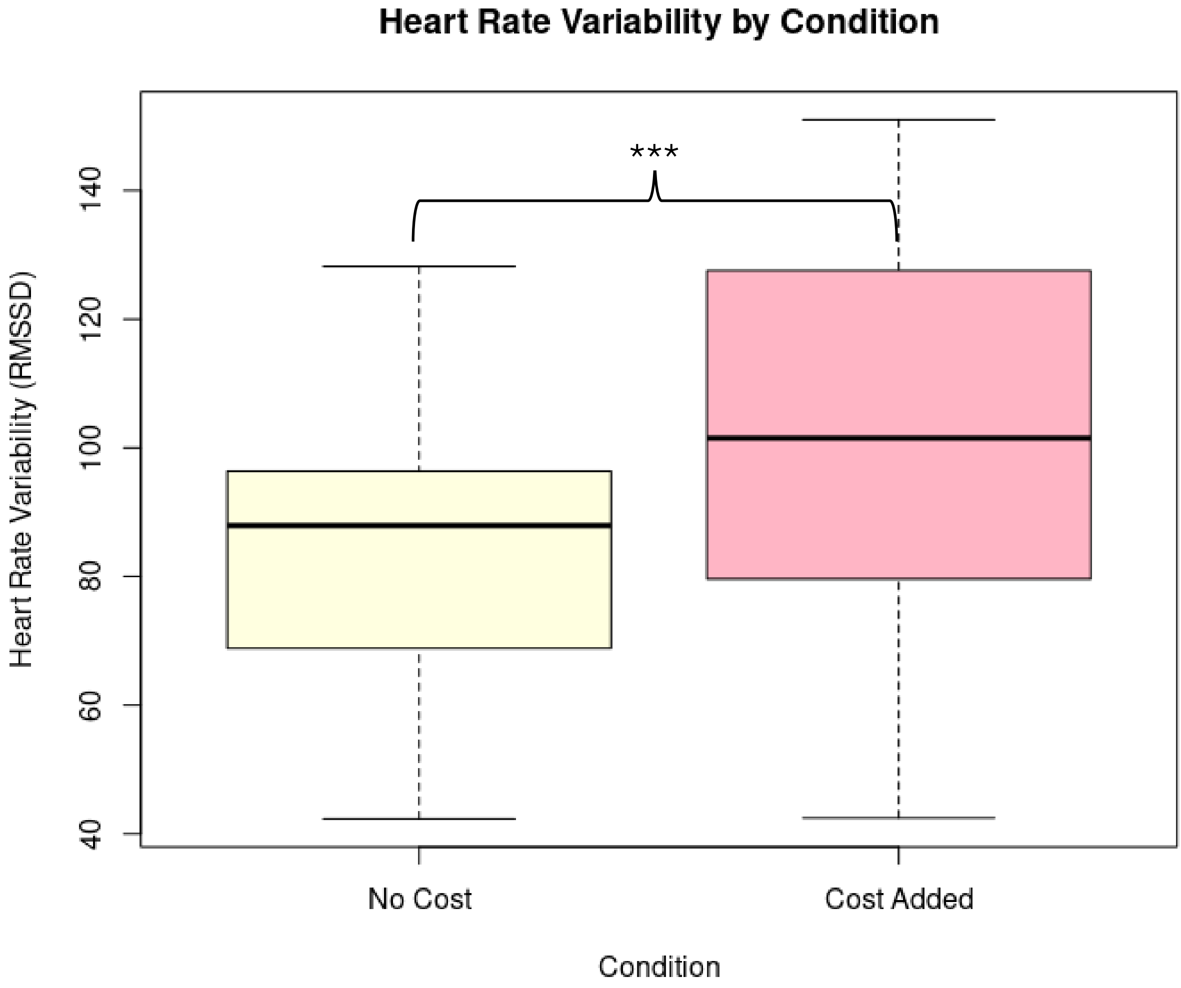

Figure 5.7. Boxplot of heart rate variability across training conditions. The yellow box (No Cost) represents the mean heart rate variability from sessions 1-3, where only positive reinforcement was used. The pink box (Cost Added) represents the mean heart rate variability from sessions 4-6, where positive

reinforcement plus negative punishment was used. HRV was significantly higher when cost was added than when there was no cost (t= 3.47, df=19, p=0.002). The boxes represent the first to the third quartile of data, with a bold line at the median value. The maximum and minimum values are indicated by the upper and lower whiskers, respectively.

3.2 GLM: Physiological predictors of performance in session 4 of the Go/No-Go task (first experience of cost added for errors of emission).

A negative binomial GLM revealed that right eye temperature, heart rate variability, baseline blink rate and baseline heart rate variability were all significant predictors of performance on the Go/No-Go task (Table 5.8). A decreased right eye temperature and lower heart rate variability during session 4 of the Go/No-Go task predicted better performance ($p<0.001$ and $p<0.05$, respectively). Higher baseline blink rate and higher baseline heart rate variability also predicted better performance ($p<0.001$ and $p<0.001$, respectively).

Table 5.8. Results of the negative binomial GLM. The outcome variable was Go/No-Go task performance in Session 4. Coefficients (listed in the first column of the table) relate to session 4 unless marked Baseline. Model estimates, standard error and significant values are also given. Significance level is coded as follows: p>0.05 = ‘not significant (n/s)’, p=0.05 = ‘·’, p<0.05 = ‘*’, p<0.01 = ‘**’, p< 0.001 = ‘***’.

Coefficients	**Estimate**	**Std. Error**	**Z value**	**p value**	**Significance**
(Intercept)	2.81	0.36	7.73	<0.001	***
Heart rate variability (RMSSD)	-0.005	0.002	-2.33	0.02	*
Right eye temperature change	-0.67	0.14	-4.88	<0.001	***
Baseline blink rate	0.07	0.02	3.9	<0.001	***
Baseline heart rate variability	0.009	0.002	3.99	<0.001	***

4. Discussion

The aims of this chapter were as follows. First, to investigate physiological responses to a Go/No-Go inhibitory task; second, to investigate physiological responses to two training methods (+R and +R/-P); and third, to investigate a statistical model for predicting inhibitory control performance using novel physiological markers.

4.1 Physiological responses to the task

There were no significant differences in blink rate and blink rate variability across control and Go/No-Go sessions. However, moderate to large effect sizes were observed from session four (Tables 5.3 and 5.4). In addition, the difference between baseline and session four blink rate was approaching statistical significance (p=0.06), so perhaps the strong correction method for multiple comparisons (Ludbrook, 1998) may have influenced this result. Upon visual inspection of the data, a decrease in blink rate and an increase in blink rate variability at session four, compared to other sessions, was observed (Figures 5.1 and 5.2, respectively). This is consistent with increased attention, as seen in human blink rate studies (Doughty, 2001; Gebrehiwot et al., 2017; Karson, 1983; Lenskiy & Paprocki, 2016; Paprocki & Lenskiy, 2017).

Blink rate decreases during periods of focus to maximise information gathering (Doughty, 2001). The effect of attention on eye blink rate in horses has been investigated (Cherry et al., 2020), however results were inconclusive due to confounding variables. The current study offers an observed trend consistent with the hypothesis of Cherry et al. (2020), that attention would be negatively associated with blink rate in horses. Blink rate variability has only been investigated in relation to cognition in a handful of human studies, all of which suggest that blink rate variability is positively associated with cognitive load (Gebrehiwot et al., 2017; Lenskiy & Paprocki, 2016; Paprocki & Lenskiy, 2017). This is due to a common pattern of long periods without blinks, followed by rapid serial blinks during periods of high cognitive load (Nomura & Maruno, 2019). Taken together, the patterns observed here appear consistent with both a blink rate and bink rate variability response to attention and cognitive load, though not statistically significant.

The observation of this pattern in session four alone may provide further evidence consistent with model-based learning (as described in Chapter 4). If horses were not demonstrating their ability to complete Go/No-Go in sessions one to three, this could explain why blink rate variability, as a potential indicator of cognitive load (Nomura and Maruno, 2019), increased at session four as horses began to attend to the task contingencies and respond accordingly. However, this increase in blink rate variability is not maintained across sessions five and six. This could be due to the task becoming easier with practice; therefore, the cognitive load is reduced. Alternatively, it could be that the Go/No-Go task itself does not present a particularly high cognitive load, but the introduction of cost at session four required horses to perform a cost-benefit analysis, which temporarily increased mental load during this session. Though it is not clear what caused this interesting result, further investigation into blink rate variability in horses during cognitive tasks is warranted.

Eye temperature decreased in response to the Go/No-Go task, though this was only statistically significant in the right eye in session two, the left and right eyes in session five, and the right eye in session six. In Chapter 3, there was also a marked decrease in eye temperature in response to the two-choice discrimination-reversal task, suggesting that this is a relatively consistent response to cognitive challenge. Abdelrahman et al. (2017) observed a decrease in nasal temperature and increase in forehead temperature in humans, in response to cognitive load across two different cognitive tasks, and four levels of difficulty. This suggests that there may be specific facial regions where vasoconstriction and vasodilation occur in response to increased cognitive load, and this is reflected in surface temperatures measured by IRT. Different facial regions are not typically used for IRT analysis in horses due to hair being a confounding factor in this species (Travain and Valsecchi, 2021). Taken with the blink rate and blink rate variability responses, this indicates a pattern of physiological responses consistent with increased attention and cognitive load during the Go/No-Go task.

HRV was significantly different between baseline and Go/No-Go sessions, and further analysis revealed a significant increase in heart rate variability between sessions one and five, and sessions one and six (Figure 3). In session one, the task was novel to subjects, which may explain why HRV appears to be lowest in this session (Figure 3). Horses are neophobic (Krueger et al., 2014), so the lower HRV in session one could indicate increased arousal in response to being introduced to the task stimulus for the first time. HRV appeared to increase over the training sessions, with a significant increase at sessions 5 and 6, consistent with this novelty effect abating. This is consistent with a positive relationship between HRV and cognitive performance (Forte et al., 2019, 2022; Grässler et al., 2020). However, only the differences in HRV between sessions 1 and 5 and sessions 1 and 6 were statistically significant following Bonferroni correction, so conclusions must be drawn with caution.

4.2 Physiological responses to cost

Heart rate variability was significantly higher when cost was added, compared to when only positive reinforcement was used, indicating that horses experienced lower arousal during sessions where they may be punished for incorrect responses. This was unexpected; the introduction of negative punishment was expected to have a mildly arousing effect due to possible surprise or frustration at the sudden rule change (Burokas et al., 2012; Pannewitz & Loftus, 2023). However, this result is consistent with Chapter 3 (Reversal). An increase in HRV during reversal learning, compared to baseline HRV, was observed. This potentially demonstrates consistent responses across tasks which may relate to flow state.

Flow state is the complete absorption in a task, where the task is intrinsically rewarding (Peifer, 2012; Hintze and Yee, 2022). In flow state, focus levels are high, but arousal remains low (Peifer, 2012; Hintze and Yee, 2022). In the current study, blink rate appeared to be lower during sessions 4-6 than sessions 1-3, which indicates increased focus (Doughty, 2001). Higher HRV indicates lower arousal, therefore physiological responses were consistent with those expected during flow state. For flow to occur, the challenge of the task must be appropriately matched by the skill of the participant (Hintze and Yee, 2022). This could explain why the increase in HRV is observed in sessions 4-6. At this point, horses may have consolidated their understanding of the task (skill), but the challenge suddenly increased due to the addition of cost which demanded increased focus.

As Chapter 4 revealed, subjects acquired knowledge of the task by session three (though potentially earlier), as an immediate strategy switch was observed when cost was added in session 4. Therefore, horses were unlikely to be frustrated by the task when cost was introduced, as they could reliably predict the outcomes. Being able to reliably predict and control outcomes is known to reduce arousal and improve emotional well-being in humans (Mirowsky & Ross, 1990; Solomon et al., 1980). It is possible that horses also experience reduced arousal when they are able to control outcomes through their acquired understanding of the task. It is well established that a lack of perceived control over outcomes can lead to learned helplessness (Maier, 1984; Wortman & Brehm, 1975), evidence of which has been seen in horses (Hall et al., 2008). As such, a certain level of agency is of value to horses in terms of their emotional state.

The increased HRV in sessions 4-6 may provide further evidence consistent with model-based learning in horses, as per Chapter 4. If horses' poor performance in sessions 1-3 was due to lack of understanding of the task, it would be reasonable to assume that punishment for errors would cause mild stress response in horses, which would be reflected in decreased HRV (Borstel et al., 2017). Considering the results of both Chapter 4 and the current chapter together, that horses' performance significantly and immediately improved and HRV was higher when punishment was added, it is therefore unlikely that horses did not understand the task in the initial three sessions. Horses calmly switched their approach from indiscriminate responding when there was no cost for errors, to discriminate responses contingent on the light cue, when cost was added. This is a surprising and novel finding of the thesis.

Blink rate variability was significantly higher in sessions 4-6 (+R/-P) than in sessions 1-3 (+R), suggesting a training method effect (Figure 7). In addition, blink rate appeared to be lower during the sessions where cost was added, compared to sessions where there was no cost, though this was not statistically significant (Figure 6). The effect sizes at session four, when cost was first introduced, were all large (Table 3), despite the non-significant result. Also, Bonferroni correction was applied to these tests due to the possible effects of multiple comparisons (Ludbrook, 1998). Therefore, there may be an effect of cost which should be investigated in future research. Blink rate variability was significantly higher when cost was added, consistent with a switch in their approach to attending to the light cue.

In humans, blink rate is observed to decrease significantly during periods of focus (Lenskiy & Paprocki, 2016), followed by rapid serial blinks (Nomura & Maruno, 2019), causing high variability during tasks which require high levels of attention. This may explain why blink rate appears to decrease, and blink rate variability increases significantly, in sessions where cost is added. As explained in Chapter 4, horses switched their approach in favour of a discriminate response to the light cue when cost was added. This requires more attention than the previous approach of indiscriminate responding, as the horse now must attend to the light cue. As the results of the previous chapter demonstrated, subjects' performance improved significantly in session four. Taken together this suggests that in session four, the increase in blink rate variability was due to increased attention towards the light cue, and this was reflected in their improved performance, evidenced in Chapter 4.

Eye temperature was not significantly different in sessions where negative punishment was added, compared with sessions where positive reinforcement only was used. IRT is a validated welfare indicator in this species (Redaelli et al., 2019; Ijichi et al., 2020; Kim et al., 2021). Therefore, this suggests that introducing a mild form of negative punishment in combination with positive reinforcement (+R/-P) did not have any negative consequences for the welfare of horses in this study. The negative punishment was only a ten second time out period, which was designed to be as mild as possible. The finding that such a short penalty was enough to have a significant and immediate effect on performance (Chapter 4), but without negative welfare consequences, is highly encouraging for efficient, ethical training.

The use of punishment in animal training must be carefully considered due to possible negative effects on welfare (Fernandez et al., 2017). For example, a recent survey found that dog owner-reported problematic behaviour was positively correlated with the incidence of punishment in training (Hiby et al., 2023). Aside from anecdotal reports of its' misuse, such as riders withholding resources following a poor performance at competition (McLean and Christensen, 2017), negative punishment is not widely used in equine training. However, if used mildly and in combination with positive reinforcement, it may serve as an effective training tool without negative welfare consequences. Further, the results of this and the previous chapter indicate that horses may find cognitive enrichment of the kind utilised in this thesis so intrinsically rewarding that even a very short time-out away from the task is sufficient punishment to motivate correct responding.

4.3 Predictors of performance

The model investigated whether baseline physiology and physiological measures taken in session four predicted performance. Session four was the first training session with cost added for errors of emission. Errors of emission were touches of the target during the No-Go contingency, which was signalled by the switching on of a light cue. In Chapter 4 it was observed that the introduction of cost had a significant effect on responses to the task, in terms of performance. Therefore, session four was the first suitable session to assess performance, as this was the first certain demonstration of understanding. It was hypothesised that horses' physiological reactions to the addition of cost may be predictive of performance. Indeed, right eye temperature, heart rate variability, baseline blink rate and baseline heart rate variability were all significant predictors of performance on the Go/No-Go task (Table 5.8).

Lower HRV was a significant predictor of better performance in this task, in contrast with McBride et al. (2022), which did not find an association between HRV and either impulsivity or compulsivity in horses during a three-choice serial reaction time task. Impulsivity within the McBride study was characterised by premature responding and compulsivity was related to continued responding after the task was complete. In the current study, errors of emission (touching the target during No-Go trials) may relate to impulsivity as described in McBride et al. (2022), as horses were prematurely touching the target before the end of the No-Go contingency in this case.

In addition to lower HRV, decreased right eye temperature also predicted better performance. This suggests that an increase in arousal (indicated by lower HRV) was adaptive for performing well in the Go/No-Go, a relatively difficult task. However, decreased right eye temperature as a predictor of performance suggests that negative valence was not triggered in high performing individuals, consistent with the valence hypothesis (Zhang et al., 2011). This potentially means that horses who were not negatively aroused by the task were better able to perform well. A possible explanation for this comes from Mendl et al. (2010), which maps core affect in a two-dimensional axis (Figure 5.8).

According to this axis, if the punishment avoidance system (high arousal, negative valence) is not triggered, this may leave an individual free to engage in reward acquisition (high arousal, positive valence; Mendl et al., 2010). Lower HRV indicates higher arousal, and decreased right eye temperature may suggest that negative valence is not triggered, though this has not been validated. If lateralised eye temperature changes during learning are indicative of hemispheric lateralisation, as proposed throughout this thesis, the decreased right eye temperature in successful horses is consistent with Mendl et al. (2010) and the valence hypothesis. This warrants further investigation because, if validated, IRT of eye temperature could offer a rapid, non-invasive indicator of hemispheric lateralisation for use in animal cognition research.

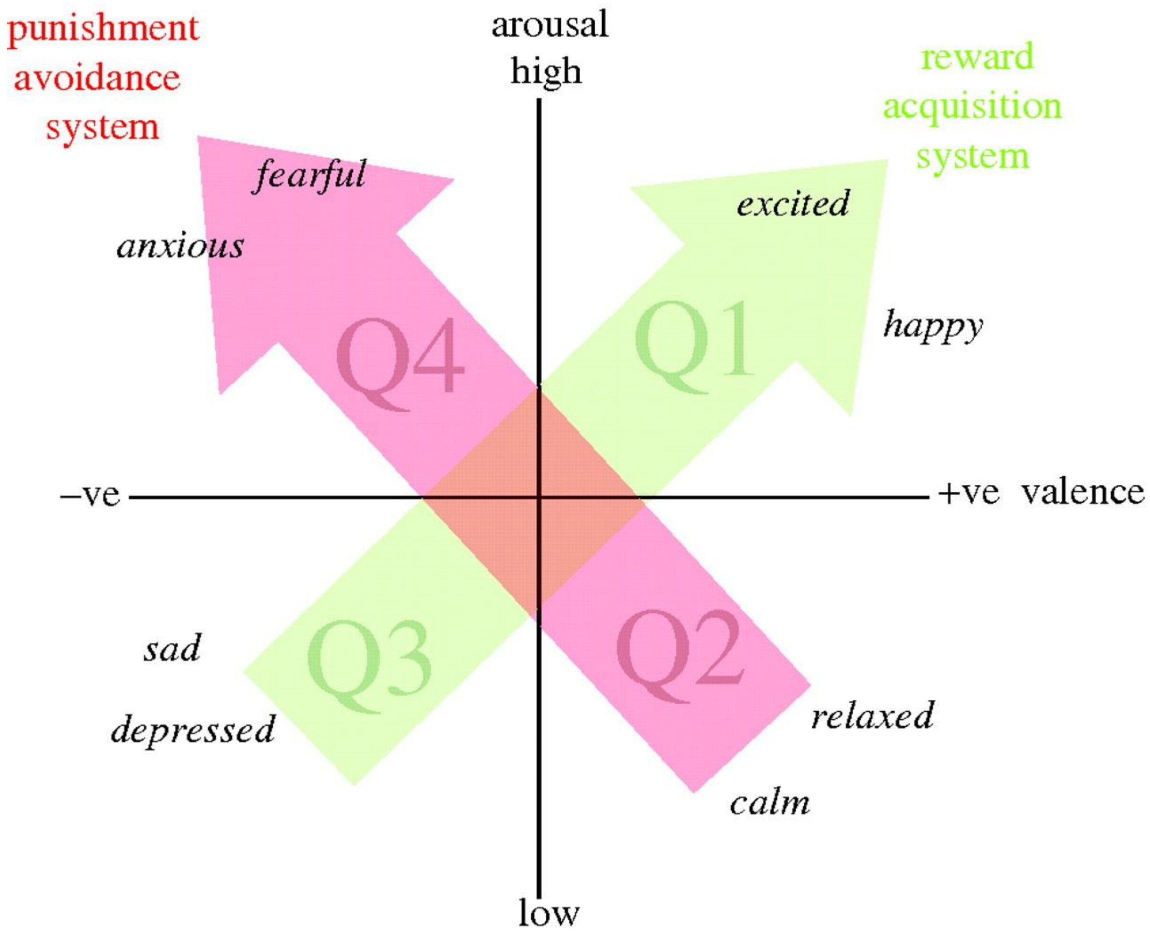

Figure 5.8. Core affect diagram from Mendl et al. (2010), which maps the two dimensions of affective state: arousal (vertical axis) and valence (horizontal axis). The four quadrants represent the four affective states. Q1 and Q2 are both positive states while Q3 and Q4 are the negative affective states. The pink arrow indicates the shift from Q2 to Q4 which can occur in the punishment avoidance system. The green arrow indicates the shift from Q3 to Q1 which is possible in the reward acquisition system.

Higher baseline blink rate predicted better performance on the Go/No-Go task. This is consistent with the positive association between tonic striatal dopamine activity and Go/No-Go performance (Frank & O'Reilly, 2006; Colzato et al.,2009). It is also consistent with higher tonic dopamine as a positive indicator of reward learning more generally (Beeler et al., 2010). However, Slagter et al (2015) were unable to establish a positive relationship between dopamine and learning from positive outcomes. In horses, McBride et al. (2022) found that increased dopamine (as measured by SEBR) was associated with increased impulsivity in the three-choice serial reaction time task. Comparably, impulsivity would relate to higher errors of emission, therefore worse performance in the current study. As such, the findings of the current study are at odds with the limited existing data in this species.

Interestingly, baseline blink rate was not a significant predictor of performance in the previous Reversal task, and it was suggested that this was because the task was not challenging enough (Chapter 3). The Go/No-Go task had the additional challenge of the light cue contingency which indicated to the horse whether they should offer their learned behavioural response to the target (Go) or whether they should inhibit this response (No-Go). Therefore, it is possible that SEBR is a valid indicator of tonic striatal dopamine activity and may be used as a proxy measure to predict individual learning performance in more challenging tasks.

Higher baseline heart rate variability predicted better performance, consistent with the findings of Chapter 3. This further indicates that horses with lower arousal in their home environment have better cognitive performance, which may relate to welfare and management (Hall et al., 2018). Taken together with the result that lower HRV during the task predicted better performance, this suggests a Yerkes-Dodson style relationship between arousal and performance (Yerkes & Dodson, 1908). Horses with higher arousal at baseline that experience reduced arousal during Go/No-Go may move to a moderate arousal state during learning, which according to Yerkes-Dodson law is optimum for performance. In contrast, those who start with higher arousal at baseline and become more aroused during learning may be too aroused to perform well. Similarly, those with lower arousal at baseline and reduced arousal during learning may be too relaxed (or under-aroused) to perform well, in accordance with the Yerkes-Dodson curve (Yerkes and Dodson, 1908). This may also relate to the Mendl et al. (2010) core effect axis (Figure X), because individuals in a relaxed and calm state may lack the motivation to move into the reward acquisition system (Mendl et al., 2010). Chapter 3 was not consistent with this pattern; lower HRV both at baseline and during learning predicted high performance. This could be due to the increased arousal required to pay attention to the light cue in Go/No-Go (Janelle, 2002; Zsidó, 2024), which was not present during Reversal.

5. Conclusions

Equestrian activities rely on successfully utilising the horse's innate behavioural responses, such as the flight instinct that is required for success in the sport of horse racing. It is also necessary, for safe and successful equestrian activities, that the horse learns to inhibit such behavioural responses in specific circumstances. In this chapter, physiological reactions to a Go/No-Go task which tests this ability were investigated. Horses appeared to show lower blink rate and higher blink rate variability when a combination of positive reinforcement and negative punishment was introduced, consistent with increased attention as the horse switched to discriminate responding to the light cue. Heart rate variability was higher in horses when cost was added, indicating that horses did not find the addition of cost to be stressful and suggests that control over outcomes may be rewarding to horses.

In addition, novel physiological predictors of inhibitory control performance were investigated. A decreased right eye temperature and lower heart rate variability during session 4 of the Go/No-Go task, when cost was first introduced, predicted better performance. This suggests that an increase in arousal was adaptive for performing well in the Go/No-Go, a relatively difficult task. However, horses that did not become too aroused by the task (indicated by a lower right eye temperature), were able to maintain good performance. Further, consistent with the valence hypothesis, this potentially suggests horses that did not experience negative valence associated with performed well. Higher baseline blink rate predicted better performance on the Go/No-Go task, consistent with an association between tonic striatal dopamine activity and Go/No-Go performance. Higher baseline heart rate variability predicted better performance, consistent with the findings of Chapter 3. The evidence of the thesis so far indicates horses with lower baseline arousal levels perform better on both simple tasks (Reversal) and more cognitively complex tasks (Go/No-Go). The applications for training and welfare will be discussed in detail within the General Discussion of this thesis.

CHAPTER 6

SORRY FOR YOUR LOSS: PHYSIOLOGICAL PREDICTORS OF SENSITIVITY TO REWARD VALUE AND LOSS

1. Introduction

Chapters 3 and 5 revealed that arousal in the home environment and during learning are significant predictors of cognitive performance. It was also demonstrated in chapters 3-5 that horses are capable of behavioural flexibility to maximise reward. For example, in Chapter 3 horses flexibly adapted their approach to the discrimination task when the reward-related contingency (rewarded target) reversed. In Chapter 4, horses appeared to adapt their approach towards the Go/No-Go task based on a cost-benefit analysis of reward.

In Chapter 5, horses were able to inhibit their learned behavioural responses to a conditioned target, to maximise their chance of reward and avoid the disappointment of losing opportunities to gain this reward. The current chapter investigated reward sensitivity in horses using two distinct test phases; 1) reward value discrimination and 2) response to reward loss. The purpose of reward value discrimination was to determine whether the sample of horses were able to discriminate between two targets of contrasting reward value evidenced by a preference for the high value target. The purpose of reward loss was to investigate horses' behavioural and physiological responses to a loss of expected reward. Additionally, in both phases, physiological measures were investigated as possible novel predictors of individual differences in reward sensitivity.

1.1 Reward sensitivity

Animals are biologically driven to seek reward and avoid punishment to increase their adaptive fitness (Spruijt et al., 2001). To this end, well-adapted animals demonstrate behavioural flexibility in their approach to reward acquisition. A lack of reward-seeking behaviour and flexibility can have dire consequences for survival. Animals seek primary reinforcers such as food, warmth, companionship, and mating, as these are the resources needed for survival. Animals experience positive affect as a result of engaging in rewarding behaviours (Gray, 1987) and this is critical for operant learning (Skinner, 1971). Reward sensitivity is the degree to which animals are responsive to reward (Cho et al., 2016) and can be tested by analysing responses towards rewards of differing values.

Investigating reward sensitivity can help us better understand the motivations underlying reward-related behaviours and utilise the most effective training methods. Reward sensitivity can also be used as a tool to indicate welfare states in animals (van der Harst & Spruijt, 2007). van der Harst & Spruijt (2007) define welfare as an interaction between stress and reward systems. For good welfare to be maintained, positive experiences (processed by the reward system) should outweigh negative experiences (processed by the stress system; van der Harst & Spruijt, 2007). Therefore, sensitivity to reward increases following negative experiences, due to the increased need for a positive experience to maintain positive welfare (Spruijt et al., 2001; van der Harst & Spruijt, 2007). Further, positive experiences can compensate for negative experiences. As such, reward sensitivity may be used as a welfare indicator because it reveals the current balance of reward versus negative experience that underpins welfare (Van der Harst & Spruijt, 2007). In this model, reward sensitivity is dependent on the valence of previous experiences. Additionally, it is proposed that repeated delivery of reward may improve welfare by shifting this balance towards the positive.

Previous chapters used binary presence/absence of reward in paradigms designed to investigate cognitive flexibility (Chapter 3) and cognitive control (Chapter 5). While this provided valuable insight into physiological predictors of adaptive learning, it could be argued that previous paradigms simply conditioned the horse to feel positive about one target and negative or neutral about the other. In the current chapter this went further, examining responses to two reinforcing stimuli, to investigate whether horses are able to make strategic choices (by preferentially selecting the high reward target), contributing further evidence in support of model-based learning (Chapter 4). In doing so, I investigate whether horses are able to hold a model of the relative value of each choice, rather than making purely associative decisions. If horses can do this, a significant preference for the higher value reward should be demonstrated.

1.2 Reward loss

In addition to reward sensitivity tests, affective states and welfare can be indicated by an individual's response to reward loss (Burman et al., 2008; Huston et al., 2013). Welfare is known to affect an individual's experience and perception of a loss of reward. For example, depression (Huston et al., 2013) and pain (Ede et al., 2023) both exacerbate responses to reward loss, whereas individuals in more positive welfare states are more robust and able to cope with loss (Burman et al., 2008). Therefore, sensitivity to reward loss is a useful indicator of emotion and welfare (Burman et al 2008). Human studies have revealed that individuals in a negative affective state are more sensitive to reward loss than those in good welfare states. As such, responses to reward loss (physiological or behavioural) may be used to indicate welfare (Burman et al., 2008).

Animal studies have investigated this (Berridge and Kringelbach, 2008; Burman et al., 2008; Huston et al., 2013), however to knowledge this has never been investigated in horses. This is despite the fact that horses are a large, highly reactive species that is potentially dangerous to humans (Budzyńska, 2014). This is relevant because reward loss can lead to frustration (Burman et al., 2008). A recent Delphi Consultation identified the following characteristics of frustration in horses, "increased locomotion, muscle tension, directed and redirected aggression, conflict or displacement behaviours; in chronic situations through the development of repetitive (stereotypic) behaviours and learned helplessness or shutdown behaviours" (Pannewitz and Loftus, 2023).

Learned helplessness is a significant welfare concern in riding school horses, who may regularly experience reward loss when handled and ridden by inexperienced people (Kaiser et al., 2006). For example, ridden activities primarily involve the use of negative reinforcement. Leg or rein pressure is removed to reward the desired locomotory response. In novice riders, the removal of pressure may be sporadic or mis-timed, resulting in the experience of reward loss for the horse. Additionally, some of the above-mentioned frustration behaviours, such as aggression, conflict or redirected behaviours, can be dangerous, (Pannewiz and Loftus, 2023). Therefore, understanding horses' responses to reward loss has applications for equine welfare, training and safety.

Successive negative contrast (SNC; Flaherty, 1996) describes the effect of an unexpected downshift in reward value on behaviour. It has been demonstrated that animals will slow down or stop responding completely to a conditioned stimulus, when reward is downshifted from expected value. However, individuals who have always been conditioned with the lower reward value will continue to respond (Crespi, 1942). This suggests a degree of sensitivity to reward loss (Tan et al., 2020). SNC studies therefore typically use two groups of animals: a 'shifted' and 'unshifted' group, who are both conditioned to give a behavioural response to a stimulus, however the 'shifted' group experience a sudden and unexpected shift (down or upwards) in reward value, whereas the 'unshifted' group are rewarded with the same value throughout. This relies on a large sample size and as such as primarily been studied using laboratory animals such as rats (Phelps et al, 2015) and zebrafish (Tan et al., 2020). The current study aimed to replicate some of the features of a SNC design but adapted for use in a relatively smaller sample of horses.

If horses can recognise and select higher rewarding stimuli, they may recognise and respond to an unexpected loss of reward value in one of three ways. First, horses in a low welfare state would avoid the reduced value target as it induces disappointment which is exacerbated by their negative affective state. This theory has been explored in successive negative contrast (SNC) experiments (Crespi 1942). Second, high welfare state individuals would persevere with the reduced value target in the hope that it may elicit the expected reward value. Such horses demonstrate positive judgement bias, indicative of their high welfare state (Mendl et al., 2009). Alternatively, horses may recognise that both targets now pay the same, therefore there is no advantage to choosing one target over the other, resulting in selecting targets at chance rate. Cognitive bias is related to stress and welfare states (Mendl et al., 2009) including in this species (Henry et al., 2017). As such, physiological measures of welfare may predict responses to reward loss.

1.3 Aims of the Chapter

This chapter investigated reward sensitivity in horses using two distinct test phases: reward value discrimination and reward loss. The aim of the chapter was 1) to determine if horses are sensitive to reward value discrepancy and loss of reward and 2) to investigate whether there were physiological responses to these two phases associated with subject choices. In particular following the results of previous chapters in which eye temperature changes predicted learning performance, this chapter investigated the valence hypothesis of hemispheric lateralisation. Physiological measures were investigated as possible novel predictors of reward sensitivity and response to reward loss. Blink rate (SEBR) and blink rate variability (BRV) were measured as potential proxy indicators of striatal dopamine activity. Heart rate variability (RMSSD) and eye temperature (IRT) were measured as indicators of arousal. Lateralised differences in ocular temperature were measured as potential proxy measures for hemispheric lateralisation.

1.4 Predictions

It was hypothesised that higher baseline heart rate variability would predict better (more strategic) performance in the reward value discrimination phase (Eysenck, 1976), consistent with previous chapters. Further, in the reward loss phase higher baseline HRV should predict increased avoidance of the reduced value target, consistent with the theory that better welfare predicts more optimistic responding (Mendl et al., 2009). However, lower heart rate variability during the reward value discrimination phase was expected to predict better performance, consistent with Chapter 5 in which increased arousal was required for a more complex task. In the reward loss phase, lower HRV was expected to predict increased avoidance of the RVT as more stressed individuals may be more susceptible to reward loss (Katahira et al., 2014).

It was hypothesised that higher baseline blink rate, as an indicator of higher tonic dopamine, would predict performance because increased tonic dopamine is associated with reward sensitivity in humans (Jongkees and Colzato, 2016). Higher blink rate was expected to predict better performance in the reward value discrimination phase and avoidance of the RVT in the reward loss phase, because increased blink rate is associated with frustration in horses (Ricci-Bonot and Mills, 2023). Further, higher blink rate variability was expected to predict better performance, consistent with data in humans (Paprocki & Lenskiy, 2017).

According to the right hemisphere hypothesis of lateralisation, it was hypothesised that the left eye temperature would increase in better performing horses in the reward value discrimination phase due to left hemisphere dominance during target selection and feeding behaviour (Rogers, 2021), and consistent with Chapter 3. According to the valence hypothesis, right eye temperature increase should predict avoidance of the reduced value target, due to right hemisphere processing of negatively valanced emotions (Schwartz et al., 1979; Killgore and Yurgelun-Todd, 2007). Also, in line with the valence hypothesis, it was predicted that horses choosing the target presented on their left first would be more likely to avoid the reduced value target in the reward loss phase, due to a negative bias processed in the right hemisphere (Schiffer et al., 2004; Marr et al., 2018).

2. Methods

Ethical Statement and Subject information are presented in the General Methods.

2.1 Experimental stimuli

The experimental stimuli consisted of two novel A3 size laminated cue cards, each presented in the display window of an A3 poster stand (1.075m H x 0.33m W x 0.24m D). Both cue card targets consisted of a white background with a black symbol at the centre. White and black are both shades known to be visible to horses (Murphy et al., 2009; Roth et al., 2007). One target displayed a symbol of a teapot (referred to in this chapter as "teapot"), and the other target displayed a symbol of a sun (referred to as "sun"). These symbols were chosen based on previous work identifying the visual discrimination abilities of the horse (Hanggi & Ingersoll, 2009; Mejdell et al., 2016).

In reward discrimination trials the targets had differing reward values, with one acting as the high value target (HVT) and the other acting as the low value target (LVT). The assignment of HVT or LVT to either the sun or teapot was randomised and counterbalanced prior to training (Appendix I). The reward value of the HVT was three pellets of a palatable mint flavoured horse feed (Bailey's Minty Treats). The feed was high in fibre, low in calories and, according to the manufacturer's website (Bailey's Horse Feeds, 2024), contained no vitamins or minerals. This meant that any effects on the nutritional composition of the diet were limited. The reward value of the LVT was one pellet of the same feed. This feed has larger pellets than Ulsakind (the food reward used in previous chapters), making it more suitable for a reward quantity discrimination task. Pellets were consistent in size and weighed approximately 4.3 grams per pellet. The decision to alter the quantity of reward rather than the quality of reward was based on a recent study which found that horses responded better to contrasts in reward quantity than reward quality (Brucks et al., 2022). Additionally, this avoided confounding results with individual differences in taste preference (van den Berg et al., 2016). Further, horses are known to discriminate between contrasting quantities of small numerical value (Uller & Lewis, 2009), therefore it was expected that horses would be able to distinguish between one and three pellets.

2.2 Pre-conditioning

All subjects underwent six sessions of target conditioning, on separate days within a two-week period in March 2023. Horses had three sessions of HVT conditioning and three sessions of LVT conditioning, in a randomised and counterbalanced order (Appendix I). Three sessions to learn each target value was selected to ensure subjects had the same opportunity to learn the task as in all previous chapters. During a conditioning session, the relevant target was presented directly in front of the horse on an A3 poster stand (Figure 6.1a). Any touch was rewarded with a whistle sound and the corresponding food reward for that target. When rewarding touches of the HVT, the trainer (standing directly behind the target) handed three pellets of the food reward, one by one, to the horse. The food delivery was staggered in this way to exaggerate the contrast in reward quantity between the HVT and LVT and increase the likelihood that the greater quantity was detected.

Horses were allowed to touch the target a maximum of 20 times before the session ended so that a maximum of 60 pellets were given in a single session. These target conditioning sessions occurred in a random order, pre-determined by a random number generator.

Appendix I gives the order of pre-conditioning for each horse, including the assigned reward values of each target.

2.4 Experimental conditions

In the week following pre-conditioning, all twenty horses were tested in a reward value discrimination task (RD). Horses were presented with the HVT and LVT side-by-side (Figure 6.1b) for the first time and given twenty opportunities to give the pre-conditioned touch response to the target of their choice. The side the targets were presented on was pseudo-randomised in a counterbalanced design, as the HVT was presented on the left for 10 subjects and on the right for 10 subjects, to account for potential side biases in the population (Appendix II). It was not suitable to randomise the position of the targets in each trial, as this would involve manually placing targets in front of the horse which could cue horses' responses, as in the Clever Hans phenomenon (Murphy and Arkins, 2007). As the targets remained stationary throughout the session, there was no set inter-trial interval. The only limitation was the latency to deliver the food reward between touches. Horses were otherwise free to pace their responses to the targets as desired.

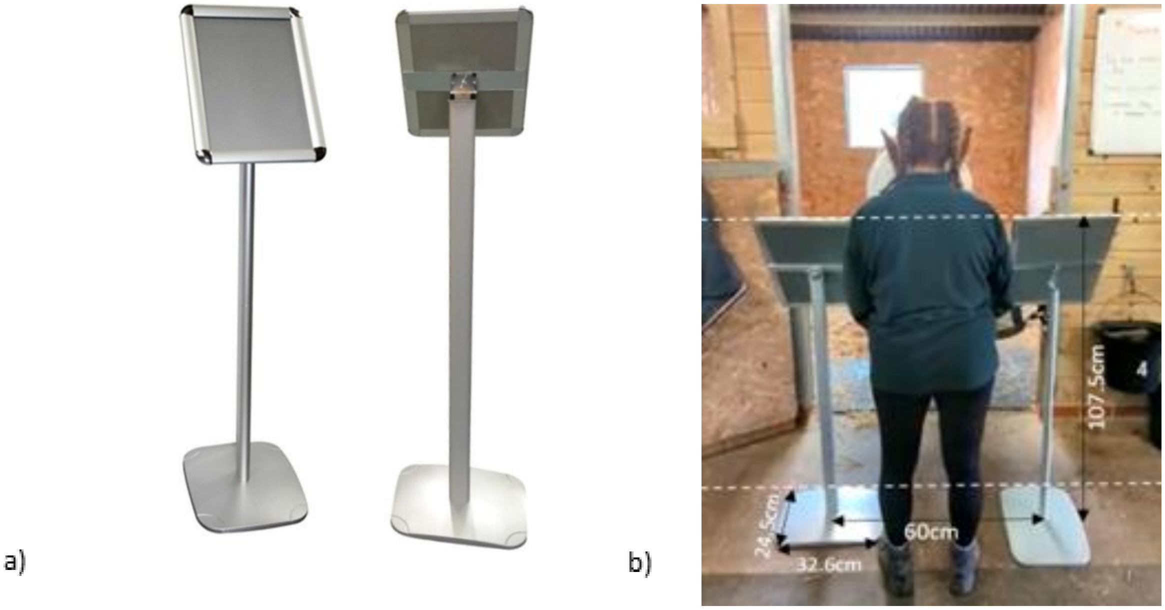

Figure 6.1a) A3 Poster stand used to present the experimental stimuli (dimensions= height 107.5cm x width 32.6cm x depth 24.5cm). b) Photograph showing the position of the targets during data collection. Targets were placed within reaching distance of the horse, level with one another so that neither target was closer to the horse than the other. The distance was not standardised due to individual differences in body size and neck length.

If the horse chose the HVT, three pellets of the food reward were given individually, as in the pre-conditioning. If the horse chose the LVT, one pellet was given. Therefore, the optimum response pattern of 20 HVT choices would result in receiving 60 food rewards in total and the least strategic response of 20 LVT responses would result in only 20 rewards in total. The limit of twenty choices prevented over-feeding, as it meant that the maximum number of pellets given was sixty, if the horse chose the HVT every time. This was equal to approximately 260 grams of food, an amount which was approved by the yard manager, and primary care technician, at Brackenhurst Equine Centre.

The following week, all horses were tested in the reward loss task (RL). Horses were presented with both targets side-by-side again and given twenty opportunities to select the target of their choice. However, this time both targets had the same reward value. Again, the HVT was presented on the left for 10 subjects and on the right for 10 subjects, but targets were presented on the opposite side to the previous RD phase, in a pseudo-randomised, counterbalanced (Appendix III). Touches of either target were rewarded with one pellet of feed. Therefore, while the LVT remained consistent, the HVT reduced in value in a manner intended to induce disappointment. Therefore, the LVT became the consistent value target (CVT) and the HVT became the reduced value target (RVT; Figure 6.2).

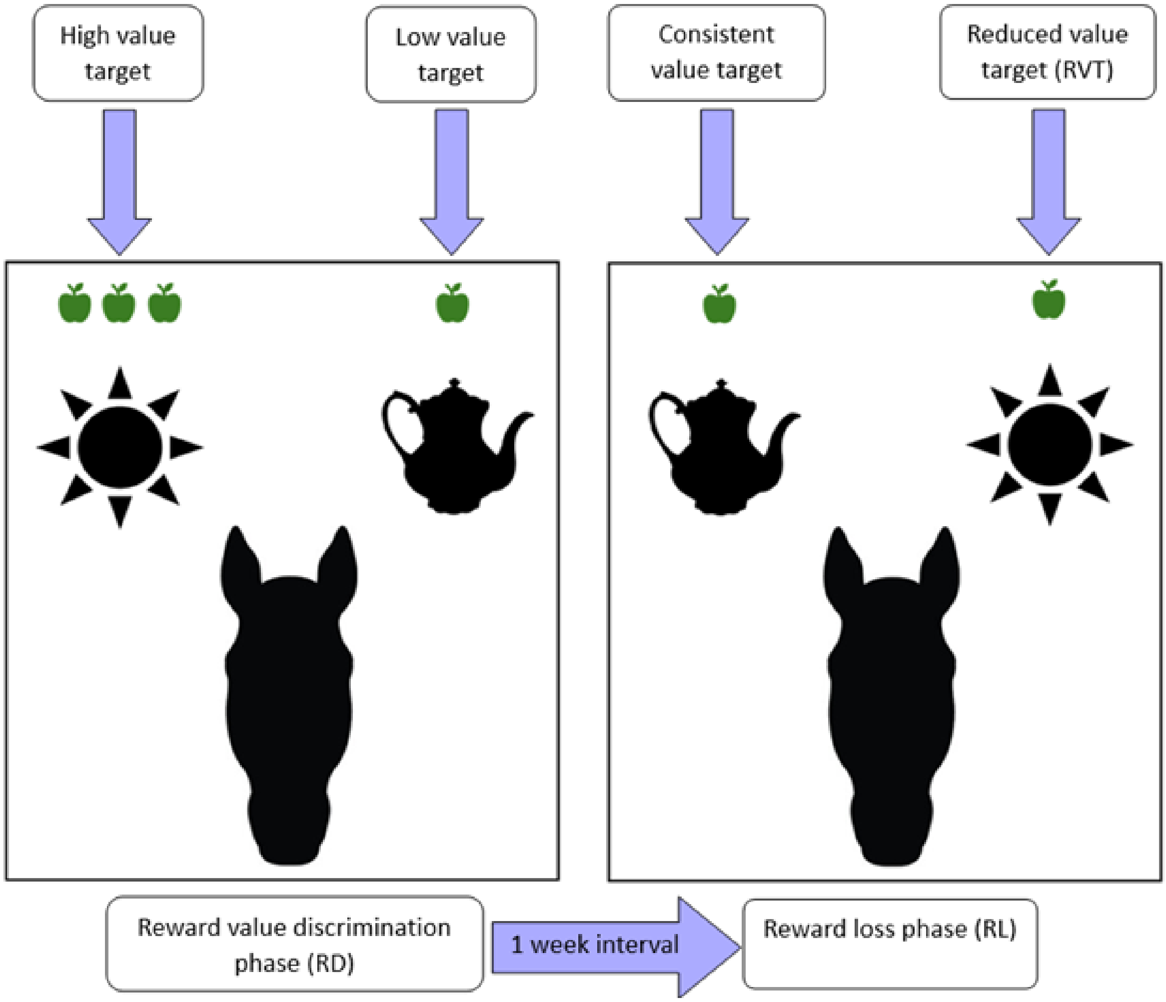

Figure 6.2. Diagram representing an example of the experimental conditions in each phase.

2.5 Physiology

Blink rate, blink rate variability, heart rate variability and eye temperature were all measured as described in General Methods, including baseline measures which were taken from the same group of horses undisturbed in their home environment. Physiological data were cleaned and analysed as previously described.

2.6 Performance

Two independent, blinded raters analysed all video footage of the reward value discrimination and reward loss trials to ensure unbiased categorisation of responses as either HVT or LVT. Raters could see whether the subjects chose targets on the left or right but could not see whether the targets were HVT or LVT, nor could they deduce this from the reward delivery (1 or 3 treats) as both were obscured from view due to the camera position. Raters recorded whether the horse chose the left or right option in each of their twenty choices (but were blind to the position of HVT).

Inter-rater reliability was calculated using Cohen's kappa. Inter-rater reliability was 0.69 (unweighted κ=0.69, weighted κ=0.97). Though 0.69 indicates substantial agreement (McHugh, 2012), the classification of Cohen's kappa is debated in the literature (O'Leary et al., 2014; Sun, 2011) and κ was lower than expected given the binary nature of the observation. This was caused by the camera angle; gentle touches of the target appeared almost identical to when they were simply inspected by the subject before making a different choice. Therefore, a third rater analysed the ten videos in which disagreement occurred. The third rater was blind to the position of either target but was present during data collection and did have a more accurate understanding of the mechanics of the target selection by subjects. The author, who was not blinded to the target position or reward value, then used the side choices, corroborated by rater three, to determine the percentage of high value target (HVT) choices in reward discrimination and reduced value target (RVT) choices in reward loss.

Performance in reward discrimination was calculated as the number of HVT choices divided by the time taken to complete the task. This was adapted from the Rate-Correct Score (Woltz and Was, 2006), which accounts for both speed and accuracy in two-choice discrimination tasks (Vandierendonck, 2018). The speed of responses can provide valuable insight into horses' individual approaches to the task such as impulsivity which may be revealed by quick and low paying responses. The Rate-Correct Score allows for this to be included in the statistical model. The successive negative contrast model also uses a combination of response latency and choices made (Phelps et al., 2015).

Performance in reward loss was calculated as the number of RVT choices divided by time taken to complete the task. The RVT was the target of interest because horses had previously been conditioned that this target was worth a higher value reward and it was now reduced in value. Therefore, sensitivity to reward loss could be evaluated by observing responses to this target. Perseverative responding to the RVT may indicate that horses were more optimistic about the higher reward value being reinstated, whereas avoidance of this target may indicate that horses were negatively affected by the loss of expected reward.

2.7 Statistical analysis

Statistical analysis was performed in R (version 4.3.2; R Core Team, 2023). Data were analysed for normal distribution using Shapiro Wilks. Friedman tests were performed to determine the effect of each condition (Baseline, Reward Sensitivity and Reward Loss) on physiology, followed by post-hoc paired t-tests and Wilcoxon signed ranks tests as appropriate for normality.

To determine whether target biases occurred, chance rates were calculated. A binomial distribution calculator revealed that the number of successful attempts required for preference above chance level was ≥15/20 (95% confidence interval). A one-sample t-test was used to determine whether group-level target choices were statistically above chance. The hypothetical mean was set at 10 to represent half of the available attempts, due to the two-choice nature of the task.

A post-hoc analysis of target responses in the final ten attempts of the reward value discrimination phase was conducted, following an observation that responses appeared to shift towards the high value target (Figure 6.2). The final ten attempts were chosen because, as seen in Figure 6.2, group level choices appeared to shift towards the high value target from attempt ten onwards. This may indicate that horses used the initial ten attempts to explore the two targets and compare their respective reward values, therefore these initial attempts may not fully reflect understanding (see *Section 4.1* for discussion of this finding). A binomial distribution calculator revealed that the number of successful attempts required for preference above chance level was ≥8/10 (95% confidence interval). A one-sample t-test was used to determine whether group-level target choices were statistically above chance. The hypothetical mean was set at 5 to represent half of the available attempts, due to the two-choice nature of the task.

Negative binomial generalised linear models were carried out using the MASS package (Venables and Ripley, 2002) to test whether any of the physiological measures could predict performance in each test phase. An additional, post-hoc model was carried out to predict performance in the final ten attempts of the reward value discrimination phase. Models were carried out according to the 10-step process recommended by Zuur and Ieno (2016). The negative binomial model is suitable for over-dispersed data (high variability in the dataset; Zuur and Ieno, 2016), which applied here. Data were examined for missing values, outliers, zeros and collinearity using variance inflation factor (VIF). A stepwise regression using Akaike Information Criterion (AIC) and significance (p) value was conducted to find the best model fit. Table 6.1 provides details of all covariates included in the models, with justification for inclusion and specific hypotheses.

Table 6.1. Details of the covariates included in the statistical models, with reasons for inclusion in the model and hypotheses.

Covariate	**Reason for inclusion in the model**	**Hypothesis**
Baseline heart rate variability (RMSSD).	As a measure of baseline stress sensitivity, which may predict learning performance (Eysenck, 1976; Yerkes and Dodson, 1908), consistent with previous chapters.	Higher baseline heart rate variability will predict better performance, consistent with previous chapters.
Heart rate variability (RMSSD) during task.	As a measure of physiological arousal during learning. Arousal may predict learning performance, as in previous chapters. HRV is negatively correlated with tendency to avoid negative emotional/unpleasant outcomes (Katahira et al., 2014).	Lower heart rate variability during RD will predict better performance, consistent with Chapter 5. This is due to the increased difficulty of the later tasks compared to Chapter 3. In RL, lower HRV will predict increased avoidance of the RVT as more stressed individuals may be more susceptible to reward loss.

Baseline blink rate (blinks per minute).	Baseline blink rate is reflective of tonic striatal dopamine (Jongkees & Colzato, 2016) which may predict individual sensitivity to reward.	Higher baseline blink rate, as an indicator of higher tonic dopamine, will predict performance because increased tonic dopamine is associated with reward sensitivity in humans.
Blink rate (blinks per minute) during task.	This study was interested in whether phasic blink rate may be a useful predictor of reward sensitivity. Increased blink rate is associated with frustration in horses (Ricci-Bonot and Mills, 2023).	Higher blink rate will predict better performance in RD and avoidance of the RVT in RL.
Blink rate variability (RMSSD) during task.	Blink rate variability may offer a more appropriate method for investigating the relationship between SEBR and learning performance. Research in humans suggests that higher blink rate variability predicts better performance (Paprocki & Lenskiy, 2017) . This is because blink frequency drops during visual	Higher BRV will predict better performance, consistent with the human literature.

	attention and then increases in characteristic blink clusters during processing.	
Left eye temperature change (°C)	As a marker of arousal in response to learning. May predict learning performance.	Left eye temperature increase will predict better performance in RD due to left hemisphere dominance during reward value discrimination.
Right eye temperature change (°C)	As a marker of arousal in response to learning. May predict learning performance.	Right eye temperature increase will predict avoidance of RVT due to right hemisphere processing of negatively valanced emotions (valence hypothesis).
First choice (Left or Right).	Subjects' first choice (left or right) was included in the model to account for preferences due to primacy bias. Laterality may also predict performance, following the valence hypothesis.	Primacy hypothesis: Horses' first choices will predict their overall preference due to primacy bias. Valence hypothesis: horses that choose left first will be more likely to avoid the reduced value target in RL.

3. Results

3.1 Performance

In the reward value discrimination phase, there was no significant preference for either target (t=0.579, df=19, p=0.572). Only 7/20 horses chose HVT ≥15/20 times (chance level). The mean±sd latency to touch in the reward sensitivity phase was 8.20±2.26 and mean±sd latency to complete the task (all 20 trials) was 164.05±45.29 seconds. Performance scores (number of HVT choices/latency to complete task) ranged from 0-6.45 (mean±sd=3.74±2.01).

Post-hoc analysis of the final ten choices of the discrimination phase revealed no significant preference for either target in the final ten choices (t=0.276, df=19, p=0.785). Only 8/20 horses chose HVT ≥8/10 times (chance level) in the final ten attempts. Additionally, there was no significant difference between the number of high value target choices in the first five and the last five attempts at the reward value discrimination phase (t=-1.412, df=19, p=0.174).

In the reward loss phase, there was a marginally significant preference for the consistent value target (t=2.1, df=19, p=0.049). Sixteen horses chose CVT ≥15/20 times (chance level). The mean±sd latency to touch in the reward loss phase was 5.13±1.09 seconds and mean±sd latency to complete the task (all 20 trials) was 102.60±21.92 seconds. Performance scores (number of RVT choices/latency to complete task) ranged from 0-10.79 (mean±sd=4.15±3.24). Figures 6.3 and 6.4 show the group average choices across the twenty attempts in reward discrimination (Figure 6.3) and reward loss (Figure 6.4).

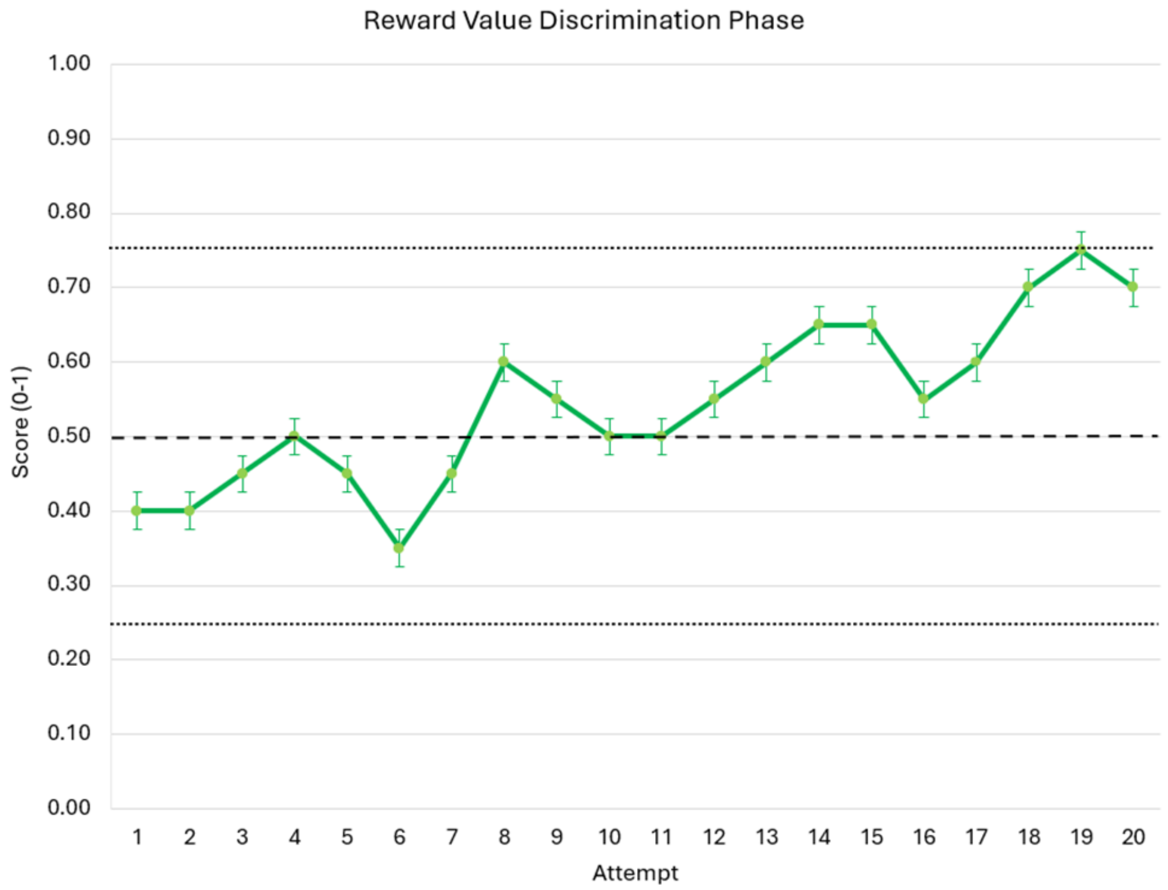

Figure 6.3. Graph showing group level choices across attempts during the reward value discrimination phase. Individual choices in each attempt were scored as 0=Low Value Target and 1= High Value Target. Points on the graph represent the mean score of all subjects in each attempt, with error bars. The dashed black line represents equal HVT and LVT choices across the sample. The dotted lines represent the above-and-below-chance levels (0.75 and 0.25, respectively). The maximum score of 1 indicates that all horses chose the HVT and the minimum score of 0 indicates that all horses chose the LVT. The graph illustrates a shift towards the high value target in the latter attempts, possibly suggesting that horses may have required the initial attempts to consolidate their previous conditioning that the HVT elicited a higher reward value. However, post-hoc analysis revealed no significant difference between the initial five attempts and the final five attempts (t=-1.412, df=19, p=0.174).

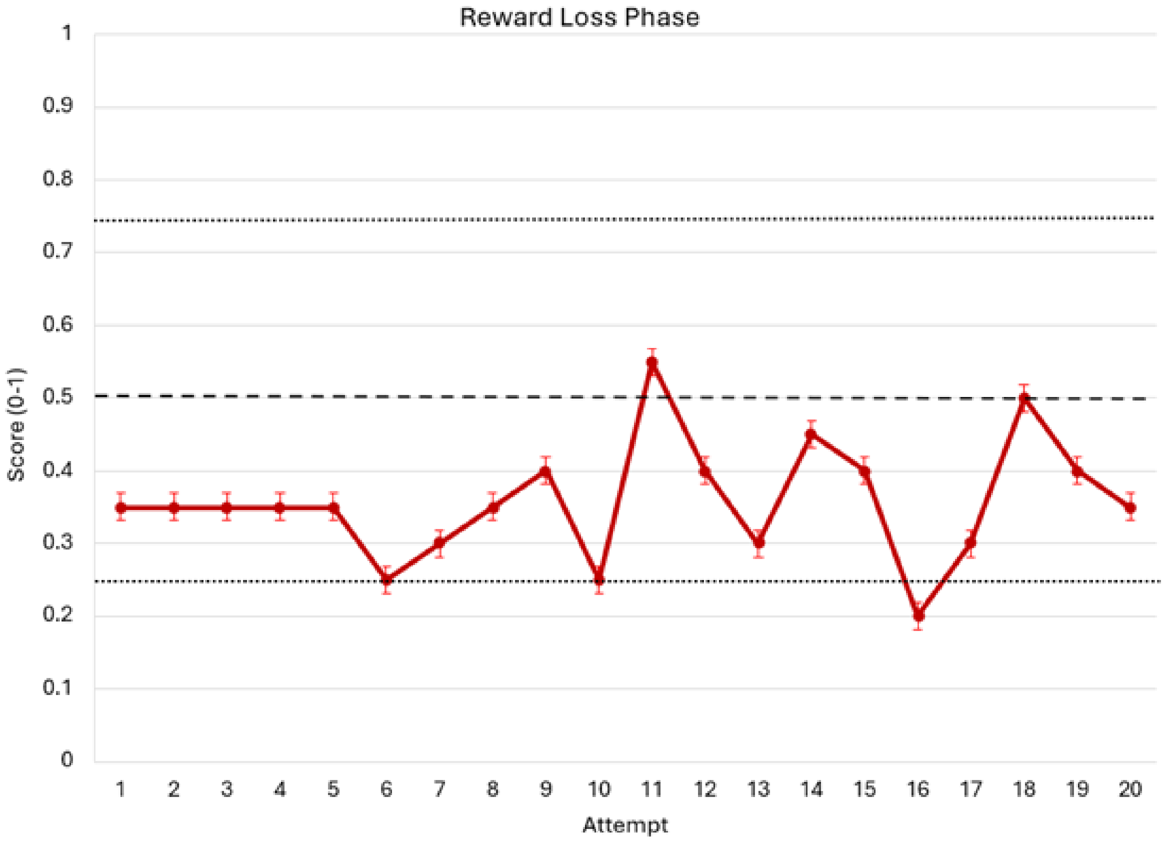

Figure 6.4. Graph showing group level choices across attempts during the reward loss phase. Individual choices were scored as 0= Consistent Value Target and 1= Reduced Value Target. Points on the graph represent the mean score of all subjects in each attempt, with error bars. The dashed black line represents equal RVT and CVT choices across the sample. The dotted lines represent the above-and-below-chance levels (0.75 and 0.25, respectively). The maximum score of 1 indicates that all horses chose the RVT and the minimum score of 0 indicates that all horses chose the CVT. The graph shows a preference for the consistent value target across attempts, indicating that horses preferred the target, which was previously low and remained low in value, rather than the target which started high and reduced in value.

3.2 Physiological responses to the task

A Friedman test revealed no significant effect of test phase (baseline, reward discrimination and reward loss) on SEBR ($X^2=3$, df=2, p=0.223; Figure 6.5). There was a significant effect of test phase on blink rate variability ($X^2=19.111$, df=2, p<0.001; Figure 6.5). Post-hoc tests revealed that BRV was significantly lower during reward discrimination than at baseline (t=4.02, df=17, p<0.001); BRV was significantly lower during reward loss than at baseline (Wilcoxon V=189, p<0.001); and BRV was significantly lower during reward loss than reward discrimination (Wilcoxon V=133, p=0.038; Figure 6.6).

A Friedman test revealed no significant effect of test phase on heart rate variability ($X^2=2.8$, df=2, p=0.247; Figure 6.7). There was no significant difference in eye temperature from pre-task to post-task in reward discrimination (left: t = -0.1, df = 18, p=0.923; right: t = -1.32, df = 18, p=0.203) or reward loss (left: Wilcoxon V=85, p=0.393; right: t = -0.44, df = 18, p=0.661; Figure 6.8).

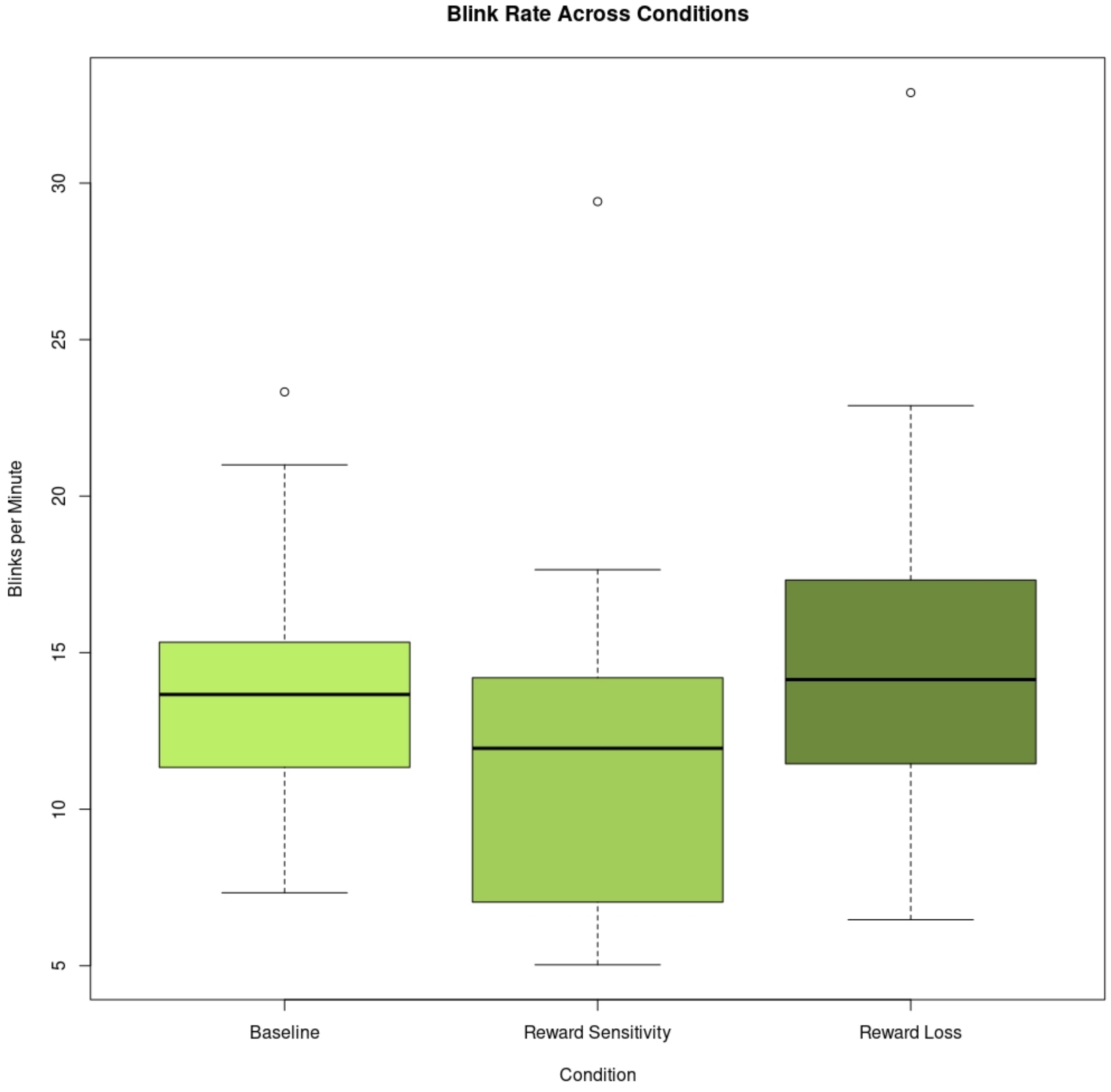

Figure 6.5. Boxplot showing SEBR (blinks per minute) across baseline, reward value discrimination and reward loss ($X^2=3$, df=2, p=0.223). Boxes represent the interquartile range and whiskers represent the minimum and maximum values. The bold line indicates the median value. Outliers are represented by circles.

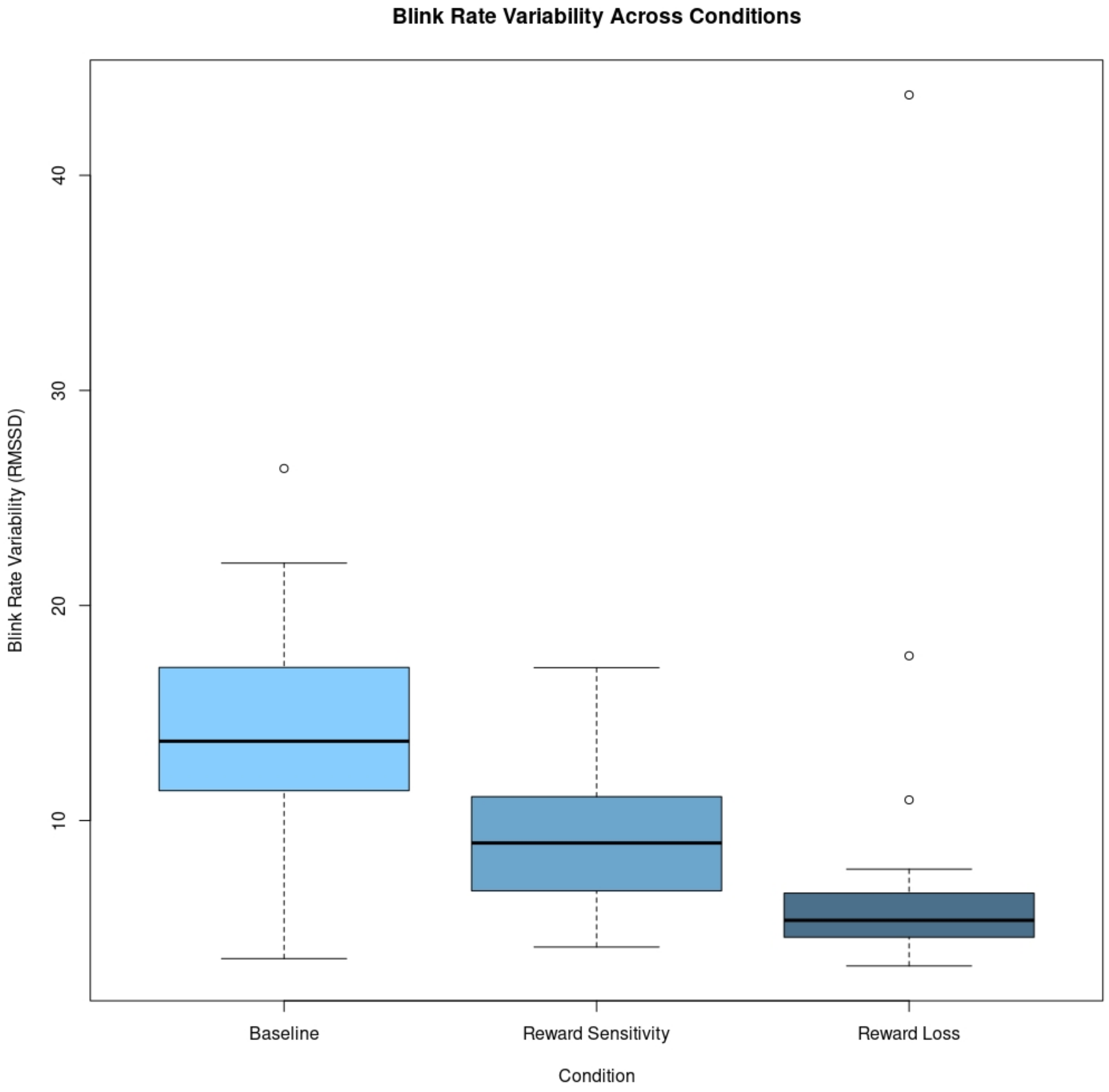

Figure 6.6. Boxplot showing blink rate variability (RMSSD) across baseline, reward value discrimination and reward loss (X^2=19.111, df=2, p<0.001). Boxes represent the interquartile range and whiskers represent the minimum and maximum values. The bold line indicates the median value. Outliers are represented by circles.

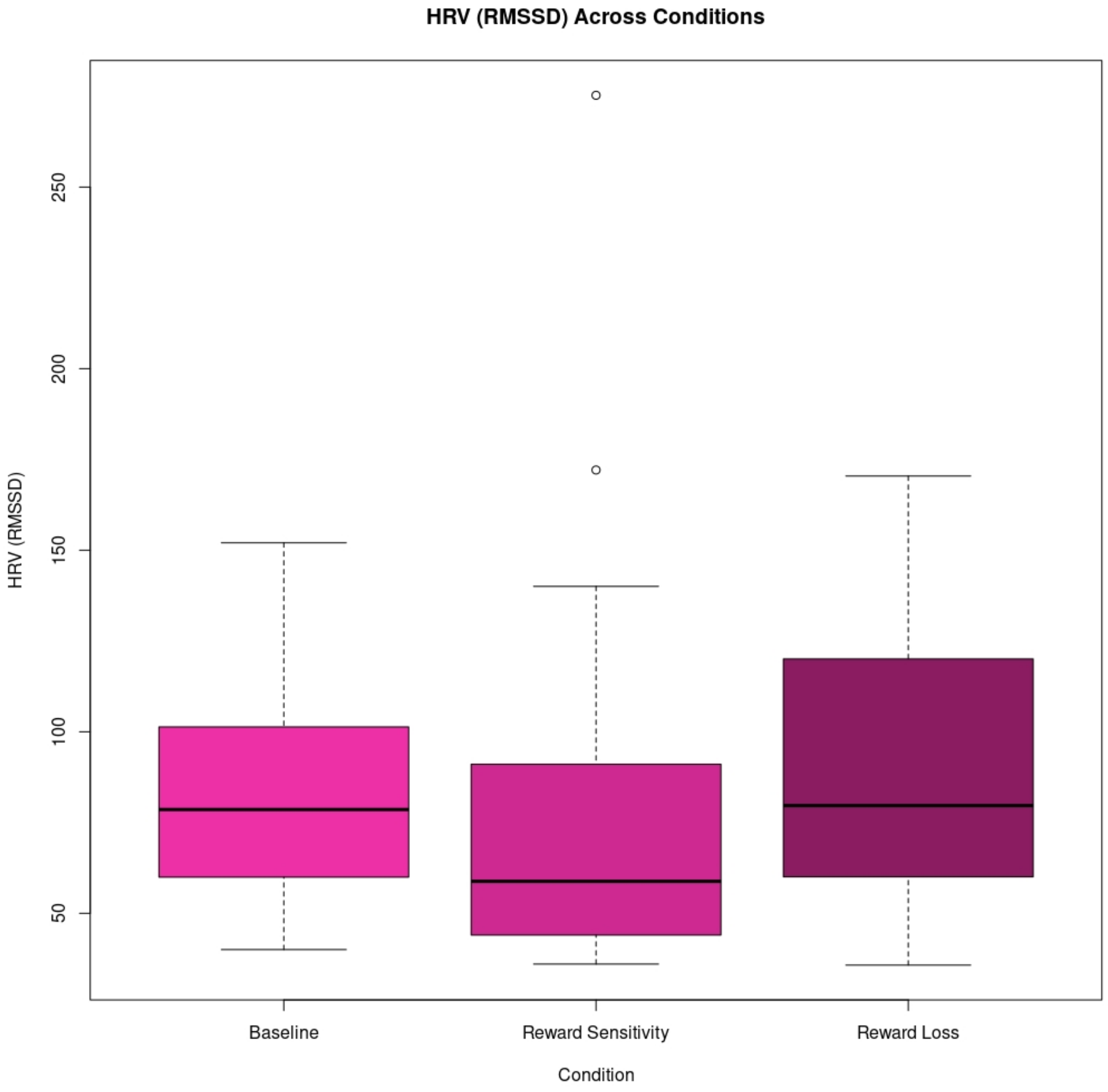

Figure 6.7. Boxplot showing heart rate variability (RMSSD) across baseline, reward value discrimination and reward loss conditions (X^2=2.8, df=2, p=0.247). Boxes represent the interquartile range and whiskers represent the minimum and maximum values. The bold line indicates the median value. Outliers are represented by circles.

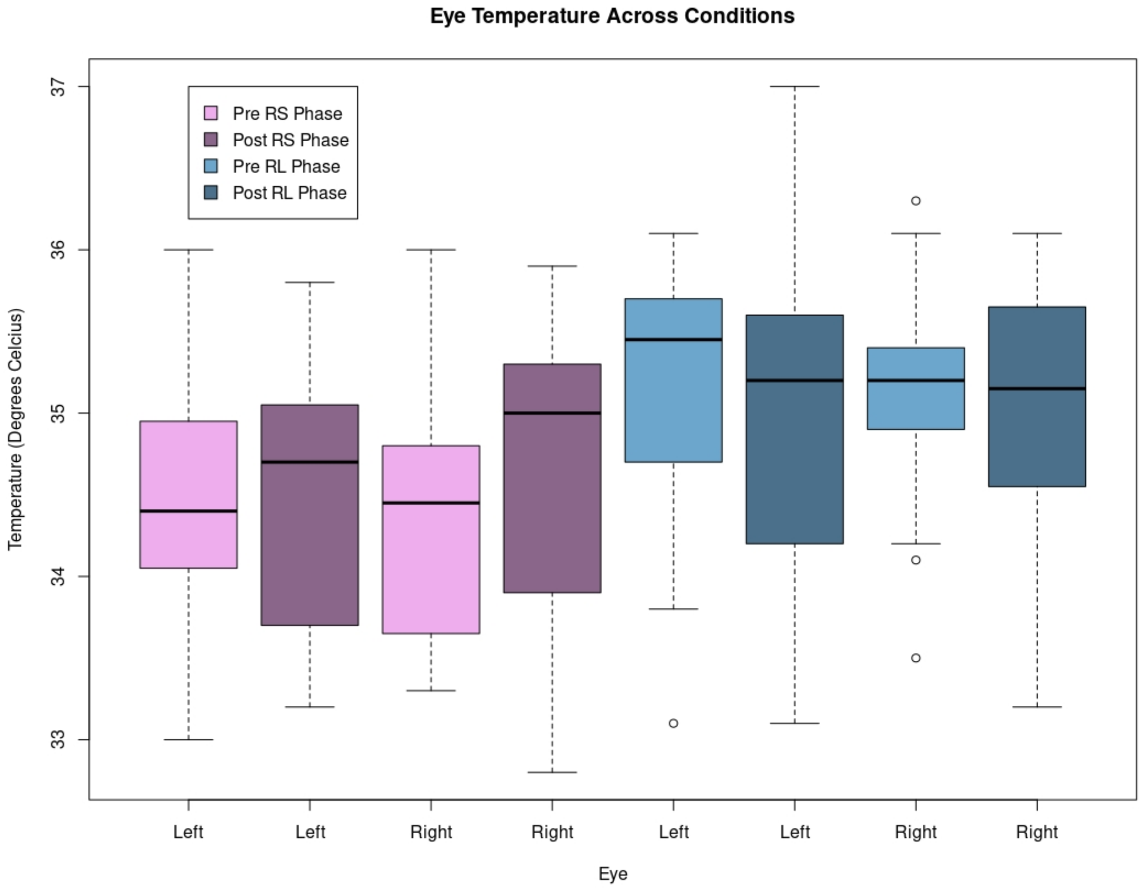

Figure 6.8. Boxplot showing eye temperature (°C) pre- and post- reward discrimination (pink; left: t = -0.1, df = 18, p=0.923; right: t = -1.32, df = 18, p=0.203) and reward loss (blue; left: Wilcoxon V=85, p=0.393; right: t = -0.44, df = 18, p=0.661) conditions. The eye (left or right) is given along the x-axis and condition is indicated by the colour key (RS= Reward Sensitivity and RL= Reward Loss). Boxes represent the interquartile range and whiskers represent the minimum and maximum values. The bold line indicates the median value. Outliers are represented by circles.

3.3 Statistical models

A negative binomial GLM revealed no significant predictors of performance in the reward value discrimination phase (Table 6.2). There were also no significant predictors of performance when only the last ten choices were included in the model (Table 6.3).

In contrast, heart rate variability (Z=2.882, p=0.004) and baseline blink rate (Z=2.406, p=0.016) were significant positive predictors of performance in the reward loss phase (Table 6.4). The lateralised location of subjects’ first choice (left) was a negative predictor of performance in the reward loss phase (Z=-2.949, p=0.003). Horses that chose the target to their left on their first choice were significantly less likely to choose the reduced value target overall.

Table 6.2. Results of the Negative Binomial GLM to predict performance in the reward value discrimination phase. Significance codes: 0 = ***, 0.001 = **, 0.01 = *, 0.05 = ‘.’, 0.1 = n/s. Bold text indicates significant predictors.

Covariate	**Estimate**	**Std. Error**	**Z value**	**p-value**	**Significance**
Intercept	1.636	1.376	1.189	0.235	n/s
Baseline heart rate variability	0.003	0.006	0.534	0.593	n/s
Heart rate variability during reward value discrimination	0.006	0.004	1.517	0.129	n/s
Baseline blink rate	-0.037	0.052	-0.693	0.488	n/s
Blink rate during reward value discrimination	0.037	0.024	1.540	0.124	n/s
Blink rate variability during reward value discrimination	-0.087	0.057	-1.541	0.123	n/s
Left eye temperature change	0.276	0.295	0.937	0.349	n/s
Right eye temperature change	0.236	0.374	0.631	0.528	n/s
First choice (Left)	-0.762	0.502	-1.518	0.129	n/s

Table 6.3. Results of the post-hoc Negative Binomial GLM to predict performance in the final ten attempts of the reward value discrimination phase. Significance codes: 0 = ***, 0.001 = **, 0.01 = *, 0.05 = ‘.’ , 0.1 = n/s. Bold text indicates significant predictors.

Covariate	**Estimate**	**Std. Error**	**Z value**	**p-value**	**Significance**
Intercept	3.846	2.225	1.729	0.118	n/s
Baseline heart rate variability	0.009	0.009	1.011	0.339	n/s
Heart rate variability during reward value discrimination	-0.003	0.005	-0.623	0.549	n/s
Baseline blink rate	-0.983	0.089	-1.096	0.302	n/s
Blink rate during reward value discrimination	-0.087	0.064	-1.345	0.211	n/s
Blink rate variability during reward value discrimination	-0.035	0.106	-0.326	0.752	n/s
Left eye temperature change	0.821	0.448	1.831	0.100	n/s
Right eye temperature change	-0.806	0.537	-1.499	0.168	n/s

Table 6.4. Results of the Negative Binomial GLM to predict performance in the reward loss phase. Significance codes: 0 = ***, 0.001 = **, 0.01 = *, 0.05 = '.' , 0.1 = n/s. Bold text indicates significant predictors.

Covariate	Estimate	Std. Error	Z value	p-value	Significance
Intercept	-0.151	1.145	-0.132	0.895	n/s
Baseline heart rate variability	-0.002	0.005	-0.364	0.715	n/s
Heart rate variability during Reward Loss	**0.021**	**0.007**	**2.882**	**0.004**	**
Baseline blink rate	**0.103**	**0.043**	**2.406**	**0.016**	*
Blink rate during Reward Loss	0.021	0.035	0.594	0.552	n/s
Blink rate variability during Reward Loss	-0.022	0.021	-1.042	0.297	n/s
Left eye temperature change	0.246	0.314	0.784	0.433	n/s
Right eye temperature change	0.059	0.346	0.171	0.864	n/s
First choice (Left)	**-1.897**	**0.643**	**-2.949**	**0.003**	**

4. Discussion

4.1 Performance

This chapter investigated reward sensitivity in horses using two distinct test phases, 1) reward value discrimination and 2) response to reward loss. The purpose of reward discrimination was to determine whether the sample of horses were able to discriminate between two targets of contrasting reward value and show a preference for the high value target. The purpose of reward loss was to investigate horses' responses, including both behavioural and physiological responses, to a loss of expected reward intended to induce disappointment. In both phases, physiological measures were investigated as possible novel predictors of responses to varying reward paradigms.

Unexpectedly, there was no significant preference for the high value target in RD at the group level, which could indicate that six conditioning sessions were not adequate to associate the targets with their respective reward value. This species has demonstrated an ability to discriminate based on quantity in small numerical contrasts (Uller and Lewis, 2009). However, it is important to note that this may not necessarily indicate higher cognitive abilities (McLean, 2001). African Grey parrots have been observed 'subitising', which is defined as an intuitive or perceptual apprehension of numbers (Pepperberg, 1987; McLean, 2001). Taken together with the results of Uller and Lewis (2009), this indicates that animals, including horses, may have an intuitive ability to apprehend quantity in small numbers.

Additionally, horses were able to discriminate based on reward value in a delayed gratification paradigm (Brucks *et al.*, 2022). Therefore, alternative explanations for the apparent lack of reward value discrimination in the current study should be explored. First, it is possible that this test phase, as it was subjects' first experience of both targets side-by-side, was required as an exploratory or learning phase, rather than an opportunity to make strategic choices (as intended by the researchers).

As seen in Figure 6.2, subjects' choices appeared to shift towards the HVT in the later attempts, indicating a learning effect throughout the test. If the initial attempts were exploratory, this could explain why there was no significant preference for the HVT overall. Post-hoc analysis revealed no significant preference above chance level in the final ten attempts of the reward discrimination phase and, despite the trend seen in Figure 6.2, there was no significant difference between HVT choices in the first and last five attempts. Therefore, initial exploration does not necessarily explain the lack of reward value sensitivity in this phase. Future research should include more attempts beyond the twenty allocated for this task, to account for exploration. However, this limitation was only discovered after conclusion of both phases of data collection, preventing any additional opportunities to conduct the reward discrimination phase with this population of horses. This would have allowed the initial attempts to be excluded from analysis on the basis that they were part of the conditioning phase.

Further, it is possible that horses require side-by-side comparison to assess relative reward value of different stimuli. During the target conditioning phase horses were presented with only one target per session to ensure that they chose (and were therefore conditioned to) each target equally. This meant that the first time the horses were presented with both targets side-by-side was during the reward value discrimination phase. Therefore, horses may have used this phase to explore both targets and directly compare the difference in reward value. This would explain why, as seen in Figure 6.2, horses did not appear to preferentially choose the high value target until the later attempts. The possible trend indicates that horses require the first few attempts to explore the targets side-by-side and only then can they discriminate and show a preference towards the high value target. However, post-hoc analysis revealed no significant preference for the high value target in the final ten attempts. Of the twenty horses, eight chose the high value target at a rate above chance level in the final ten attempts of the reward value discrimination phase. While this was not a significant preference at the group level, it does show that some individuals can successfully discriminate between the two reward values and are sensitive to reward value discrepancy. It is possible that, given more opportunities, the whole sample may be able to do this.

In Uller and Lewis' (2009) study, horses spontaneously chose the larger of two quantities from watching contrasting quantities of artificial apples being placed into buckets. The apples were no longer visible once placed into the buckets, yet horses could select the bucket containing more apples. This demonstrates that horses can maintain two sets of representations in their working memory (the quantity of apples placed in two separate buckets). It also indicates that horses can conceptualise relative size difference (for example, that 3 is more than 1). However, unlike the targets in the current study, the buckets were side-by-side during the acquisition phase. Perhaps horses have difficulty holding a model of the reward value of different stimuli when conditioned separately but can process and respond to contrasting reward value with relative speed when stimuli are presented together. An additional reason for conditioning the targets separately in the current study was to investigate whether horses can hold a model of a target's reward value and apply that knowledge when faced with a choice. The finding that horses did not initially show any significant preference when faced with the targets side-by-side for the first time indicates that this may be an ability that horses do not possess, or at least is challenging. It may also provide further insight into the differences between working memory and long-term memory for holding information about reward value. This is an interesting finding because it suggests that horses require direct comparison to be able to make strategic decisions. This could explain why this same sample of horses performed very well in the Discrimination-Reversal task in Chapter 3, where targets were presented together for direct comparison in three training sessions.

It is also possible that horses do not pay attention to differences in reward value until they need to, for example when stimuli are presented side-by-side for direct comparison. Previous work has shown that horses can perform complex discrimination (Flannery, 1997; Fiske and Potter, 1979; Hanggi, 2003; Stone, 2010). In Flannery (1997) horses were even able to generalise their learning to a novel situation. In Chapter 3, horses quickly learned to discriminate between the two targets. However, the current chapter was unique because horses were not trained to discriminate between a conditioned and unconditioned stimulus; both stimuli were rewarded. In the training phase, horses were rewarded for any choice they made, differing only in the quantity of the reward. Therefore, there may have been no particular need to attend to the value at that point. This is interesting because, as evidenced in Chapter 4, horses may only use more complex cognitive processes when there is a pay-off for doing so. This may provide additional evidence consistent with prospective cost-benefit analysis in horses.

As discussed above, a key difference between the Discrimination-Reversal task in Chapter 3 and the Reward Sensitivity task was that in the former, there was a clear ‘correct’ and ‘incorrect’ response, as target selection was either rewarded or not. However, in the current task, both targets were ‘correct’ and elicited some level of reward. Perhaps a combination of the lack of side-by-side comparison and the relative ambiguity about what the ‘correct’ answer was made the Reward Sensitivity task more challenging for horses, as intended. Further, individual differences may have influenced subjects’ perceptions of what was the ‘correct’ target. The high value reward was set at three pellets of food and the low value reward was set at one pellet. This gave a reward quantity contrast; however, it did not necessarily provide a reward quality contrast when considering individual perception of the reward value. What is considered high value may not apply consistently across a sample or population. In this study, delivery of the three pellets for the high value reward was deliberately staggered as a means of exaggerating the increased quantity of food relative to the low value reward. While this may have achieved the objective of helping subjects to conceptualise the contrast in reward quantity, it also had the unavoidable effect of lengthening the time taken to complete the reward delivery. This meant that when horses chose the HVT, it took longer to deliver the reward and therefore there was a higher latency to resume choice-making. This trade-off between higher reward quantity and higher latency to continue interacting with the task may have turned the HVT into a less desirable option for some individuals. Some horses, particularly those with impulsive personality traits (MacKillop et al., 2016), may value the speed at which they can return to playing the game more highly than the food associated with the target.

In previous chapters, the physiological data suggested that certain horses found the target selection tasks rewarding. For example, in Chapter 3, HRV was higher during the cognitive task than at baseline, indicating reduced arousal. In Chapter 5, HRV data indicated a possible flow state, which is defined as a state of immersion in an intrinsically rewarding activity (Hintze and Yee, 2023). As such, some horses may find interacting with the task itself more rewarding than the food. This would mean the more rewarding strategy was to choose the LVT for faster reward delivery and return to target selection. This highlights the importance of considering reward value when designing cognitive paradigms for animals.

There was a significant preference for the consistent value target in the reward loss phase. Since the two targets were worth the same reward value, this preference suggests that horses were sensitive to reward loss because there is no other reason to avoid the RVT and so choices should be made at chance level. In human literature, sensitivity to reward loss is thought to be greater than sensitivity to reward gain (Dreher, 2007), which may explain why there was a significant preference in this phase but not in the reward value discrimination phase. Sensitivity to reward loss is attributed to cognitive bias and used as a welfare indicator (Burman et al., 2008). It is not possible to conclude from this study alone that horses felt disappointed *per se*, but it is possible that horses attributed a negative experience to the RVT due to its reduced reward value, and this may explain why horses showed a significant bias towards the CVT.

Interestingly, horses' initial attempts (Figure 6.3) did not continue the pattern seen at the end of reward discrimination (Figure 6.2). Initial attempts were slightly more biased towards the CVT, whereas at the end of reward discrimination horses appeared to be showing a trend towards the HVT. If horses had learned to strategically select the HVT towards the end of the discrimination phase, it would be expected that they would initiate the reward loss phase by choosing the RVT, since they have not yet learned this no longer pays the higher price. Upon learning, they may then shift to neutral choices, or a preference for CVT if disappointed but this was not observed. This indicates that perhaps the apparent preference for HVT towards the end of the discrimination phase were random or that horses struggled to maintain long-term memory of this phase, which took place one week earlier. The latter explanation is unlikely though since the same sample of horses retained a model of the Go/No-Go task for the three-week fallow period in Chapter 4. The slow improvement towards HVT and loss of this response in the initial reward loss phase suggest horses had not yet consolidated the relative values and further reward value discrimination sessions were possibly required.

4.2 Physiological responses

Spontaneous eye blink rate was not significantly different to baseline during either task phase. This is in agreement with previous Chapters 3 and 5, where blink rate was not significantly different across Reversal or Go/No-Go tasks. This is now the third piece of evidence that SEBR is not a reliable tool for measuring responses to cognitive tasks in this species. On visual inspection of Figure 6.3, however, blink rate appears higher during the reward loss phase than the reward value discrimination phase. This is interesting because recent evidence suggests that horses blink more during disappointment (Ricci-Bonot and Mills, 2023). Ricci-Bonot and Mills (2023) induced disappointment by conditioning horses to anticipate a food reward which then did not appear. In the current study, there was never an absence of reward, only a reduced value reward. Horses' disappointment may have been appeased by this, which could explain the non-significant trend here.

Blink rate variability was significantly different across the task phases, with the following pattern: baseline > reward value discrimination > reward loss. SEBR decreases and BRV increases significantly in humans during periods of high visual attention such as reading or viewing salient stimuli (Gebrehiwot et al., 2016). However, during more complex processing such as memory tasks (Fukunda et al., 2015) and communication tasks (Bentivoglio et al., 2004), blink rate increases significantly beyond the resting rate and BRV decreases (Gebrehiwot et al., 2016). This indicates that while attention has the effect of increasing variability, more complex mental processing has the opposite effect (Gebrehiwot et al., 2016) and this is supported by the results of the current study. This was the first chapter in which a significant reduction in BRV was observed, and it was also the most challenging task, as demonstrated by the performance outcomes. The nature of the relationship between BRV and cognitive performance remains uncertain. However, these results are promising in that they are consistent with the limited human literature available on blink rate variability and cognitive processes. As proposed in Chapter 3 and investigated thereafter, BRV appears to be more reliable than blink rate as a proxy indicator of learning in horses.

Unlike previous chapters, there was no significant effect of the cognitive task on heart rate variability. Previously, HRV increased during learning, compared to baseline. This suggests that the previous tasks were intrinsically rewarding and may indicate flow state (Hintze and Yee, 2023). In the current study however, the same effect was not found. Flow state is reliant on subjective experiences; a balance between perceived capacity (skill) and perceived opportunity (challenge) is required for flow state to occur (Csikszentmihalyi, 1975; 1990; Hintze and Yee, 2023). According to Csikszentmihalyi (1975; 1990), when skill is high, but challenge is low, this induces a state of boredom (Figure 6.9). This cohort of horses has demonstrated that they can perform discrimination-reversal (Chapter 3), model-based reasoning (Chapter 4) and inhibitory control contingent on a light cue (Chapter 5) within a relatively short training period. Therefore, to accept that horses experienced boredom in the current study is contingent on low levels of perceived challenge.

From the performance results, it appears that horses did find the task challenging. Only 7/20 horses preferentially chose the high value target in the reward value discrimination phase. However, it is important to acknowledge the difference between perceived challenge and actual challenge. For example, the actual challenge of this task was correctly discriminating between the two reward values and making strategic choices based on relative quantity discrimination. However, the perceived challenge of this task to a horse that has not recognised the difference between the two reward values was to touch either target for reward every time, as both targets were rewarded in this study. HRV in either phase was not significantly different to baseline, which was taken while horses were undisturbed in their stables. Perhaps this reflects under-arousal or boredom in the current study if horses perceive the task as easy because they interpret both targets as “correct” responses due to being rewarded for both. This is supported by the observation they were relatively insensitive to differences in reward quantity.

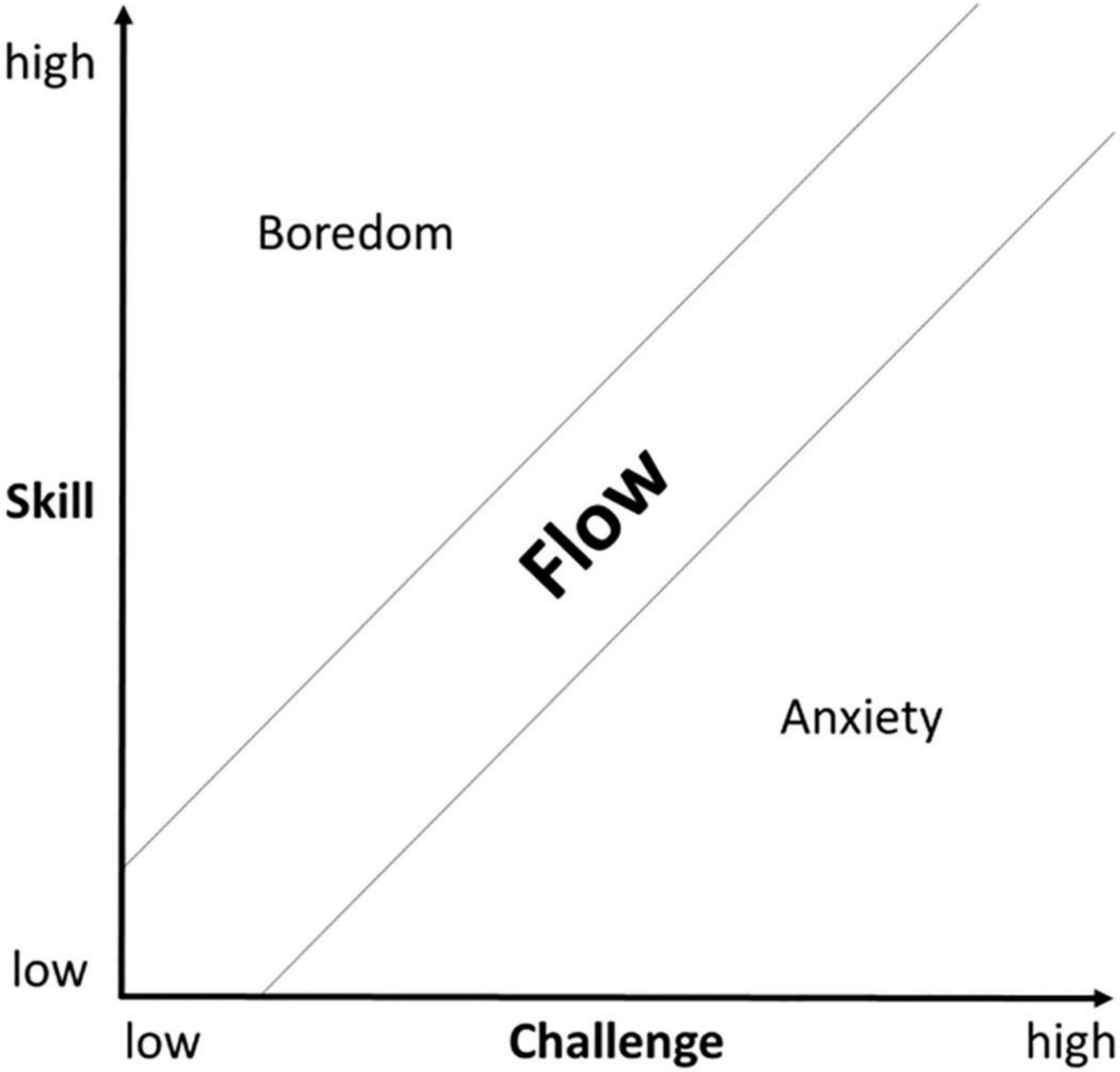

Figure 6.9. A graphical representation of flow state, taken from Hintze and Yee (2023). When skill and challenge are well balanced, flow state can occur. When challenge is high and skill is low, this can induce anxiety responses. When challenge is low and skill is high, boredom can occur.

There was also no significant effect on eye temperature, further indicating that horses were not aroused above control by either phase of the study. This was the first chapter in which eye temperature was not significantly affected by the cognitive task. Figure 6.7 illustrates that there was high variation in eye temperature values throughout, with no clear direction. Eye temperature is known to increase in response to arousal, in particular stress, in horses (Valera et al., 2012; Bartolome et al., 2013; Fenner et al., 2016; Borstel et al, 2017; Ijichi et al., 2020). This is due to increased blood flow to the eyes during sympathetic nervous system activation (Cook et al., 2001; Blessing, 2003). As such, the current results indicate that horses were not aroused by the task, which could further support under-stimulation.

4.3 Physiological predictors of performance

Individual performance in the reward value discrimination task was not predicted by any of the physiological measures. Although at the group level it appeared that horses didn't correctly select the HVT, in fact, 7/20 subjects made choices at above chance level when considering all 20 choices. This may have indicated that these particular horses had learned the strategic response and differences in individual ability to do so could be expected to correlate with physiology. However, individual performance in the complete reward value discrimination task was not predicted by any of the physiological measures. This suggests that horses' sensitivity to contrasting reward values is not significantly associated with their blink rate or arousal either at baseline or during the task. However, the model accounted for all twenty trials (attempts) of the reward value discrimination task whereas, illustrated in Figure 6.1, horses may have only demonstrated discrimination in later attempts, and this may have influenced the results of the model. As discussed, horses may require further trials to allow for side-by-side comparison prior to sensitivity testing. Therefore, outcomes may be different with some small adjustments to the study design, such as an additional side-by-side target exploration period prior to the test. However, the post-hoc model (Table 6.3) revealed no significant predictors of performance in the final ten attempts, when horses appeared to shift towards the HVT (Figure 6.2), suggesting that physiology did not predict reward value discrimination performance. Alternatively, if some individuals understood the task but were intentionally selecting the LVT target to return to an intrinsically rewarding activity faster, their performance scores might look similar to individuals that had not understood the task. This may confound associations with physiology and learning performance.

Performance on the reward loss task was predicted by heart rate variability. Individuals with higher HRV (indicating lower arousal) were more likely to choose the reduced value target. This is in agreement with work in humans, identifying that lower HRV (indicating higher arousal) is associated with tendency to avoid unpleasant outcomes (Katahira et al., 2014). Depending on the valence of arousal, this may be consistent with the theory that individuals in a more positive welfare state are more robust in response to reward loss and therefore less likely to avoid disappointing stimuli. If the arousal was negative (i.e., disappointment or frustration towards the loss of expected reward value), this would explain why those individuals with higher arousal were more likely to choose the consistent value target which did not disappoint them. This adds further evidence that physiological arousal is an important component of cognitive performance in this species.

The model also indicates that horses' first choice was a significant predictor of performance. Horses who chose the target on the left on the first attempt were less likely to choose the reduced value target overall. This supports the valence hypothesis, which states that the right hemisphere of the brain is associated with processing negative emotions (Ahern and Schwartz, 1979; Reuter-Lorenz and Davidson, 1981; Rodway et al., 2003). Motor behaviour is thought to manifest contralateral to the respective brain hemisphere. Therefore, left-biased movements indicate right brain hemisphere processing. Taken together with the HRV result, this may indicate that horses that were more aroused, with negative valence, were more likely to avoid the reduced value target. Interesting to note, this lateralized response was not associated with lateralized eye temperature differences. This does not support the findings of earlier chapters, in which eye temperature differences are proposed to be related to hemispheric lateralization because greater activation of the right hemisphere would be expected to contribute increased heat in the right eye which was not observed.

Performance in the reward loss phase was also predicted by baseline blink rate. Baseline SEBR was higher in horses who were more likely to choose the reduced value target. Higher baseline blink rate is argued to indicate increased striatal dopamine function (Jongkees and Colzato, 2016). Choosing the reduced value target even when it is no longer worth more suggests an inflexible approach. Increased dopamine D3 receptor availability is associated with inflexible decision-making (Groman et al., 2016). Therefore, the results of the current chapter indicate that high baseline blink rate, as a proxy indicator of increased tonic dopamine activity, is associated with an inflexible approach to reward loss. This is interesting because schizophrenia in humans has been associated with perseverative responding and general cognitive inflexibility (Frith and Done, 1983). Schizophrenic individuals are known to have increased SEBR at rest (Karson et al., 1982; Chen et al., 1996). Therefore, there seems to be an association between increased resting SEBR and cognitive inflexibility, consistent with the current chapter's findings.

5. Conclusions

At a group level, horses did not demonstrate a significant preference for the high value target in the reward value discrimination phase when considering the full 20 attempts and the final 10 attempts. However, some individuals did demonstrate an above-chance-level preference for the high value target, suggesting individual differences in reward sensitivity. Some horses may require side-by-side comparison to develop a model of relative reward value and make strategic choices. However, there was a significant preference for the consistent value target in the reward loss phase. This suggests that some horses may be able to discriminate between the two reward values, and therefore recognised when one target reduced in value, and avoided it. It is possible that in the reward value discrimination phase, some horses understood the task but acted impulsively or simply preferred a quick return to the game over the additional food reward.

Other horses may have understood the task and preferred three pellets of food over one pellet. Alternatively, some horses may not have understood or recognised the difference in reward value. The data presented here is not sufficient to determine individual motivations or understanding. This was the most complex cognitive challenge of the thesis, and this is reflected in the somewhat inconsistent performance outcomes. Further investigation is required to determine horses' abilities in this area. I suggest that future research accounts for personality, to determine whether traits such as impulsivity might influence horses' responses to the task. Additionally, individual responses to Reversal and the Go/No-Go task (Chapters 3 and 5) may be correlated with responses in the current task, to better understand how individual horses respond across different tasks. This was beyond the scope of the current project but should be considered in future work.

Blink rate appeared to be higher during the reward loss phase, consistent with disappointment, though this was not statistically significant. Blink rate variability was significantly reduced in both phases, possibly indicating complex mental processing and reflecting the challenging nature of the task. There were no significant predictors of performance in the reward value discrimination task, but response to reward loss was predicted by increased HRV, laterality of first choice and baseline blink rate. I suggest that taken together, this model is consistent with the valence hypothesis and higher tonic dopamine in reward sensitive individuals.

CHAPTER 7

GENERAL DISCUSSION

1. Introduction and Overview of the Thesis

This thesis investigated spontaneous eye blink rate and physiological arousal as predictors of cognitive performance in an equine model. This chapter will start by providing an overview of the thesis. Then, it will compare physiological results across chapters, to determine which hypotheses are supported by the data. Critical evaluation of the thesis with suggestions for future work will be discussed. Applications for horse training and welfare will be considered throughout.

1.1 Spontaneous eye blink rate

Spontaneous eye blink rate (SEBR) has been associated with striatal dopamine activity in humans (Jongkees and Colzato, 2016). Dopamine is a neurotransmitter involved in operant learning and responsible for marking reward as it occurs (Wise and Rompre, 1989; Schultz, 2002; Flagel et al., 2011). As such, it was proposed that SEBR, as a proxy indicator of striatal dopamine activity, may offer a novel predictor of learning performance. In contrast to this theory, it has also been suggested that SEBR is simply a measure of visual attention (Maffei and Angrilli, 2019) with no relationship to striatal dopamine activity (Nahar et al., 2007). It has also been suggested that SEBR relates to emotional stress (Hirokawa et al., 2001) and this has been studied in non-human animals including horses (Merkies et al., 2019; Mott et al., 2020). Further, recent work in humans has found that SEBR increases by up to 40% with exposure to everyday environmental stressors (dry air associated with offices and aircraft cabins, for example; Tabernero et al., 2021). As such, this thesis contributed to the current debate by investigating SEBR as a proxy indicator of cognitive performance. By following the same subjects through a series of cognitive tasks with increasing complexity, this thesis aimed to determine the reliability of SEBR as a predictor of cognitive performance in an equine model.

1.2 Arousal

Physiological arousal is a state of physiological activation. Arousal may increase during learning due to increased mental effort (Mehler et al., 2009). Further, arousal is known to influence cognitive processes such as attention, learning and memory in humans (Eysenck, 1976; Storbeck and Clore, 2008; Ma et al., 2018). In this thesis physiological arousal measures included heart rate variability (HRV) and infrared thermography of eye temperature (IRT). Heart rate variability, calculated using root mean square of successive differences (RMSSD), is a measure of the variation in time between successive heart beats, also known as the inter-beat-interval (Dias et al., 2016). HRV is regulated by the autonomic nervous system, through activity of the parasympathetic and sympathetic branches (Cygankiewicz and Zareba, 2013).

Infrared thermography of eye temperature is a validated welfare indicator in several animals including horses (Ijichi et al., 2020; Kim and Cho, 2021). IRT measures emissive heat from changes in blood flow as a result of sympathetic nervous system activity (Blessing, 2003). During physiological arousal, blood flow is increased to the critical organs such as the eyes, causing an increase in emissive temperature (Stewart et al., 2007). Using both methods in this thesis, the objective was to investigate validated measures of arousal as possible indicators of cognitive performance in horses, for the first time. The research reported in this thesis is novel because 1) it investigated multiple measures of physiological arousal 2) within-individual physiology and cognitive performance were examined in a longitudinal study involving multiple learning paradigms 3) statistical models for predicting cognitive performance in this thesis included both during-task and baseline physiology.

1.3 Adaptive learning

1.3.1 Cognitive flexibility

Adaptive learning was measured in terms of cognitive flexibility, cognitive control and reward sensitivity. Chapter 3 involved a two-choice discrimination-reversal task (referred to as Reversal Learning) which challenged horses' cognitive flexibility by asking them to switch their attention and behavioural responses away from a previously rewarded target and towards a previously unrewarded target, following a period of target conditioning. Cognitive flexibility is a core executive function (Diamond, 2012) which has been associated with dopamine activity (van Holstein et al., 2011; Klanker et al., 2013; Berry et al., 2016), therefore making it a suitable challenge for investigating SEBR as a proxy indicator of dopamine activity during learning. Further, horses are regularly expected to demonstrate cognitive flexibility in their working roles. For example, domestic horses typically experience a variety of riders, trainers and handlers throughout their working lives. Many regularly experience changes to their home environments such as travel to competition (Houpt and Wickens, 2014), changing yards (Dashper, 2014) and moving between owners (Heijtel, 2012).

1.3.2 Cognitive control

In Chapters 4 and 5, horses were asked to inhibit a learned response to target, contingent on a light cue. This has not previously been demonstrated in horses. Chapter 4 investigated whether horses used a model-based approach to inform their decision-making during this task, while Chapter 5 investigated SEBR and arousal as performance predictors. Success in the Go/No-Go task relied on effective response inhibition, another core executive function (Diamond, 2012). Striatal dopamine activity is thought to mediate response inhibition in humans (Robertson et al., 2015). Further, the Go/No-Go task was expected to induce a moderate increase in physiological arousal, due to the increased cognitive load required to attend to the light cue, plus possible frustration effects in early No-Go trials when the expected reward did not match the actual reward (Schultz, 2017). Domestic horses are routinely required to demonstrate good inhibitory control, including at the basic level reliably responding to “stop” and “go” signals (Starling et al., 2016; McLean et al., 2017).

1.3.3 Reward sensitivity

In Chapter 6, horses’ reward sensitivity was investigated using two distinct paradigms. Following a period of conditioning, horses were asked to discriminate between two targets of differing reward value. Then, in the second phase, horses’ responses to reward loss were investigated by reducing the value of one target so that both targets were now rewarded with equal value. Reward sensitivity has been validated in other species as an emotion and welfare indicator (Parker et al., 2008), but not previously investigated in horses. Further, sensitivity to reward loss indicates how robust an animal is to experiences of loss or disappointment, and this is a validated indicator of overall welfare (Parker et al., 2008). In a large, potentially dangerous animal like the horse, being able to predict sensitivity to disappointment or frustration has consequences for both horse and human safety and welfare. Chapter 6 of this thesis investigated arousal and blink rate as physiological predictors of reward sensitivity in horses. Horses' physiological arousal during the task provided an indication of emotional response to reward value differences and loss of expected reward (Lane et al., 2009). Dopamine is considered an integral moderator of reward sensitivity (Schultz, 2002; Hahn et al., 2011; Muhammed et al., 2016), therefore SER as a possible proxy indicator of striatal dopamine activity was a valuable measure to investigate here.

1.4 Predictors of performance

Horses' physiological responses were recorded in all tasks and analysed using predictive statistical models to determine whether physiological response to learning and phasic dopamine activity (indicated by SEBR during learning; Jongkees and Colzato, 2016), could predict performance. Baseline physiology was also included in the statistical models, to investigate whether horses' baseline arousal levels and tonic dopamine activity (as indicated by baseline SEBR; Maffei and Angrilli, 2018), could predict learning performance. This is novel because baseline arousal has only been considered as a possible predictor of cognitive performance once in the literature (Bray et al., 2015), and never in horses.

Physiological responses to the task were investigated by comparing control physiology, taken from horses while at rest in their home environment, with physiology during the task. The purpose of this was to determine the effect of tasks of varying difficulty and skill on spontaneous eye blink rate and physiological arousal. In each chapter, specific hypotheses were given about how horses may respond physiologically to the task and how physiological arousal and SEBR may predict learning performance. The first section of this general discussion chapter evaluates the results of the physiological analyses and predictive models. In the second section of this general discussion chapter, these insights are discussed in relation to current understanding of equine cognitive abilities and their applications.

2. Physiological predictors of learning

2.1 Overview of results

This thesis demonstrated that it was possible to predict learning performance using non-invasive physiological measures. The same subjects were studied throughout this project, which meant that the reliability of physiological measures could be evaluated across multiple cognitive experiments. Previously, there have been no longitudinal studies of cognition in horses, testing the same subjects in a variety of cognitive tasks. This is novel and important; any physiological and performance differences observed between chapters are not due to differences in the sample and are therefore likely to reflect task difficulty. Each task was designed to challenge different cognitive skills, and gradually increased in demand. This meant that physiological response to tasks varying in both skill type and difficulty could be observed and modelled against performance in the tasks. The results of the thesis as whole are summarised below (Table 7.1) before being discussed in depth.

Table 7.1. A summary table of all physiological results across studies, including physiological response to the task and predictive statistical model results. Physiological measures included spontaneous eye blink rate (SEBR), heart rate variability (HRV), blink rate variability (BRV), left eye temperature change and right eye temperature change. Statistically significant results are highlighted in bold text. Blink rate variability was not applicable in the two-choice discrimination-reversal study because BRV was not introduced as a measure until Chapter 5 (see *Section 2.2* for full justification).

Study (Chapter)	**Physiological measure**	**Response to the task (difference from baseline/control)**	**Model results (predictors of performance)**
Two-Choice Discrimination-Reversal (3)	SEBR	No difference (p=0.31)	n/s
	BRV	Not applicable	**Not applicable**
	HRV	Marginal increase (p=0.06)	**Positive predictor during task (p=0.002)** **Positive predictor at baseline (p=0.009)**
	Left eye temperature change	**Decrease (p=0.002)**	**Positive predictor during task (p<0.001)**
	Right eye temperature change	**Decrease (p=0.05)**	n/s

Go/No-Go (4 and 5)	SEBR	No difference from baseline (p=0.07) or between +R and +R/-P conditions (p=0.22)	Not significant during task **Positive predictor at baseline (p<0.001)**
	BRV	No difference from baseline (p=0.28) **Significantly lower during +R than during +R/-P (p=0.04)**	n/s
	HRV	**Increase from baseline (p=0.008)** **Significantly lower during +R than during +R/-P**	**Negative predictor during task (p=0.02)** **Positive predictor at baseline (p<0.001)**
	Left eye temperature change	**Decrease from control (p<0.001)** No difference between +R and +R/-P conditions (p=0.28)	n/s
	Right eye temperature change	**Decrease from control (p<0.001)** No difference between +R and +R/-P conditions (p=0.26)	**Negative predictor during task (p<0.001)**

Reward value discrimination (6)	SEBR	No difference (p=0.223)	n/s
	BRV	**Decrease (p<0.001)**	n/s
	HRV	No difference (p=0.247)	n/s
	Left eye temperature change	No difference (p=0.923)	n/s
	Right eye temperature change	No difference (p=0.203)	n/s
Reward loss (6)	SEBR	No difference (p=0.223)	Not significant during task **Positive predictor at baseline (p=0.016)**
	BRV	**Decrease (p<0.001)**	n/s
	HRV	No difference (p=0.247)	**Positive predictor during task (p=0.004)** Not significant at baseline.
	Left eye temperature change	No difference (p=0.923)	n/s
	Right eye temperature change	No difference (p=0.203)	n/s

2.2 Spontaneous eye blink rate

Spontaneous eye blink rate (SEBR) was investigated as a possible proxy measure of striatal dopamine activity, based on research in humans (Jongkees and Colzato, 2016) and some non-human animals such as monkeys and rats (Taylor et al., 1999; Kaminer et al., 2011) identifying a convincing positive relationship. This relationship is supported by drug studies; administration of dopamine agonist drugs increases blink rate while dopamine antagonist drugs decrease blink rate (Elsworth et al., 1991; Lawrence and Redmond, 1991). Further, the equine striatum is almost identical to the human striatum (Hemmings et al., 2018), therefore it was expected that the association between SEBR and striatal dopamine activity well-documented in humans would also be present in horses. However, other studies have found contradictory effects of dopamine agonist and antagonist drugs on SEBR (Kleven and Koek, 1996), while others have failed to establish a correlation between SEBR and dopamine activity (Dang et al., 2017).

Maffei and Angrilli (2018) suggest that SEBR reflects attention, decreasing significantly during periods of high mental load and focus. The work to date in horses has identified SEBR as an indicator of attention, reporting significant decreases in SEBR during activities requiring increased attention (Merkies et al., 2019; Mott et al., 2020). Cherry et al. (2020) found significant increase in SEBR in horses during periods of high attentional focus, though they attribute this to locomotory activity during the test. Horses were stationary in all tasks in the current thesis to control for this limitation. McBride et al. (2022) found a significant positive correlation between SEBR and impulsivity scores in horses, and they relate this to underlying dopamine activity, in agreement with a rodent study by Jeffrey et al. (2011). However, Mott et al. (2020) found significant positive correlations between SEBR and cortisol, and a significant negative correlation between SEBR and HRV. Similarly, Merkies et al. (2019) found evidence consistent with SEBR as a stress indicator in horses. It is possible that more impulsive horses are also more stress-sensitive, which could explain the finding by McBride et al. (2022).

This thesis aimed to investigate two conflicting hypotheses about SEBR in this species. The first hypothesis was that SEBR would increase during learning, relative to baseline, due to increased dopamine activity associated with effective reward learning. The second hypothesis was that SEBR would decrease during learning, due to increased cognitive load, attention, and focus. However, there were no significant effects on SEBR in any cognitive tasks in this thesis (Table 7.1), contradicting both hypotheses and in disagreement with previous literature in this species.

SEBR during learning did not predict performance in any of the tasks, which was unsurprising given that there were no effects of learning on SEBR. However, baseline SEBR was a positive predictor of performance on both the Go/No-Go and Reward Loss tasks (Table 7.1). This suggests that baseline SEBR may provide a useful predictor of performance on some tasks, but not others. The Go/No-Go and Reward Loss tasks both involved elements of disappointment, and potentially frustration, though the physiological data indicates that horses were not frustrated during the Go/No-Go task. In the second half of the Go/No-Go task, horses experienced a time-out period for errors of emission. In this time, they could not access the experimental stimuli or food reward. In the Reward Loss task, horses experienced a loss of expected reward, as the previous high-value target was reduced in value.

If SEBR is a measure of stress (Merkies et al., 2019; Mott et al., 2020), this would mean that horses with a higher baseline level of stress were more likely to perform well on a Go/No-Go task where there is risk of punishment. This is consistent with a recent study in rats, which found that stress increases risk-aversion and punishment sensitivity (Chiavegatti and Floresco, 2024). However, in the Reward Loss task, the more stress-sensitive horses were the ones who were more likely to choose the reduced value target, which directly contradicts this. Therefore, it was not possible to accept either hypothesis based on the results of this thesis, and the relationship between SEBR and learning performance in horses remains unclear.

Several explanations for inconclusive SEBR results in this thesis have been identified. First, the accurate and reliable measuring of SEBR is challenging; in particular there is a lack of consensus on a standardised non-invasive method. Previous work has used video analysis taken from a fixed location, such as a surveillance camera or tripod. This relies on the subject’s eyes remaining in shot throughout, achieved by restraining the animal or applying other restrictions to movement. In the current project, light restraint was used for safety purposes in compliance with the University equestrian centre policy. However, it was important that restriction be kept to a minimum. The reasons for this were twofold: (1) It was important from an ethical standpoint that cognitive tasks were free choice. This meant that the horse could choose not to take part by moving away from the task if desired. (2) Minimal restrictions meant that subjects were able to interact with the various cognitive tasks without the influence of a handler. To this end, the method used here was to mount a GoPro camera on the horse's headcollar, aimed towards the face to capture both eyes simultaneously. The mounted position of the camera meant that subjects were able to move their heads without either eye leaving the shot. However, this did mean that horses had to be habituated to having the camera equipment attached to their headcollar which would have been novel to them, at least in the initial experiments.

It is possible that the presence of a camera on the horses' faces influenced their spontaneous blinking patterns and/or contributed to a higher number of voluntary or reflexive blinks, which were indistinguishable from spontaneous blinks in analysis. Spontaneous blinks can be identified by measuring blink amplitude (Cruz et al., 2011; Kaminer et al., 2011), however this was beyond the scope of the current project and would have required electromyography (EMG; Frigero et al., 2014) or contact lens sensor (CLS) technology (Gisler et al., 2015). Both methods require specialist equipment which makes direct contact with either the eyeball itself (CLS) or the surrounding eye area (EMG), whereas the current project used non-invasive methods only. Non-invasive methods were used for two main reasons. First, in line with the three Rs of ethical research involving animals, the methods were refined to minimis the risk of discomfort or distress as far as possible. Second, as this thesis was interested in physiological responses to learning, it was important to reduce the risk of confounding these results with physiological reactions to invasive procedures.

2.3 Blink rate variability

While previous studies present a convincing relationship between SEBR and striatal dopamine activity (Elsworth et al., 1991; Lawrence and Redmond, 1991; Jongkees and Colzato, 2016), this is not reflected in the results of this thesis. Blink rate did not significantly alter in response to learning, nor was SEBR during learning a significant predictor of performance in any task. It is possible that blink rate (number of blinks per minute) is not the most useful proxy measure of phasic striatal dopamine activity, due to the temporal variation in blink patterns in response to various cognitive processes (Fukunda et al., 2005; Siegle et al., 2008; Paprocki and Lenskiy, 2017). This may explain why blink rate was not a significant predictor of performance in any task.

In Chapter 3, it was proposed that blink rate variability (BRV) may offer a more reliable method of predicting cognitive performance, due to human evidence suggesting that fluctuations in temporal blink patterns are associated with different cognitive tasks (McLean et al., 2009; Lenskiy and Paprocki, 2016). For example, it has been demonstrated that blink rate slows significantly during periods of high attention such as reading (Lenskiy and Paprocki, 2016) and increases significantly during reward anticipation (Peckham and Johnson, 2016). Therefore, an average of the number of blinks occurring across a task involving periods of high attention and reward anticipation may not adequately represent SEBR patterns during learning. In each cognitive task, visual stimuli (cue card targets) were presented and horses had to pay attention to which target was conditioned stimulus and which was the unconditioned stimulus. In the Go/No-Go task, horses were also required to pay attention to an additional light cue to signal whether it was a Go or No-Go trial. As such, it is assumed that all tasks involved periods of increased attention, relative to baseline. Due to the thorough pre-conditioning, horses learned to associate correct behavioural responses (touches of the conditioned stimulus) with subsequent reward. Therefore, periods of reward anticipation were also present during tasks and so it is possible that these blink patterns (Lenskiy and Paprocki, 2016; Peckham and Johnson, 2016) were lost when average blink rate (blinks per minute) was calculated. In response to this early finding, subsequent chapters included blink rate variability (BRV) analysis.

Blink rate variability is the variance in time between successive blinks and so better reflects temporal patterns in blinking than an average of the number of blinks across a time period. BRV was calculated by root mean square of successive differences (RMSSD). RMSSD is a calculation commonly used for heart rate variability analysis (Stucke et al., 2015), however it has also been used in other contexts for variability analysis (Abellán-Aynés et al, 2022; Boullosa et al., 2023). Due to the time constraints of the thesis, blink rate variability analysis was not retrospectively conducted for Chapter 3, however all future chapters included this analysis.

Blink rate variability was not a significant predictor of performance in the Go/No-Go task (Chapter 5) or in the Reward Sensitivity task (Chapter 6). However, there were some promising findings, which suggest that this novel and preliminary method may be useful. For example, in Chapter 5, BRV was significantly higher when cost was introduced (+R/-P), than when there was no cost for errors (+R). In Chapter 6, BRV was significantly lower during the reward value discrimination phase than at baseline, and significantly lower again during the reward loss phase. Taken together, these results could indicate that BRV is lower when horses are in an uninterrupted flow-like state of response-reward. For example, in the Go/No-Go task no cost meant that horses were able to repeatedly touch the target with minimal interruption, therefore making the experience of playing the game fast and rhythmical. When cost was introduced, the flow of the game was disrupted with time out periods when errors occurred. Even successful horses who made minimal errors in the +R/-P phase were interrupted because success in a Go/No-Go task requires periods of stopping and waiting to resume target selection. Therefore, the finding that BRV was lower during no cost sessions than sessions with cost added may be due to the rhythm of the task itself being less variable, which may have contributed to a flow-like experience.

Similarly, the reward value discrimination task in Chapter 6 did not involve any time-out period, as only positive reinforcement was used. Since both targets were rewarded, horses could enter a consistent flow of response-reward and, indeed, responses were offered quickly. The reward loss phase offered further potential for a flow-like state to occur because horses were rewarded with just one pellet every time, so the latency between attempts was consistent. This could explain why BRV decreased during reward value discrimination and then decreased further during reward loss.

The choices that horses made in Chapter 6 may also indicate a flow-like experience. Horses did not show a significant preference for the target intended as the high value option, which could be due to an influential number of individuals favouring the target which allowed them to return to the task more quickly (LVT). Some horses may derive intrinsic value from the act of completing this kind of task, similar to a flow state in humans (Nakamura and Csikszentmihalyi, 2009; Csikszentmihalyi et al., 2014) and this could explain why so many horses appeared to favour the low reward value option. In all three cases of reduced blink rate variability (Go/No-Go, reward value discrimination and reward loss), horses were experiencing response-reward in a consistent temporal pattern and making choices which would allow them to continue this pattern (repeatedly touching the target in +R Go/No-Go sessions and favouring the low value target in Reward Sensitivity). This is a novel contribution because flow state, which has not previously been demonstrated in horses, may have been identified on three separate occasions within this thesis. This is a fascinating finding which warrants further investigation. Blink rate variability, calculated by RMSSD of the inter-blink interval, may offer a novel, non-invasive method of identifying flow-like states in animals. The applications of this interesting finding will be discussed later in the General Discussion chapter.

2.4 Heart rate variability

HRV was a significant predictor of performance in all chapters. This means that HRV is not only an excellent, non-invasive measure of arousal, it can be used as a predictor of performance in a range of cognitive tasks. Valuable insights into the relationship between arousal and different aspects of adaptive learning were gathered. In Chapter 3, higher HRV during learning was associated with better performance on a two-choice discrimination-reversal task (Reversal). This meant that horses in a lower arousal state were better able to flexibly switch to the unconditioned stimulus when the conditioned stimulus was no longer rewarded. Subjects appeared to find this task relatively simple, as all horses reached learning criterion within two to three short training sessions. In Chapter 5, however, lower HRV during learning predicted better performance on a Go/No-Go inhibitory control task.

This indicated that increased physiological arousal was required for success in this task. There were some key differences between the Reversal task and the Go/No-Go task which may have contributed to this inconsistency. The Go/No-Go task was more cognitively demanding than the Reversal task because it involved attending to an additional cue (the light) and adapting their responses to the conditioned stimulus contingent on that cue. Therefore, the attention load during the Go/No-Go task is expected to be higher than in the Reversal task, which only required horses to attend to the targets themselves. This additional load may have required a higher level of arousal, as seen in humans (Gellatly and Meyer, 1992). This could explain why horses with lower HRV (indicating higher arousal) performed better on this task.

HRV was also a significant predictor of performance in Reward Loss. Horses with lower HRV (higher arousal) were less likely to choose the reduced value target intended to illicit disappointment. Depending on the valence of arousal, this could be consistent with the theory that more anxious individuals are more likely to have a negative response to reward loss (Burman et al., 2008), demonstrated by an avoidance of the stimulus associated with loss. Taken together, these results suggest that (1) performance on relatively simple tasks relies on lower arousal during training, whereas for more cognitively demanding tasks an increase in arousal is required (2) maintaining lower arousal may help individuals cope better with perceived loss or disappointment during training.

Broad emotional state has been studied in relation to cognitive flexibility in horses once before, in a between-subjects design (Fortin et al., 2018). In Fortin et al. (2018), horses in a positive affect group performed better on a cognitive flexibility task than those in a negative or neutral affect group. However, physiological arousal within-subjects has not previously been investigated as a predictor of cognitive performance in horses. Further, while arousal during cognitive tasks has been linked with performance in other animals previously (Baldi and Bucherelli, 2005; Starling et al., 2013), associations between an individual's tendency to be aroused at baseline and performance are little studied and never in horses. In the current thesis, Baseline heart rate variability was a significant positive predictor of performance throughout this project. This has previously been observed in rats (Howell and Hamilton, 2022), but never in horses. In the current thesis, horses with higher heart rate variability (indicating lower arousal) at baseline performed significantly better on both Reversal and Go/No-Go tasks. Interestingly, HRV during Go/No-Go training sessions was negatively associated with performance, yet baseline HRV was positively associated with performance. This suggests that even in more demanding tasks, where increased physiological arousal is required for success, maintaining lower arousal at baseline in the home environment is important. For high-level competition horses, learning complex responses to cues from the rider may require increased arousal. However, the results of the current project suggest that even when an increased level of arousal is required for better performance (Yerkes and Dodson, 1908), horses should be managed under conditions designed to reduce physiological arousal in the home environment. This highlights that the starting point of arousal is important. For example, if a horse with high tendency to be aroused at baseline experiences further arousal during learning, this is likely to result in over-arousal, which ultimately has a detrimental effect on performance according to the Yerkes-Dodson model (Yerkes and Dodson, 1908). Conversely, if a horse with good welfare and relatively low baseline arousal experiences increased arousal during learning, they may enter the arousal "sweet spot", in which performance is optimal (Yerkes and Dodson, 1908). The application of this could have positive implications for the welfare of competition horses.

2.5 Infrared thermography of eye temperature

Infrared thermography of eye temperature (IRT) is a validated welfare indicator in horses (Valera et al., 2012; Ijichi et al., 2020; Soroko et al., 2021). Increased blood flow to the eyes during sympathetic nervous system activation can be measured rapidly and non-invasively using IRT and is very well tolerated by domestic horses. The results of the current project revealed potential insights into differential hemispheric processing. In Chapters 3 and 5 eye temperature was affected by the cognitive tasks but not as expected. It was hypothesised that eye temperature would increase during learning, compared to baseline measures taken immediately before each training session. This is because an increase in eye temperature is indicative of increased blood flow in response to sympathetic nervous system activation (Blessing, 2003; Stewart et al., 2008). It was predicted that the cognitive tasks would induce a level of arousal that would increase eye temperature, due to the anticipation of food reward, novel enrichment and the increased visual attention required.

Furthermore, it was observed in training sessions that horses showed behavioural signs of increased arousal when the researcher entered the stable area with the task stimuli. Some examples of behaviour observed included moving to the front of the stable, positioning of the head and neck over the stable door, increased vocalisation, occasional stamping of the feet, pawing at the ground and kicking the stable door. This indicated a level of physiological activation, compared to the resting behaviours usually observed. It was interesting, then, that the IRT results showed consistent eye temperature decrease in both eyes and in all training sessions of both Reversal and Go/No-Go. This was particularly surprising given that these were tasks involving visual cues, including cue cards and an additional light cue in the Go/No-Go task. The IRT results indicated that horses experienced increased relaxation during training sessions, which was supported by the increased heart rate variability observed in Reversal and Go/No-Go. This suggests that cognitive enrichment (Clark, 2017) may be relaxing for horses and could be used to support improved welfare in stabled horses.

Cognitive enrichment is known to improve the welfare of zoo-housed (Krebs and Watters, 2017; Clark et al., 2019; Scheer et al., 2019), laboratory-housed (Leidinger et al., 2017) and farmed animals (Manteuffel et al., 2009; Zebunke et al., 2013). However, there is a lack of research on cognitive enrichment in stabled horses, perhaps due to perceived cognitive limitations in this species (Cooper, 2007; Brubaker and Udell, 2016). Zoo-housed animals kept in enclosures, housed with conspecifics and with enough space to perform natural behaviours are arguably managed in less restrictive conditions than a stabled horse with little or no access to field turnout. While not a substitute for adequate turnout time with conspecifics, cognitive enrichment may provide a welfare-positive addition to the environment of a stabled horse.

In addition to changing in response to cognitive tests, IRT predicted performance in both Reversal and Go/No-Go tasks. A left eye temperature increase was associated with better performance in Reversal, and this was discussed in terms of hemispheric lateralisation (Chapter 5). Similarly, a decrease in temperature of the right eye was a significant predictor of performance in Go/No-Go. IRT measures heat transfer due to changes in blood flow (Blessing, 2003). Therefore, it was suggested that lateralised eye temperature changes may reflect hemispheric blood flow during learning reflected as a proxy in ocular temperature. There are three main theories concerning hemispheric lateralisation: the right hemisphere hypothesis (Gainotti, 1972; Smith and Bulman-Fleming, 2004), the valence hypothesis (Ahern and Schwartz, 1979) and the BIS/BAS hypothesis (Carver and White, 1994). This project measured eye temperature changes using IRT in a variety of contexts in which one hemisphere may be dominant over the other. There are different hypotheses about the roles of each hemisphere, as discussed here. However, this could provide a useful starting point from which to investigate the potential of IRT as a rapid, non-invasive indicator of hemispheric activation.

The right hemisphere hypothesis suggests that the right hemisphere is dominant in processing emotional stimuli, regardless of valence (Smith and Bulman-Fleming, 2005). This is based on evidence that in humans, damage to the right hemisphere significantly impairs emotional perception, whereas damage to the left hemisphere has little or no effect on an individual's ability to perceive and respond to emotional stimuli (Borod, 1992; Smith and Bulman-Fleming, 2005). According to this theory, the left hemisphere is responsible for logical reasoning and problem-solving. Within this thesis, the Reversal task (Chapter 3) required horses to respond to a conditioned target in exchange for a food reward. Following the right hemisphere hypothesis (Gainotti, 1972), the left hemisphere should be dominant during Reversal. Therefore, the finding that increased left eye temperature was a positive predictor of performance in this task supports the right hemisphere hypothesis. IRT has not been validated as an indicator of hemispheric activation, so this is a speculative theory. However, it is possible that increased left eye temperature was a by-product of increased blood flow towards the left hemisphere of the brain during reversal learning, and this is why individuals with increased left eye temperature were more successful in this task. This theory requires further investigation but, if supported, could offer a rapid, non-invasive method of detecting hemispheric lateralisation in mammals with anterolaterally placed eyes. Currently the only way to detect such activity is with the use of EEG, which requires laboratory conditions and is not yet practical for use in horses.

Whereas the right hemisphere hypothesis suggests that all emotion is processed in the right hemisphere, the valence hypothesis argues that emotional processing is divided by valence. According to the valence hypothesis, the left hemisphere is dominant in processing positive emotions and the right hemisphere processes negative emotions (Schwartz et al., 1979; Killgore and Yurgelun-Todd, 2007). The Behavioural Inhibition System/ Behavioural Activation System theory (BIS/BAS) suggests that approach and avoidance behaviour are processed in opposite hemispheres (Goursot et al., 2021). According to BIS/BAS, behavioural activation (approach behaviour) is left lateralised and behavioural inhibition (withdrawal or avoidance behaviour) is right-lateralised (Goursot et al., 2021). Within this thesis, the Reversal task challenged behavioural activation (approach) because success in this task relied on horses' willingness to approach the previously unconditioned stimulus when the conditioned stimulus was no longer rewarded. To discover that they would be rewarded for reversal, horses had to demonstrate behavioural activation.

By contrast, the Go/No-Go task relied on effective behavioural inhibition. Horses had to inhibit their learned response towards the conditioned stimulus when the light cue was on. Applying BIS/BAS to the current project, Reversal and Reward Sensitivity should be processed in the left hemisphere, while Go/No-Go should be processed in the right hemisphere. Therefore, if IRT can be used as an indicator of hemispheric activity, it would follow that increased left eye temperature would be associated with success in Reversal; increased right eye temperature would be associated with success in Go/No-Go and increased left eye temperature would predict more strategic choices in Reward Sensitivity. In Reversal, increased left eye temperature was a significant predictor of performance, supporting BIS/BAS. In Go/No-Go, the temperature of the right eye predicted performance, but it was a decrease in temperature rather than an increase. Therefore, this did not support BIS/BAS, but it was interesting that this was the only task in which the right eye was a predictor of performance.

In Reward Sensitivity neither eye temperature predicted performance, which also did not support BIS/BAS. However, as discussed in Chapter 6, early attempts in this task were not necessarily a true reflection of performance, because horses appeared to use the initial attempts as a side-by-side comparison of the targets and did not begin to show a clear preference for the high value target until the final few attempts. In this chapter it was also discussed that assigning high value status to the target paying three pellets and low value status to the target paying one pellet was not necessarily a reflection of horses' perception of relative value. This is because paying three pellets of food meant a longer latency to return to the task, which may have held more intrinsic value than the food reward for some individuals. This was a significant limitation of Chapter 6 because it meant that it was very difficult to determine what constituted the more strategic choice. This may have contributed to the lack of significant predictors of performance in the reward value discrimination phase. Despite these methodological limitations, evidence for BIS/BAS was not consistent across all tasks. Therefore, alternative theories such as the right hemisphere and valence hypotheses should not be discounted.

3. Equine cognition: insights and contribution to knowledge

3.1 The equine model

The horse was an ideal study species for this project for several reasons. First, domestic horses are adept at operant conditioning and are regularly expected to learn, reverse and extinguish behavioural responses to cues (McLean and Christensen, 2017). In their working roles, horses demonstrate cognitive flexibility, to flexibly adapt to cues from different riders and trainers, and novelty in their environments (McGreevy and McLean, 2007; Brubaker and Udell, 2016). On the other hand, horses are also required to maintain cognitive control so that their responses to specific cues such as ‘stop’ or ‘slow’ are reliable (McGreevy and McLean, 2007). Understanding more about how horses learn to be both flexible and reliable is undoubtedly important and relevant to horse owners, trainers and riders.

Second, there are validated, non-invasive methods of measuring arousal and welfare in horses, and these methods are generally tolerated well. Heart rate variability is considered the gold standard and is worn in a similar way to other equipment regularly used on horses. IRT has been validated as a welfare indicator in this species and its use requires no physical contact with the animal. Further, horses have large eyes which allow for easier blink rate analysis. Therefore, horses are an ideal species for measuring physiological responses to arousal and modelling predictors of performance.

3.2 Approaches to training

This project demonstrated that horses could learn cognitively complex tasks within a few short training sessions using positive reinforcement. Some of the tasks, including Go/No-Go and Reward Loss, were never demonstrated in horses before, so it was unknown how horses would respond and if they would be successful. All subjects completed Reversal to learning criteria within two to three short sessions of positive reinforcement training, showing that this was a relatively easy task for horses. This is unsurprising given the regularity with which domestic horses demonstrate flexibility, as they are moved around different homes, ridden and handled by different people.

It is important to note that the sample used here were university-owned horses, accustomed to being handled by new students each year. They were also a group regularly exposed to novel objects and scenarios as part of their roles in research at the university. As such, this sample may have been more cognitively flexible than the average horse in their response to Reversal and to the novelty of the task in general. This could be due to their habituation to novelty and inconsistent handling by students. Alternatively, cognitive flexibility could be a trait that is selected for when choosing riding school horses (Lerch et al., 2021), therefore this sample may be predisposed to this.

Positive reinforcement training is known to be an effective training method, which this species responds well to (Innes and McBride, 2008). However, Chapter 4 revealed interesting insights about how horses may respond to positive reinforcement. Horses appeared to use a cost-benefit analysis in their decision-making when only positive reinforcement was used in the first three sessions of Go/No-Go. It was observed that horses were indiscriminately responding to the target when there was no cost for errors of emission. However, when cost was introduced, there was an immediate and significant reduction in errors, as horses began to attend to the Go/No-Go signal (a light cue).

The cost for making errors was a short time-out period of only ten seconds, a form of negative punishment (McLean, 2005). The length of the time-out period was determined by trial and error in a pilot; horses appeared to immediately respond to very short periods in time-out. This demonstrated that not only did the previous positive reinforcement training facilitate horses learning complex cognitive tasks, it also provided such high motivation to engage with the training that even a time-out period of only ten seconds served as an effective negative punishment. Therefore, this type of training is rewarding and a positive experience which may be used as cognitive enrichment.

The physiological data from Chapter 5, which was taken during Go/No-Go training sessions, demonstrated that the negative punishment did not induce a stress response in the horses. On the contrary, heart rate variability was significantly higher when cost was added (+R/-P) than when there was no cost for errors (+R). It was hypothesised that HRV would decrease in response to negative punishment, since the aim of punishment is to invoke a mild stress response which deters the animal from repeating undesired behaviour (Poling et al., 2002). The increased HRV in response to cost revealed that a mild form of negative punishment (ten second time-out period in which the horse could not access task stimuli) did not induce a physiological stress response, indicating that it was not aversive. Therefore, this method of negative punishment had no negative impact on welfare but had an immediate and significant positive effect on performance, which is the goal for any ethical animal training. Chapter 4 revealed that horses appeared to learn the Go/No-Go task by session three, and in subsequent sessions they switched strategy from indiscriminate responding to avoid wasting mental effort, to discriminate responding to avoid time-out. In doing so, horses were able to reliably predict the outcome of their behaviour. As discussed in Chapter 5, a possible reason for reduced arousal during sessions 4-6 of the Go/No-Go task was a perceived sense of control over outcomes (Mirowsky & Ross, 1990; Solomon et al., 1980). As horses learned that they could avoid punishment and continue taking part in the task if they responded according to the Go/No-Go contingencies, this increased sense of agency may have had a positive effect on welfare (Littlewood et al., 2023).

Similarly, in Chapter 3 HRV increased during Reversal sessions, relative to baseline. Perhaps this was due to horses' perceived control over outcomes which they were able to exercise by selecting the rewarded target and reliably receiving reward (Špinka, 2023). For a captive animal such as the domestic horse, having control over outcomes may be especially valuable, since their day-to-day management, training and interactions with humans are largely out of their control (Mills and Clarke, 2007). This could explain why physiological arousal consistently decreased with reward-based training throughout this project.

3.3 Model-based learning in horses

Chapter 4 revealed that horses are capable of model-based learning (MBL), a phenomenon that had not previously been demonstrated in the horse. Decision making during learning is either model-based or model-free. MBL uses prospection to build a mental map or model of the task and related outcomes, whereas model-free learning is much simpler as it is achieved by repetition of previously rewarded behaviour (Doll et al., 2015). MBL requires good executive function, particularly for an animal with an underdeveloped prefrontal cortex (PFC), such as the horse (Hausberger et al., 2019; Schmidt et al., 2019). The PFC is thought to be instrumental in model-based learning (Bunge et al., 2003; Mcdannald et al., 2012), due to its role in processing cause-and-effect relations between choices and outcomes (Tsujimoto et al., 2011). As such, it was assumed that horses would not be good at MBL, therefore the results of Chapter 4 revealed that horses may possess a cognitive capability not previously identified (Hanggi, 2005).

Chapter 4 also highlighted the need to consider alternative strategies that animals may be using, consciously or unconsciously, during cognitive experiments. Though in their initial responses it appeared that horses were unable to complete a Go/No-Go task, it became clear that they did understand the task but had chosen an alternative approach to avoid wasting mental effort (Chapter 4). This is a significant contribution to knowledge, because it may demonstrate a complex form of prospective reasoning, not previously thought to be available to the horse (McLean and Christensen, 2017).

This study also highlighted the value of mild negative punishment. Negative punishment was the main driving factor for the reduction in errors in this study. This was deliberate; the punishment acted as the ‘cost’ in the cost-benefit analysis used to inform responses to the task. The negative punishment used here was a ten second removal of the target. This was effective at reducing errors of emission (touching the target when inappropriate to do so, i.e. during No-Go contingencies). It has long been seen in experimental psychology that positive punishment is extremely effective, even in a single trial. This is due, however, to the severity of the positive punishment seen in one trial learning: typically pain induced by electric shock (e.g. Hudson, 1950). In the current study, by contrast, such a mild punisher was used that it is highly unlikely that the sudden reduction of errors at session four when punishment was introduced was a one trial learning effect.

Further, punishment is only immediately effective when the incorrect response is known. Horses' immediate improvement in performance when punishment was added indicated that they understood what the light cue meant. If horses were unaware of the rules of the task and simply responded in the moment to the punishment, they wouldn't necessarily start following the rules of the light cue immediately. It would be more likely that if horses were just responding to punishment, without prior understanding of the task, they would continue to perform errors of emission. Errors of emission would occur due to the 'trial and error' responding that follows punishment when the 'correct' answer is unknown. Errors of omission may also occur if the punishment is so severe that horses are discouraged from responding completely. However, this is highly unlikely (and did not happen) in this case, where the punishment was so mild (10-second removal of targets).

Subjects had already experienced a level of having food withheld for incorrect responses in the previous chapter, and in the first session if they touched during No-Go contingencies. This is because it is not possible to use positive reinforcement without an element of negative punishment – in order to offer reward for only the desirable behaviour, it must be withheld when incorrect responses are offered. More than the withholding of food, it appears that the time spent unable to access the targets and 'play the game' was particularly effective in encouraging horses to switch to discriminate responding. Interestingly, this appeared to also happen in the later Reward Sensitivity chapter, in which several horses chose the quick option of one treat over three treats, for a faster delivery and return to the task. This points to the intrinsically rewarding nature of certain cognitive tasks for horses, particularly when welfare-friendly training methods are used.

It could be argued that stepping back from the horse during time-out, whilst still in close proximity with the food reward could cause frustration. All horses in all phases were monitored for their physiological responses (heart rate, heart rate variability, infrared thermography of ocular temperature and spontaneous eye blink rate) to the tasks in real time. This was to satisfy the primary aim of the thesis – to determine how arousal is associated with cognitive performance – and to safeguard their welfare during testing. Data presented within Chapter 5 objectively demonstrates that horses were less aroused during this experiment than during rest in their home stable. Since frustration sits on the highly aroused framework of emotion and mood (Mendl et al, 2010) lower arousal is incompatible with the determination that horses were frustrated by this methodology.

The physiological data demonstrates that not only were horses less aroused during training than at rest in their home stable, but that when the negative punishment was introduced arousal was significantly lower than when positive reinforcement alone was used. This indicates the subjects did not find this frustrating. Further, the trainer was not holding the food during ‘time out’, the targets were lowered and the food was out of sight behind the trainer’s back in a closed treat bag.

Additionally, had the horses found the 10 second time out frustrating, a decrease in inhibitory control would be expected as frustration sits on the arousal end of the spectrum (Mendl, 2010) triggering excitatory pathways associated with perseverance (Ijichi et al 2013), not compatible with the improved ability to inhibit the touch response I so markedly observed here.

The importance of considering alternative explanations for data in any scientific study was highlighted in this chapter. On first analysis, it appeared that horses were unable to complete the Go/No-Go task, demonstrating either poor inhibitory control, inability to recognise and respond to a light signal, or a combination of both. Instead, on further investigation, it was revealed that not only were horses capable of effective inhibitory control and attending to a light as a Go/No-Go signal, but they also demonstrated abilities consistent with prospective model-based learning. This fascinating contribution to knowledge of equine cognitive abilities would not have been revealed had the initial data been accepted without further question.

3.4 Flow state in horses

As discussed above (*Section 2.3*) horses appeared to show signs of a flow-like state in some tasks. The six characteristics of flow identified by Nakamura and Csikszentmihalyi (2009) are (1) Intense and focused concentration; (2) Merging of action and awareness; (3) Loss of reflective self-consciousness; (4) Sense of agency over the situation or activity; (5) Distortion of temporal experience; (6) Experience of the activity as intrinsically rewarding. Flow has been identified and studied in some non-human animals (Hintze and Yee, 2022 for review), but has never been specifically identified in horses. This is despite the fact that horses take part in a range of activities involving a combination of physical and cognitive challenge that would make them ideal for flow state to occur. For example, performing a dressage test and jumping technical combinations on a cross-country course are both activities involving high levels of physical and cognitive challenge. Provided the horse also possesses the skill-level to match the level of challenge, these activities may provide optimum conditions for flow state to occur.

In fact, flow state has been identified in horse riders, including race jockeys during flat racing (Jackman et al., 2015) and national hunt (jump) racing (Jackman et al., 2019). Identification of flow-like states in animals is not only an interesting insight into animal cognition, but it offers the opportunity to consider positive welfare states in animals and quantitatively measure a positive experience (Hintze and Yee, 2022). Making demands of animals that have the appropriate level of difficulty is crucial from both an enrichment and welfare perspective and has the potential to protect the Social Licence to Operate within horse sports (McLean and McGreevy, 2010; Duncan et al., 2018).

The Social License to Operate (SLO) refers to the public perception of an industry or activity (Heleski, 2023). The equestrian industry is currently facing a challenge regarding the SLO of equestrian sports and leisure activities, due to increased pressure from the public to demonstrate that horses are willing participants in such activities (Douglas et al., 2022; Fiedler and Slater, 2024). The public typically demand greater protections for species with higher cognitive capabilities, and the scientific evidence indicates that cognition relates strongly to animal welfare (Rogers, 2010). The findings presented in this thesis, particularly those indicating the possibility of model-based learning, would likely reduce the public's tolerance of any suffering of horses in the name of sport or leisure. A significant shift of public perception in this direction may result in revoking the SLO. For example, the public are more likely to turn against racing with its very visible use of the whip or eventing with its horse falls (69 horse fatalities at competition in the last 10 years; Cameron-Whytock et al., 2024), if they feel that horses have higher cognitive capabilities.

However, this thesis also presents evidence that humane training results in reduced stress (compared to baseline), even in relatively cognitively challenging tasks. It also presents evidence consistent with flow-like state in horses, indicating intrinsic reward is possible when humane methods are used. Further, the finding in Chapter 4 that horses were so motivated to take part in the Go/No-Go task that they found a ten-second time out to be sufficiently punishing to alter their approach to the task, suggests that they find this kind of interaction rewarding. The use of horses for sport and leisure may not be a problem for the public *per se*, but the methods used to train and interact with horses may be under greater scrutiny. According to the results of Chapter 3, horses in a lower arousal state both at baseline and during training perform better. Therefore, welfare-friendly training methods can protect horse welfare, improve performance and maintain the social license to operate.

4. Critical considerations and suggestions for future work

It is important to acknowledge the limitations of the research presented in this thesis. This section will review the limitations and critical considerations and suggest directions for future work.

4.1 Statistical models

The research in this thesis identified novel physiological predictors of cognitive performance in horses across a range of tasks, using linear predictive models. Heart rate variability, lateralised eye temperature changes and spontaneous eye blink rate were identified as significant predictors of performance. A limitation of this statistical approach was that non-linear associations were not directly tested. For example, the Yerkes-Dodson law (Yerkes and Dodson, 1908) suggests an inverted-U-shaped relationship between arousal and performance, which was not directly tested by the linear models. Despite this limitation, physiological arousal was consistently a highly significant predictor of performance across tasks, even using linear models.

The negative binomial model used consistently throughout this project was selected because it accounts for over-dispersion (high variability in the dataset; Zuur and Ieno, 2016). Following the ten-step protocol set out by Zuur and Ieno (2016) for conducting predictive models, overdispersion was identified in the datasets, therefore this model type was selected. The overdispersion was likely due to high individual variability between subjects, common in ecological, biological and physiological data (Harrison, 2015; Darryl et al., 2018) The results of the statistical models revealed that physiological arousal, both in the home environment and during training is a strong, significant predictor of performance in a range of tasks in horses. It would be interesting for future work to investigate non-linear relationships between arousal and learning performance in horses, following the interesting and novel results reported here.

4.2 Measuring physiology

There were some challenges associated with the physiological measures used in this thesis. For example, accurately measuring spontaneous eye blink rate non-invasively in horses was challenging for several reasons. First, it was necessary to use a video recording method which could follow both eyes as the horse moved and interacted with the task stimuli. The GoPro method was adapted from Cherry et al. (2020) and solved the problem of eyes moving out of shot. However, this was novel to the sample and great care had to be taken to ensure that horses were properly habituated to the GoPro camera before data collection could start.

As the pilot study presented in Chapter 2 (General Methods) revealed, tolerance to the equipment varied between individuals. Most horses tolerated the GoPro for a total of around thirty minutes, including six minutes of habituation time. However, some horses showed behavioural signs of intolerance at 25 minutes and one horse only tolerated the equipment for five minutes. This indicates that the GoPro is not a well-tolerated method of measuring blink rate in horses and should only be used for short recording periods. This is possibly due to the novelty of the equipment's placement on the face. Many horses demonstrated exploratory behaviour towards the camera, such as hitting it against stable walls and fixtures. Some horses demonstrated headshaking resulting in the removal of the device. As proposed earlier (Section 2.3), blink rate variability may have potential as an indicator of flow state in animals. Therefore, future work should aim to refine the method of collecting blink data non-invasively in horses.

A limitation of the thesis was that heart rate variability was not measured using the same mode consistently. In Chapter 3, HRV was measured in IBI monitoring mode due to an oversight when using the new ECG equipment for the first time. While the IBI mode is valid and acceptable for HRV data collection (Mott et al., 2020), the more optimal method would have been to use the Full Waveform mode, as was implemented in subsequent chapters. The implication of this is that potentially the HRV data in Chapter 1 may have contained artefacts that were missed by the in-built artefact correction in Kubios. As the data were not collected in Full Waveform mode it was not possible to visually inspect the waveform and manually correct these artefacts should they exist. A strong artefact correction was applied to account for this, and the HRV data from subsequent chapters was collected in the more optimal Full Waveform mode.

4.3 Clever Hans

The Clever Hans effect is an important consideration for research of the kind presented in this thesis. The Clever Hans effect is an experimenter effect in which animals detect and respond to subtle, unintended cues from the experimenter which may confound the results of behavioural and cognitive experiments (Gould, 2004; Schmidjell et al., 2012; Rosas, 2022). Horses are sensitive to very subtle cues (Proops and McComb, 2010; Proops et al., 2010; 2013); indeed, Clever Hans (whom the phenomenon was named after) was a horse who appeared to be able to count but was responding to subtle cues from his handler (Gould, 2004). The clever Hans phenomenon raises important questions about whether animals' cognitive abilities are valid or whether they are simply responding to human cues to find the ‘correct’ answer to a cognitive challenge. In this thesis, all experiments involved the presence of a human handler/trainer. It is possible that the handler unintentionally cued horses towards the ‘correct’ response. However, this should not affect the validity of the results presented in this thesis, for the following reasons. Importantly, this thesis was primarily interested in physiological responses to training and predictors of performance. How horses learned which target was ‘correct’, for example, was not as important as their physiological responses to learning. Studies which aim to investigate the mechanisms by which horses learn skills such as reversal, inhibition and reward sensitivity should seek to eliminate the Clever Hans effect from their experiments. However, in the current project it was accepted that horses may learn from unintended cues rather than the task stimuli, but this should not affect the physiological data and validity of these results.

The same handler/trainer (LE) trained all horses in all experiments, for consistency. By the nature of training animals, the trainer was not blinded to the experimental conditions. Despite this, horses did not successfully complete all the cognitive tasks. For example, in Chapter 6 (Reward Sensitivity) most horses did not demonstrate a preference for the high value target, suggesting that horses were not following cues from the handler about which target was ‘correct’. As explored in Chapter 4 (Model-Based Learning), horses did not always respond as expected, again suggesting that the handler’s bias did not significantly influence horses’ decision-making. This thesis provides evidence that horses may be capable of reversal, inhibitory control, model-based learning and some individuals may be sensitive to reward value discrepancies. However, future research should seek to determine how horses acquire these skills, to eliminate the possibility that they are purely responding to human cues. An automated system for conducting cognitive experiments in horses is required to rule out the Clever Hans effect (Kangas and Bergman, 2017). This was beyond the scope of the current thesis and, for the reasons presented here, was not necessary to meet the aims.

4.4 Sample

The sample used in this project were twenty-five university-owned horses, all housed at Nottingham Trent University. University owned horses offer a unique sample because they are regularly used for research, including undergraduate student projects, and teaching purposes. As such, they are accustomed to novel objects, stimuli and conditions, and are regularly handled by different people. It is therefore likely that this sample is more robust in their response to novelty and possibly more cognitively flexible than the average horse. Further, horses such as these may have an improved ability to 'learn to learn' (McGreevy et al., 2018; Lansdell and Kording, 2019), due to their regular exposure to novel experimental conditions. This may have contributed to their performance on the cognitive tasks.

Results of the research presented in this thesis suggest that horses may have certain cognitive abilities. However, due to the unique and relatively small sample, this cannot necessarily be generalised to the entire species. While it has been identified that at least some horses are capable of skills such as Go/No-Go and model-based learning, conclusions about equine cognitive abilities must be drawn with caution. Future research should seek to investigate cognitive performance and arousal in different horse populations, to provide more generalisable data. It would be interesting to understand more about how these findings may relate to wild or semi-feral horse populations. The selection pressures driving some of the behavioural responses seen in this thesis are unclear. It is likely that factors relating to the horse-human relationship play a key role in how domesticated horses respond to tasks such as those presented in this thesis. This is a critical consideration for future work which should seek to replicate some of these studies in different equine populations.

In this thesis, the sample of horses acted as their own controls, to answer questions about individual differences in physiological responses to cognitive challenge and predictors of performance. A separate control group would have been interesting, but I argue that it would have answered a slightly different question. Horses acted as their own controls which generated data on individual responses across tasks. For some of the physiological measures, a control population would not have been appropriate. For example, eye temperature must be taken immediately before and after a manipulation as it is confounded by other factors such as humidity, wind and environmental temperature. It was for this reason that the baseline eye temperature measures were taken immediately before each training session, rather than at one baseline timepoint, as with the other physiological baselines. Further, the sample was too small to split into a control and experimental group, and there were not another twenty horses available at the study site, where a control group of horses would have the same management, routine and environment as the experimental group. Therefore, scientific value aside, it would not have been practically possible to have a control population.

5. General Conclusions

The primary aim of this research was to determine whether spontaneous eye blink rate, as a proxy measure of striatal dopamine activity, could provide a reliable indicator of adaptive learning in an equine model. Spontaneous eye blink rate during learning was not a reliable indicator of cognitive performance in horses, contrary to the findings of human studies. Baseline SEBR did predict performance on some tasks, but not others. This indicates that SEBR might provide a proxy marker for tonic, but not phasic dopamine activity. However, this was not consistent despite all tasks being designed with dopamine function in mind.

There were several factors which made this a thorough and robust investigation into SEBR as a tool in horses. First, in following the same cohort through a variety of cognitive tasks and using standardised data collection methods, I was able to robustly test the value of SEBR as an indicator of learning performance in this species. Second, by using other, well-validated methods (HRV and IRT), the possible confounding effects of arousal were accounted for. Further, as SEBR is a novel method in horses, habituation to the test equipment was investigated in a pilot study. This ensured the validity of SEBR data was not confounded by horses' responses to novel equipment or testing procedures. Though the results of this pilot should be generalised with caution (as discussed in the General Methods chapter), this thesis provides an approximative habituation period for future use of SEBR in horses. In addition, following a lack of positive results in Chapter 1, it was decided that an additional method, blink rate variability, should be investigated. By measuring both the average number of blinks per minute and the variation in time between successive blinks, I was able to further investigate the relationship between blinking and reward learning in horses, taking a two-pronged approach. Taken together, the research presented in this thesis provided a robust and thorough investigation into SEBR and was able to conclude that it was not a useful or reliable tool for assessing cognitive performance in horses. In addition, there were significant challenges associated with measuring SEBR in horses, which are discussed in this thesis and recommendations given.

A second aim of this work was to investigate novel physiological predictors of cognitive performance in horses, to better understand the relationship between arousal and learning in this species. Heart rate variability, both during learning and at baseline, was a consistent predictor of performance across tasks. This demonstrated that arousal, both in the home and training environment, significantly affects cognitive performance in horses. This finding is particularly significant from an applied perspective because it means that, using heart rate variability, it is possible to predict cognitive performance in horses. There are many possible applications for this, for example in predicting the outcomes of re-training of former racehorses into different disciplines, and in the charity re-homing sector more broadly. It may be possible to predict which horses will be successful in their re-training based on baseline HRV.

Further, it may be possible to predict outcomes of individual training sessions based on arousal during the session, therefore potentially avoiding frustration-related effects on the behaviour and welfare of the animals. For a large, potentially dangerous animal such as the horse, and with the risks involved in equestrian activities at all levels, this could have a significant impact on human safety and equine welfare across the sector.

The effects of arousal on cognitive performance may depend on the type of task and/or level of complexity. Though a consistent predictor of performance, the direction of the relationship between HRV and performance varied across tasks. For example, increased HRV during learning (indicating lower arousal) predicted better performance in the Reversal task, whereas lower HRV during learning (increased arousal) predicted better performance in the Go/No-Go task. Therefore, for more simple tasks, maintaining lower arousal in training sessions may be beneficial, whereas for more complex tasks a higher level of physiological activation is required. Interestingly, though, the relationship between baseline HRV and performance was always positive. This indicates that maintaining lower arousal in the home environment, possibly through improved welfare, could have a significant positive effect on the cognitive performance of horses more generally. This requires further research beyond the aims and scope of this thesis; for example, directly measuring the impact of different management conditions on learning performance. The findings presented in this thesis call for such future work to be conducted.

Further, this research has provided training protocols for a series of equine-appropriate cognitive tests, and appropriate physiological measures which could be implemented in any future work. The potential impact of this across the equine industry is significant. For example, if the management and welfare of competition horses is demonstrated to have positive effects on training outcomes and performance, this could be instrumental in driving welfare initiatives in equine sport.

Valuable insights into equine learning and cognition were gained through this research. It was revealed that horses are capable of learning complex tasks, some not previously demonstrated in this species, within a few short training sessions using mostly positive reinforcement. Where negative punishment was used (Chapter 4), only a mild application of this training method was required to have significant effects on performance, without increasing arousal. In fact, arousal was demonstrated to be lower in training sessions where a short (ten–second) time out period was introduced. The implication of this is that negative punishment, when used in moderation, is an effective and ethical training tool.

In Chapter 4 horses demonstrated model-based learning, a higher-order cognitive skill not previously reported in this species. This was a significant finding because it suggests that horses use prospection in their decision-making, rather than simple stimulus-response. This study identified a potential cognitive ability of the horse that would have been missed if the performance data in the first three training sessions was accepted without further investigation. Instead, this work identified that horses are capable of the inhibitory control required for successful Go/No-Go performance; further, it revealed that horses may alter their approach to a task based on a cost-benefit analysis of the reward-related contingencies.

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APPENDICES

Appendix I. Table providing the assignment of targets to HVT and LVT for each horse in Chapter 6, and schedule of pre-conditioning sessions.

Horse ID	**High Value Target**	**Low Value Target**	**Session 1**	**Session 2**	**Session 3**	**Session 4**	**Session 5**	**Session 6**
1	Teapot	Sun	High	Low	Low	Low	High	High
2	Sun	Teapot	High	High	Low	Low	High	Low
3	Sun	Teapot	Low	High	Low	Low	High	High
4	Sun	Teapot	High	Low	High	Low	High	Low
5	Sun	Teapot	Low	Low	High	High	High	Low
6	Teapot	Sun	Low	High	Low	High	High	Low
7	Sun	Teapot	High	Low	Low	Low	High	High
8	Sun	Teapot	High	High	High	Low	Low	Low
9	Sun	Teapot	Low	High	Low	Low	High	High
10	Teapot	Sun	Low	High	Low	High	High	Low
11	Teapot	Sun	Low	High	Low	Low	High	High
12	Teapot	Sun	Low	High	Low	High	Low	High
13	Sun	Teapot	Low	High	Low	High	Low	High
14	Sun	Teapot	High	Low	Low	High	High	Low
15	Sun	Teapot	Low	Low	High	Low	High	High

16	Teapot	Sun	Low	Low	Low	High	High	High
17	Teapot	Sun	Low	High	High	Low	High	Low
18	Teapot	Sun	High	High	Low	High	Low	Low
19	Teapot	Sun	Low	Low	High	High	Low	High
20	Teapot	Sun	High	Low	Low	High	Low	High

Appendix II. Table providing the position of each target during the reward value discrimination phase of Chapter 6, for each subject. The position of the HVT is given in column 4.

Horse ID	**Left side**	**Right side**	**Position of HVT**
1	Sun	Teapot	Right
2	Sun	Teapot	Left
3	Teapot	Sun	Right
4	Teapot	Sun	Right
5	Teapot	Sun	Right
6	Sun	Teapot	Right
7	Sun	Teapot	Left
8	Sun	Teapot	Left
9	Sun	Teapot	Left
10	Teapot	Sun	Left
11	Sun	Teapot	Right

12	Teapot	Sun	Left
13	Teapot	Sun	Right
14	Sun	Teapot	Left
15	Teapot	Sun	Right
16	Teapot	Sun	Left
17	Teapot	Sun	Left
18	Teapot	Sun	Left
19	Sun	Teapot	Right
20	Sun	Teapot	Right

Appendix III. Table providing the position of each target during the reward loss phase of Chapter 6, for each subject. The position of the RVT (the HVT from the previous Reward Sensitivity condition) is given in column 4.

Horse ID	**Left side**	**Right side**	**Position of RVT**
1	Teapot	Sun	Left
2	Teapot	Sun	Right
3	Sun	Teapot	Left
4	Sun	Teapot	Left
5	Sun	Teapot	Left
6	Teapot	Sun	Left

7	Teapot	Sun	Right
8	Teapot	Sun	Right
9	Teapot	Sun	Right
10	Sun	Teapot	Right
11	Teapot	Sun	Left
12	Sun	Teapot	Right
13	Sun	Teapot	Left
14	Teapot	Sun	Right
15	Sun	Teapot	Left
16	Sun	Teapot	Right
17	Sun	Teapot	Right
18	Sun	Teapot	Right
19	Teapot	Sun	Left
20	Teapot	Sun	Left

Appendix IV. R Script for statistical analysis. This example is the analysis from Chapter 5, but the same model and analysis techniques were used throughout the thesis.

```
install.packages("carData")
install.packages("ggplot2")
install.packages("ggpubr")
install.packages("car")
install.packages("lattice")
install.packages("latticeExtra")
install.packages("MASS")
install.packages("tidyverse")
install.packages("lme4")
```

```
install.packages("car")
install.packages("ggplot")
install.packages("rtools")
library(rtools)
library(tidyverse)
library(lme4)
library(car)
library(ggplot)
library(carData)
library(ggplot2)
library(ggpubr)
library(car)
library(lattice)
library(latticeExtra)
library(MASS)

################ Normality ############################

### Blink rate

shapiro.test(MODEL1_AGAIN$`BR GNG1`)

histogram(MODEL1_AGAIN$`BR GNG1`,
        xlab = "Session 1 BR",
        main= "Histogram")

### Blink rate variability

shapiro.test(MODEL1_AGAIN$`BRV GNG1`)

histogram(MODEL1_AGAIN$`BRV GNG1`,
        xlab = "Session 1 BRV",
        main= "Histogram")

### HRV

shapiro.test(MODEL1_AGAIN$RMSSD.1)

histogram(MODEL1_AGAIN$RMSSD.1,
        xlab = "Session 1 HRV RMSSD",
        main= "Histogram")

### Left eye temperature change

shapiro.test(MODEL1_AGAIN$`L change 1`)

histogram(MODEL1_AGAIN$`L change 1`,
        xlab = "Session 1 Left eye temp change",
```

```
        main= "Histogram")


### Right eye temperature change

shapiro.test(MODEL1_AGAIN$`R change 1`)

histogram(MODEL1_AGAIN$`R change 1`,
        xlab = "Session 1 Right eye temp change",
        main= "Histogram")

### Performance
shapiro.test(MODEL1_AGAIN$Performance4)

histogram(MODEL1_AGAIN$Performance4,
        xlab = "Session 4 Performance",
        main= "Histogram")

########################################

# DATA EXPLORATION


# 1. Are there missing values?

colSums(is.na(MODEL1_AGAIN))

# 2. Are categorical covariates balanced?

### no categorical covariates ########

# 3.OUTLIERS

# 4. Make a vector of continuous covariates

Var <- c("L.change.1", "R.change.1", "BR.GNG1", "BRV.GNG1",

       "RMSSD.1", "Performance4")

Var

# 5. The code below makes a multi-panel Cleveland dotplot. It shows the order and

# range of each of the numerical variables
#Fig 1.

dotplot(as.matrix(as.matrix(MODEL1_AGAIN[,Var])),
```

```
    groups=FALSE,

    strip = strip.custom(bg = 'white',

               par.strip.text = list(cex = 1.2)),

    scales = list(x = list(relation = "free", draw = TRUE),

           y = list(relation = "free", draw = FALSE)),

    col=1, cex  = 0.6, pch = 16,

    xlab = list(label = "Data range", cex = 1.2),

    ylab = list(label = "Data order", cex = 1.2))
 ########################################

# 6. CALCULATE NUMBER OF ZEROS IN THE RESPONSE VARIABLE
 # 7. What is the percentage of zeros for taxa?
 sum(MODEL1_AGAIN$taxa == 0)

# no zeros
 ########################################

#8. COLLINEARITY
 #Plot covariates against each other and check collinearity

Coll <- c("L.change.1", "R.change.1", "BR.GNG1", "BRV.GNG1",

     "RMSSD.1", "BR.Baseline", "HRV.Baseline", "Performance4")
 #Fig 2.

panel.cor <- function(x, y, digits=1, prefix="", cex.cor = 9)

{usr <- par("usr"); on.exit(par(usr))

par(usr = c(0, 1, 0, 1))

r1=cor(x,y,use="pairwise.complete.obs")

r <- abs(cor(x, y,use="pairwise.complete.obs"))

txt <- format(c(r1, 0.1), digits=digits)[1]

txt <- paste(prefix, txt, sep="")

if(missing(cex.cor)) { cex <- 0.6/strwidth(txt) } else {
```

```
  cex = cex.cor}

text(0.5, 0.5, txt, cex = cex * r)}

pairs(MODEL1_AGAIN["Coll",], lower.panel = panel.cor, cex.labels = 1.5)

#Look for correlations
 #9. Use VIF

vif(glm(Performance4 ~  MODEL1_AGAIN$L.change.1 + MODEL1_AGAIN$R.change.1
        + MODEL1_AGAIN$BR.GNG1 + MODEL1_AGAIN$BRV.GNG1

        + MODEL1_AGAIN$RMSSD.1,

        family = poisson,

        data = MODEL1_AGAIN))

################## Physiology Analysis ##########################

################### Normality ########################

#### Baseline blink rate

shapiro.test(Physiology_data_chapters_2_amp_3_CSV$`BR baseline`)

histogram(Physiology_data_chapters_2_amp_3_CSV$`BR baseline`,
          xlab = "Baseline blink rate",
          main= "Histogram")

#### Baseline blink rate variability

shapiro.test(Physiology_data_chapters_2_amp_3_CSV$`BRV Baseline`)

histogram(Physiology_data_chapters_2_amp_3_CSV$`BRV Baseline`,
          xlab = "Baseline blink rate variability",
          main= "Histogram")

#### Baseline heart rate variability

shapiro.test(Physiology_data_chapters_2_amp_3_CSV$`HRV Baseline`)

histogram(Physiology_data_chapters_2_amp_3_CSV$`HRV Baseline`,
          xlab = "Baseline heart rate variability",
          main= "Histogram")

#### Blink rate no cost
```

```
shapiro.test(Physiology_data_chapters_2_amp_3_CSV$`BR 1-3 Mean`)

histogram(Physiology_data_chapters_2_amp_3_CSV$`BR 1-3 Mean`,
        xlab = "Blink rate no cost",
        main= "Histogram")

#### Blink rate variability no cost

shapiro.test(Physiology_data_chapters_2_amp_3_CSV$`BRV 1-3 Mean`)

histogram(Physiology_data_chapters_2_amp_3_CSV$`BRV 1-3 Mean`,
        xlab = "Blink rate variability no cost",
        main= "Histogram")

#### Blink rate cost

shapiro.test(Physiology_data_chapters_2_amp_3_CSV$`BR 4-6 Mean`)

histogram(Physiology_data_chapters_2_amp_3_CSV$`BR 4-6 Mean`,
        xlab = "Blink rate cost",
        main= "Histogram")

#### Blink rate variability cost

shapiro.test(Physiology_data_chapters_2_amp_3_CSV$`BRV 4-6 Mean`)

histogram(Physiology_data_chapters_2_amp_3_CSV$`BRV 4-6 Mean`,
        xlab = "Blink rate variability cost",
        main= "Histogram")


#### HRV no cost

shapiro.test(Physiology_data_chapters_2_amp_3_CSV$`HRV 1-3 Mean`)

histogram(Physiology_data_chapters_2_amp_3_CSV$`HRV 1-3 Mean`,
        xlab = "HRV no cost",
        main= "Histogram")

#### HRV cost

shapiro.test(Physiology_data_chapters_2_amp_3_CSV$`HRV 4-6 Mean`)

histogram(Physiology_data_chapters_2_amp_3_CSV$`HRV 4-6 Mean`,
          xlab = "Heart rate variability no cost",
        main= "Histogram")

#### Left eye temp change no cost
```

```
shapiro.test(Physiology_data_chapters_2_amp_3_CSV$`LCh 1-3 Mean`)

histogram(Physiology_data_chapters_2_amp_3_CSV$`LCh 1-3 Mean`,
        xlab = "Left eye temp change no cost",
        main= "Histogram")

#### Left eye temp change cost

shapiro.test(Physiology_data_chapters_2_amp_3_CSV$`LCh 4-6 Mean`)

histogram(Physiology_data_chapters_2_amp_3_CSV$`LCh 4-6 Mean`,
        xlab = "Left eye temp change cost",
        main= "Histogram")

#### Right eye temp change no cost

shapiro.test(Physiology_data_chapters_2_amp_3_CSV$`RCh 1-3 Mean`)

histogram(Physiology_data_chapters_2_amp_3_CSV$`RCh 1-3 Mean`,
        xlab = "Right eye temp change no cost",
        main= "Histogram")

#### Right eye temp change cost

shapiro.test(Physiology_data_chapters_2_amp_3_CSV$`RCh 4-6 Mean`)

histogram(Physiology_data_chapters_2_amp_3_CSV$`RCh 4-6 Mean`,
        xlab = "Right eye temp change cost",
        main= "Histogram")

##### Blink Rate

t.test(Physiology_data_chapters_2_amp_3_CSV$`BR baseline`,
      Physiology_data_chapters_2_amp_3_CSV$`BR 1-3 Mean`,
      paired = TRUE)

wilcox.test(Physiology_data_chapters_2_amp_3_CSV$`BR baseline`,
      Physiology_data_chapters_2_amp_3_CSV$`BR 4-6 Mean`,
      paired = TRUE)

wilcox.test(Physiology_data_chapters_2_amp_3_CSV$`BR 1-3 Mean`,
          Physiology_data_chapters_2_amp_3_CSV$`BR 4-6 Mean`,
          paired = TRUE)

boxplot(Physiology_data_chapters_2_amp_3_CSV$`BR 1-3 Mean`,
       Physiology_data_chapters_2_amp_3_CSV$`BR 4-6 Mean`,
       xlab= "Condition",
```

```
        names = c("No Cost", "Cost Added"),
        ylab="Blink Rate (blinks per minute)",
        col = c("lightyellow", "pink1"),
        main= "Blink Rate by Condition")


#### Blink rate variability

wilcox.test(Physiology_data_chapters_2_amp_3_CSV$`BRV Baseline`,
       Physiology_data_chapters_2_amp_3_CSV$`BRV 1-3 Mean`,
       paired = TRUE)

wilcox.test(Physiology_data_chapters_2_amp_3_CSV$`BRV Baseline`,
            Physiology_data_chapters_2_amp_3_CSV$`BRV 4-6 Mean`,
            paired = TRUE)

wilcox.test(Physiology_data_chapters_2_amp_3_CSV$`BRV 1-3 Mean`,
            Physiology_data_chapters_2_amp_3_CSV$`BRV 4-6 Mean`,
            paired = TRUE)
boxplot(Physiology_data_chapters_2_amp_3_CSV$`BRV 1-3 Mean`,
     Physiology_data_chapters_2_amp_3_CSV$`BRV 4-6 Mean`,
     ylab="Blink rate variability",
     xlab="Training method",
     names = c("No cost (R+)", "Cost added (R+/P-)"),
     main= "Blink rate variability by training method")

boxplot(Physiology_data_chapters_2_amp_3_CSV$`BRV 1-3 Mean`,
        Physiology_data_chapters_2_amp_3_CSV$`BRV 4-6 Mean`,
        xlab= "Condition",
        names = c("No Cost", "Cost Added"),
        ylab="Blink Rate Variability (RMSSD)",
        col = c("lightyellow", "pink1"),
        main= "Blink Rate Variability by Condition")



#### Heart rate variability
t.test(Physiology_data_chapters_2_amp_3_CSV$`HRV Baseline`,
            Physiology_data_chapters_2_amp_3_CSV$`HRV 1-3 Mean`,
            paired = TRUE)

t.test(Physiology_data_chapters_2_amp_3_CSV$`HRV Baseline`,
            Physiology_data_chapters_2_amp_3_CSV$`HRV 4-6 Mean`,
            paired = TRUE)

t.test(Physiology_data_chapters_2_amp_3_CSV$`HRV 1-3 Mean`,
            Physiology_data_chapters_2_amp_3_CSV$`HRV 4-6 Mean`,
            paired = TRUE)
boxplot(Physiology_data_chapters_2_amp_3_CSV$`HRV 1-3 Mean`,
```

```
        Physiology_data_chapters_2_amp_3_CSV$`HRV 4-6 Mean`,
        ylab="Heart rate variability",
        xlab="Training method",
        names = c("No cost (R+)", "Cost added (R+/P-)"),
        main= "Heart rate variability by training method")

boxplot(Physiology_data_chapters_2_amp_3_CSV$`HRV 1-3 Mean`,
        Physiology_data_chapters_2_amp_3_CSV$`HRV 4-6 Mean`,
        xlab= "Condition",
        names = c("No Cost", "Cost Added"),
        ylab="Heart Rate Variability (RMSSD)",
        col = c("lightyellow", "pink1"),
        main= "Heart Rate Variability by Condition")

####### Left eye temperature change
t.test(Physiology_data_chapters_2_amp_3_CSV$`LCh 1-3 Mean`,
       Physiology_data_chapters_2_amp_3_CSV$`LCh 4-6 Mean`,
       paired = TRUE)

boxplot(Physiology_data_chapters_2_amp_3_CSV$`LCh 1-3 Mean`,
        Physiology_data_chapters_2_amp_3_CSV$`LCh 4-6 Mean`,
        xlab= "Condition",
        names = c("No Cost", "Cost Added"),
        ylab="Left eye temperature change (degrees celcius)",
        col = c("lightyellow", "pink1"),
        main= "Left Eye Temperature Change by Condition")

####### Right eye temperature change
t.test(Physiology_data_chapters_2_amp_3_CSV$`RCh 1-3 Mean`,
       Physiology_data_chapters_2_amp_3_CSV$`RCh 4-6 Mean`,
       paired = TRUE)

boxplot(Physiology_data_chapters_2_amp_3_CSV$`RCh 1-3 Mean`,
        Physiology_data_chapters_2_amp_3_CSV$`RCh 4-6 Mean`,
        xlab= "Condition",
        names = c("No Cost", "Cost Added"),
        ylab="Right eye temperature change (degrees celcius)",
        col = c("lightyellow", "pink1"),
        main= "Right Eye Temperature Change by Condition")
########## Physiology across sessions #################

### HRV

boxplot(Physiology_data_chapters_2_amp_3_CSV$`HRV Baseline`,
        Physiology_data_chapters_2_amp_3_CSV$`RMSSD 1`,
        Physiology_data_chapters_2_amp_3_CSV$`RMSSD 2`,
        Physiology_data_chapters_2_amp_3_CSV$`RMSSD 3`,
        Physiology_data_chapters_2_amp_3_CSV$`RMSSD 4`,
```

```
       Physiology_data_chapters_2_amp_3_CSV$`RMSSD 5`,
       Physiology_data_chapters_2_amp_3_CSV$`RMSSD 6`,
       names = c("Baseline", "Session 1", "Session 2", "Session 3",
                "Session 4", "Session 5", "Session 6"),
       main= "Heart Rate Variability by Session",
       xlab= "Condition",
       ylab= "Heart rate variability (RMSSD)",
       col = c("lightyellow3", "lightblue2", "lightblue2", "lightblue2",
              "lightgreen", "lightgreen", "lightgreen"))

install.packages("broman")
library(broman)
pink <- brocolors("crayons")["Sunset Orange"]
palegreen <- brocolors("crayons")["Granny Smith Apple"]
yellow <- brocolors("crayons") ["Lemon Yellow"]
paleblue <- brocolors("crayons") ["Cornflower"]


### BRV

boxplot(Physiology_data_chapters_2_amp_3_CSV$`BRV Baseline`,
       Physiology_data_chapters_2_amp_3_CSV$`BRV GNG1`,
       Physiology_data_chapters_2_amp_3_CSV$`BRV GNG2`,
       Physiology_data_chapters_2_amp_3_CSV$`BRV GNG3`,
       Physiology_data_chapters_2_amp_3_CSV$`BRV GNG4`,
       Physiology_data_chapters_2_amp_3_CSV$`BRV GNG5`,
       Physiology_data_chapters_2_amp_3_CSV$`BRV GNG6`,
       names = c("Baseline", "Session 1", "Session 2", "Session 3",
                "Session 4", "Session 5", "Session 6"),
       main= "Blink Rate Variability by Session",
       xlab= "Condition",
       ylab= "Blink rate variability (RMSSD)",
       col = c("lightyellow3", "lightblue2", "lightblue2", "lightblue2",
              "lightgreen", "lightgreen", "lightgreen"))

### BR
boxplot(Physiology_data_chapters_2_amp_3_Outliers_Removed$`BR baseline`,
       Physiology_data_chapters_2_amp_3_Outliers_Removed$`BR GNG1`,
       Physiology_data_chapters_2_amp_3_Outliers_Removed$`BR GNG2`,
       Physiology_data_chapters_2_amp_3_Outliers_Removed$`BR GNG3`,
       Physiology_data_chapters_2_amp_3_Outliers_Removed$`BR GNG4`,
       Physiology_data_chapters_2_amp_3_Outliers_Removed$`BR GNG5`,
       Physiology_data_chapters_2_amp_3_Outliers_Removed$`BR GNG6`,
       names = c("Baseline", "Session 1", "Session 2", "Session 3",
                "Session 4", "Session 5", "Session 6"),
       main= "Blink Rate  by Session",
       xlab= "Condition",
       ylab= "Blink rate (blinks per minute",
```

```
      col = c("lightyellow3", "lightblue2", "lightblue2", "lightblue2",
          "lightgreen", "lightgreen", "lightgreen"))

### Left eye temp change
boxplot(Physiology_data_chapters_2_amp_3_CSV$`L change 1`,
      Physiology_data_chapters_2_amp_3_CSV$`L change 2`,
      Physiology_data_chapters_2_amp_3_CSV$`L change 3`,
      Physiology_data_chapters_2_amp_3_CSV$`L change 4`,
      Physiology_data_chapters_2_amp_3_CSV$`L change 5`,
      Physiology_data_chapters_2_amp_3_CSV$`L change 6`,
      names = c("Session 1", "Session 2", "Session 3",
           "Session 4", "Session 5", "Session 6"),
      main= "Left Eye Temperature Change by Session",
      xlab= "Condition",
      ylab= "Left eye temperature change (degrees celcius)",
      col = c("lightblue2", "lightblue2", "lightblue2",
          "lightgreen", "lightgreen", "lightgreen"))

### Right eye temp change
boxplot(Physiology_data_chapters_2_amp_3_CSV$`R change 1`,
      Physiology_data_chapters_2_amp_3_CSV$`R change 2`,
      Physiology_data_chapters_2_amp_3_CSV$`R change 3`,
      Physiology_data_chapters_2_amp_3_CSV$`R change 4`,
      Physiology_data_chapters_2_amp_3_CSV$`R change 5`,
      Physiology_data_chapters_2_amp_3_CSV$`R change 6`,
      names = c("Session 1", "Session 2", "Session 3",
           "Session 4", "Session 5", "Session 6"),
      main= "Right Eye Temperature Change by Session",
      xlab= "Condition",
      ylab= "Right eye temperature change (degrees celcius)",
      col = c("lightblue2", "lightblue2", "lightblue2",
          "lightgreen", "lightgreen", "lightgreen"))


################## citation for varian package ################################
install.packages("varian")
library(varian)
citation("varian")
################################################################################

###### Kruskal Wallis test for difference in physiology across sessions

install.packages("ggpubr")
library(ggpubr)

####  Blink rate  #####

kruskal.test(BR ~ Group, data = GNG_GROUPED )
```

```
ggboxplot(GNG_GROUPED, x = "Group", y = "BR",
      color = "Group", palette = c("#E7B800","#00AFBB","#00AFBB","#00AFBB" ,"#FC4E07",
"#FC4E07","#FC4E07"),
      order = c("Baseline", "GNG1", "GNG2", "GNG3", "GNG4", "GNG5", "GNG6"),
      ylab = "Blink Rate", xlab = "Session")

wilcox.test(Physiology_data_chapters_2_amp_3_CSV$`BR GNG3`,
       Physiology_data_chapters_2_amp_3_CSV$`BR GNG4`,
       paired = TRUE)


pairwise.wilcox.test(GNG_GROUPED$BR, GNG_GROUPED$Group,
            p.adjust.method = "bonferroni")

wilcox_effsize(data = GNG_GROUPED,
        formula = BR~Group,
        paired = TRUE)
effsizeBR<-wilcox_effsize(data = GNG_GROUPED,
              formula = BR~Group,
              paired = TRUE)
effsizeBR%>%print(n=21)

###### Blink rate variability

kruskal.test(BRV ~ Group, data = GNG_GROUPED )


pairwise.wilcox.test(GNG_GROUPED$BRV, GNG_GROUPED$Group,
            p.adjust.method = "bonferroni")

wilcox_effsize(data = GNG_GROUPED,
        formula = BRV~Group,
        paired = TRUE)
effsizeBRV<-wilcox_effsize(data = GNG_GROUPED,
              formula = BRV~Group,
              paired = TRUE)
effsizeBRV%>%print(n=21)

####### HRV

kruskal.test(HRV ~ Group, data = GNG_GROUPED )


pairwise.wilcox.test(GNG_GROUPED$HRV, GNG_GROUPED$Group,
            p.adjust.method = "bonferroni")

wilcox_effsize(data = GNG_GROUPED,
```

```
            formula = HRV~Group,
            paired = TRUE)
effsizeHRV<-wilcox_effsize(data = GNG_GROUPED,
                    formula = HRV~Group,
                    paired = TRUE)
effsizeHRV%>%print(n=21)

######### Left eye
kruskal.test(`L Change` ~ Group, data = GNG_GROUPED_BASELINEXCLUDED )


pairwise.wilcox.test(GNG_GROUPED_BASELINEXCLUDED$`L Change`,
GNG_GROUPED_BASELINEXCLUDED$Group,
                p.adjust.method = "bonferroni")

wilcox_effsize(data = GNG_GROUPED_BASELINEXCLUDED,
            formula = `L Change`~Group,
            paired = TRUE)
effsizeLChange<-wilcox_effsize(data = GNG_GROUPED_BASELINEXCLUDED,
                    formula = `L Change`~Group,
                    paired = TRUE)
effsizeLChange%>%print(n=21)

######## Right eye
kruskal.test(`R Change` ~ Group, data = GNG_GROUPED_BASELINEXCLUDED )


pairwise.wilcox.test(GNG_GROUPED_BASELINEXCLUDED$`R Change`,
GNG_GROUPED_BASELINEXCLUDED$Group,
                p.adjust.method = "bonferroni")

wilcox_effsize(data = GNG_GROUPED_BASELINEXCLUDED,
            formula = `R Change`~Group,
            paired = TRUE)
effsizeRChange<-wilcox_effsize(data = GNG_GROUPED_BASELINEXCLUDED,
                      formula = `R Change`~Group,
                      paired = TRUE)
effsizeRChange%>%print(n=21)


glm.nb(MODEL1_AGAIN$Performance4 ~ MODEL1_AGAIN$`BR baseline`+
        MODEL1_AGAIN$`BRV Baseline`+ MODEL1_AGAIN$`HRV Baseline`+
        MODEL1_AGAIN$`BR GNG1`+ MODEL1_AGAIN$`BRV GNG1`+
        MODEL1_AGAIN$`RMSSD 1`+ MODEL1_AGAIN$`L change 1`+
        MODEL1_AGAIN$`R change 1`,
       data = MODEL1_AGAIN,
       link = log())
```

```
############ eye temp missing analysis ################
par(mfrow=c(2,3))
### Session 1

shapiro.test(GNG_IRT$`Pre L 1`)
shapiro.test(GNG_IRT$`Pre R 1`)
shapiro.test(GNG_IRT$`Post L 1`)
shapiro.test(GNG_IRT$`Post R 1`)

### All Normal

t.test(GNG_IRT$`Pre L 1`,
      GNG_IRT$`Post L 1`,
      paired = TRUE)
boxplot(GNG_IRT$`Pre L 1`,
       GNG_IRT$`Post L 1`,
       xlab= "Data collection point",
       ylab= "Eye Temperature (Degrees Celcius)",
       main= "Left eye Session 1",
       names = c("Pre-Session", "Post-Session"),
       col = c("orange1","purple"))

## effect size ##
library(lsr)
cohensD(GNG_IRT$`Pre L 1`,
       GNG_IRT$`Post L 1`,
       method = "paired")

t.test(GNG_IRT$`Pre R 1`,
      GNG_IRT$`Post R 1`,
      paired = TRUE)
boxplot(GNG_IRT$`Pre R 1`,
       GNG_IRT$`Post R 1`,
       xlab= "Data collection point",
       ylab= "Eye Temperature (Degrees Celcius)",
       main= "Right eye Session 1",
       names = c("Pre-Session", "Post-Session"),
       col = c("orange1","purple"))

cohensD(GNG_IRT$`Pre R 1`,
       GNG_IRT$`Post R 1`,
       method = "paired")

### Session 2

shapiro.test(GNG_IRT$`Pre L 2`)
shapiro.test(GNG_IRT$`Pre R 2`)
```

```
shapiro.test(GNG_IRT$`Post L 2`)
shapiro.test(GNG_IRT$`Post R 2`)

### All Normal

t.test(GNG_IRT$`Pre L 2`,
      GNG_IRT$`Post L 2`,
      paired = TRUE)
boxplot(GNG_IRT$`Pre L 2`,
        GNG_IRT$`Post L 2`,
        xlab= "Data collection point",
        ylab= "Eye Temperature (Degrees Celcius)",
        main= "Left eye Session 2",
        names = c("Pre-Session", "Post-Session"),
        col = c("orange1","purple"))

cohensD(GNG_IRT$`Pre L 2`,
        GNG_IRT$`Post L 2`,
        method = "paired")

t.test(GNG_IRT$`Pre R 2`,
      GNG_IRT$`Post R 2`,
      paired = TRUE)
boxplot(GNG_IRT$`Pre R 2`,
        GNG_IRT$`Post R 2`,
        xlab= "Data collection point",
        ylab= "Eye Temperature (Degrees Celcius)",
        main= "Right eye Session 2",
        names = c("Pre-Session", "Post-Session"),
        col = c("orange1","purple"))

cohensD(GNG_IRT$`Pre R 2`,
        GNG_IRT$`Post R 2`,
        method = "paired")

### Session 3

shapiro.test(GNG_IRT$`Pre L 3`)
shapiro.test(GNG_IRT$`Pre R 3`)
shapiro.test(GNG_IRT$`Post L 3`)
shapiro.test(GNG_IRT$`Post R 3`)

### All Normal

t.test(GNG_IRT$`Pre L 3`,
      GNG_IRT$`Post L 3`,
      paired = TRUE)
boxplot(GNG_IRT$`Pre L 3`,
```

```
        GNG_IRT$`Post L 3`,
        xlab= "Data collection point",
        ylab= "Eye Temperature (Degrees Celcius)",
        main= "Left eye Session 3",
        names = c("Pre-Session", "Post-Session"),
        col = c("orange1","purple"))

cohensD(GNG_IRT$`Pre L 3`,
        GNG_IRT$`Post L 3`,
        method = "paired")

t.test(GNG_IRT$`Pre R 3`,
       GNG_IRT$`Post R 3`,
       paired = TRUE)
boxplot(GNG_IRT$`Pre R 3`,
        GNG_IRT$`Post R 3`,
        xlab= "Data collection point",
        ylab= "Eye Temperature (Degrees Celcius)",
        main= "Right eye Session 3",
        names = c("Pre-Session", "Post-Session"),
        col = c("orange1","purple"))

cohensD(GNG_IRT$`Pre R 3`,
        GNG_IRT$`Post R 3`,
        method = "paired")

### Session 4

shapiro.test(GNG_IRT$`Pre L 4`)
shapiro.test(GNG_IRT$`Pre R 4`)
shapiro.test(GNG_IRT$`Post L 4`)
shapiro.test(GNG_IRT$`Post R 4`)

### All Normal except pre R

t.test(GNG_IRT$`Pre L 4`,
       GNG_IRT$`Post L 4`,
       paired = TRUE)
boxplot(GNG_IRT$`Pre L 4`,
        GNG_IRT$`Post L 4`,
        xlab= "Data collection point",
        ylab= "Eye Temperature (Degrees Celcius)",
        main= "Left eye Session 4",
        names = c("Pre-Session", "Post-Session"),
        col = c("orange1","purple"))

cohensD(GNG_IRT$`Pre L 4`,
        GNG_IRT$`Post L 4`,
```

```
        method = "paired")

wilcox.test(GNG_IRT$`Pre R 4`,
       GNG_IRT$`Post R 4`,
       paired = TRUE)
boxplot(GNG_IRT$`Pre R 4`,
        GNG_IRT$`Post R 4`,
        xlab= "Data collection point",
        ylab= "Eye Temperature (Degrees Celcius)",
        main= "Right eye Session 4",
        names = c("Pre-Session", "Post-Session"),
        col = c("orange1","purple"))

install.packages("rstatix")
install.packages("coin")
library(rstatix)
library(coin)
wilcox_effsize(data = CH_5_SESSION_4_IRT,
               formula = Temperature~Condition,
               comparisons = NULL,
               ref.group = NULL,
               paired = TRUE,
               alternative = "two.sided",
               mu = 0,
               ci = FALSE,
               conf.level = 0.95,
               ci.type = "perc",
               nboot = 1000)

### Session 5

shapiro.test(GNG_IRT$`Pre L 5`)
shapiro.test(GNG_IRT$`Pre R 5`)
shapiro.test(GNG_IRT$`Post L 5`)
shapiro.test(GNG_IRT$`Post R 5`)

### All Normal

t.test(GNG_IRT$`Pre L 5`,
       GNG_IRT$`Post L 5`,
       paired = TRUE)
boxplot(GNG_IRT$`Pre L 5`,
        GNG_IRT$`Post L 5`,
        xlab= "Data collection point",
        ylab= "Eye Temperature (Degrees Celcius)",
        main= "Left eye Session 5",
        names = c("Pre-Session", "Post-Session"),
        col = c("orange1","purple"))
```

```
cohensD(GNG_IRT$`Pre L 5`,
       GNG_IRT$`Post L 5`,
       method = "paired")

t.test(GNG_IRT$`Pre R 5`,
          GNG_IRT$`Post R 5`,
          paired = TRUE)
boxplot(GNG_IRT$`Pre R 5`,
       GNG_IRT$`Post R 5`,
       xlab= "Data collection point",
       ylab= "Eye Temperature (Degrees Celcius)",
       main= "Right eye Session 5",
       names = c("Pre-Session", "Post-Session"),
       col = c("orange1","purple"))

cohensD(GNG_IRT$`Pre R 5`,
       GNG_IRT$`Post R 5`,
       method = "paired")

### Session 6

shapiro.test(GNG_IRT$`Pre L 6`)
shapiro.test(GNG_IRT$`Pre R 6`)
shapiro.test(GNG_IRT$`Post L 6`)
shapiro.test(GNG_IRT$`Post R 6`)

### All Normal except pre r

t.test(GNG_IRT$`Pre L 6`,
      GNG_IRT$`Post L 6`,
      paired = TRUE)
boxplot(GNG_IRT$`Pre L 6`,
       GNG_IRT$`Post L 6`,
       xlab= "Data collection point",
       ylab= "Eye Temperature (Degrees Celcius)",
       main= "Left eye Session 6",
       names = c("Pre-Session", "Post-Session"),
       col = c("orange1","purple"))

cohensD(GNG_IRT$`Pre L 6`,
       GNG_IRT$`Post L 6`,
       method = "paired")

wilcox.test(GNG_IRT$`Pre R 6`,
          GNG_IRT$`Post R 6`,
          paired = TRUE)
boxplot(GNG_IRT$`Pre R 6`,
```

```
    GNG_IRT$`Post R 6`,
    xlab= "Data collection point",
    ylab= "Eye Temperature (Degrees Celcius)",
    main= "Right eye Session 6",
    names = c("Pre-Session", "Post-Session"),
    col = c("orange1","purple"))

wilcox_effsize(data = CH_6_SESSION_6_IRT,
        formula = Temperature~Condition,
        comparisons = NULL,
        ref.group = NULL,
        paired = TRUE,
        alternative = "two.sided",
        mu = 0,
        ci = FALSE,
        conf.level = 0.95,
        ci.type = "perc",
        nboot = 1000)
```