

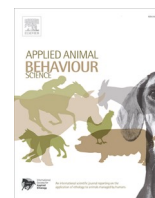
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Title	Eye understand: physiological measures as novel predictors of adaptive learning in horses.
Type	Article
URL	<a href="https://clock.uclan.ac.uk/50196/">https://clock.uclan.ac.uk/50196/</a>
DOI	##doi##
Date	2024
Citation	Evans, Louise, Cameron-Whytock, Heather orcid iconORCID: 0000-0003-0760-2584 and Ijichi, Carrie (2024) Eye understand: physiological measures as novel predictors of adaptive learning in horses. Applied Animal Behaviour Science, 271 . ISSN 0168-1591
Creators	Evans, Louise, Cameron-Whytock, Heather and Ijichi, Carrie

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# Eye understand: Physiological measures as novel predictors of adaptive learning in horses

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## ARTICLE INFO

### Keywords:

Cognition  
Heart rate variability  
Hemispheric asymmetry  
Infrared thermography  
Reversal learning  
Spontaneous eye blink rate

## ABSTRACT

Striatal dopamine is a neurotransmitter that marks reward and mediates reward learning. Spontaneous Eye Blink Rate (SEBR) reflects striatal dopamine activity and could offer a novel, proxy measure of learning performance. Additionally, arousal affects performance in a range of cognitive tasks, but there is less evidence for the relationship between baseline arousal and learning. This study investigated whether SEBR predicts learning performance in an equine model. Further, it investigated novel physiological predictors of learning performance (eye temperature and heart rate variability). Nineteen horses completed a two-choice discrimination reversal learning (RL) task. Performance was measured using a Performance Index. SEBR and Heart Rate Variability (HRV) were measured at rest (home stable) and during RL. Infrared Thermography of eye temperature (IRT) was measured immediately before and after RL. SEBR did not change in response to RL despite being thought to reflect striatal dopamine activity. HRV was higher during RL than at rest though was not statistically significant ( $p = 0.06$ ). Additionally, eye temperature decreased significantly during trials (left eye:  $p = 0.002$ ; right eye:  $p = 0.05$ ). These results indicate lower arousal in response to training, possibly similar to a 'flow state' in humans. Results of a negative binomial GLM revealed that SEBR was not associated with performance, however, positive predictors of learning performance included resting HRV ( $p = 0.009$ ), HRV during the task ( $p = 0.002$ ), and left eye temperature change ( $p < 0.0001$ ). The association between left eye temperature and learning performance is consistent with lateralised blood flow to the left-brain hemisphere (responsible for learning targeted responses) and thought to be the first observation of this phenomenon during learning. Lower arousal may facilitate left-hemisphere dominance, creating the cognitive space to respond adaptively in RL. Left hemispheric dominance controls parasympathetic activity, potentially explaining the interactions observed here. Results suggest arousal both in the home and training environment may have important impacts on learning.

## 1. Introduction

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 and 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 and 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 and Conceição, 2017), thus increasing an individual's tendency to perform a behavioural response when there is opportunity for reward (Berridge, 2007; Maia and 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

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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 and 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 and Jackson, 1970; Wascher, 2021) and lower heart rate variability (Applehans and 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 and 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 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. 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 and 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 and 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.

Domesticated horses provide a suitable model species for exploring the link between SEBR, arousal and reversal learning for several reasons. They are adept at operant conditioning and readily learn novel tasks when reinforced correctly (McGreevy and 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 and 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). 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). In fact, horses have been used to investigate stress-induced alterations to striatal dopamine physiology (McBride and Hemmings, 2005) and the relationship between SEBR at rest and reduced behavioural extinction in an operant task (Kirsty et al., 2015). Finally, multiple measures of arousal are well validated in this species (Garnett and Merkies, 2019; Hall et al., 2011; Ijichi et al., 2020; Merkies et al., 2019; Rietmann et al., 2004; Stucke et al., 2015).

It has been generally accepted that SEBR is reflective of striatal dopamine activity (Jongkees and 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 and 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

### 2.1. Ethical note

Ethical approval was granted by the Nottingham Trent University Ethical Review Committee (ethical approval code: ARE202129). This study was conducted in compliance with UK and EU laws relating to the use of animals in research. The training was a free-choice paradigm in that subjects could choose not to engage with a training session. Subjects were tested in their home environment with their usual companion nearby to prevent isolation stress. Only currently healthy subjects were used throughout the study.

### 2.2. Subjects

Subjects were twenty-two domestic horses (*Equus caballus*) aged between 8 and 22 years (mean = 15.8 years s.d. 3.06) and included 8 females and 14 castrated males. Subjects were housed and managed at Nottingham Trent University. Subjects' general routines were unchanged by this study; their regular exercise and turnout times were unaffected and their usual night-time sleeping and resting hours were uninterrupted. Subjects received *ad libitum* access to forage and water with concentrate feeds appropriate for their individual needs. Subjects had regular access to free outdoor movement and socialisation and a

workload tailored to their age and physical ability.

### 2.3. Learning protocols

The protocol was a two-choice discrimination reversal learning task. Subjects first undertook pre-conditioning, 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. The same trainer (LE) trained all 22 horses, using the same method and training schedule (Fig. 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.

### 2.4. Pre-conditioning

A whistle was used as a secondary reinforcer as this left the researcher's hands free for target presentation and prompt delivery of reward. Subjects completed a minimum of two 15-minute whistle conditioning sessions, conducted over consecutive days. Positive reinforcement was used to condition the whistle sound. Every time the whistle sounded the horse was given a small handful of palatable Ulsakind cubes (approximately 5–6 small pellets). This conditioning protocol was repeated continuously for three minutes, followed by a two-minute break, and then repeated for a further three minutes. This training schedule was repeated for a total of 15 minutes.

### 2.5. Discrimination training

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

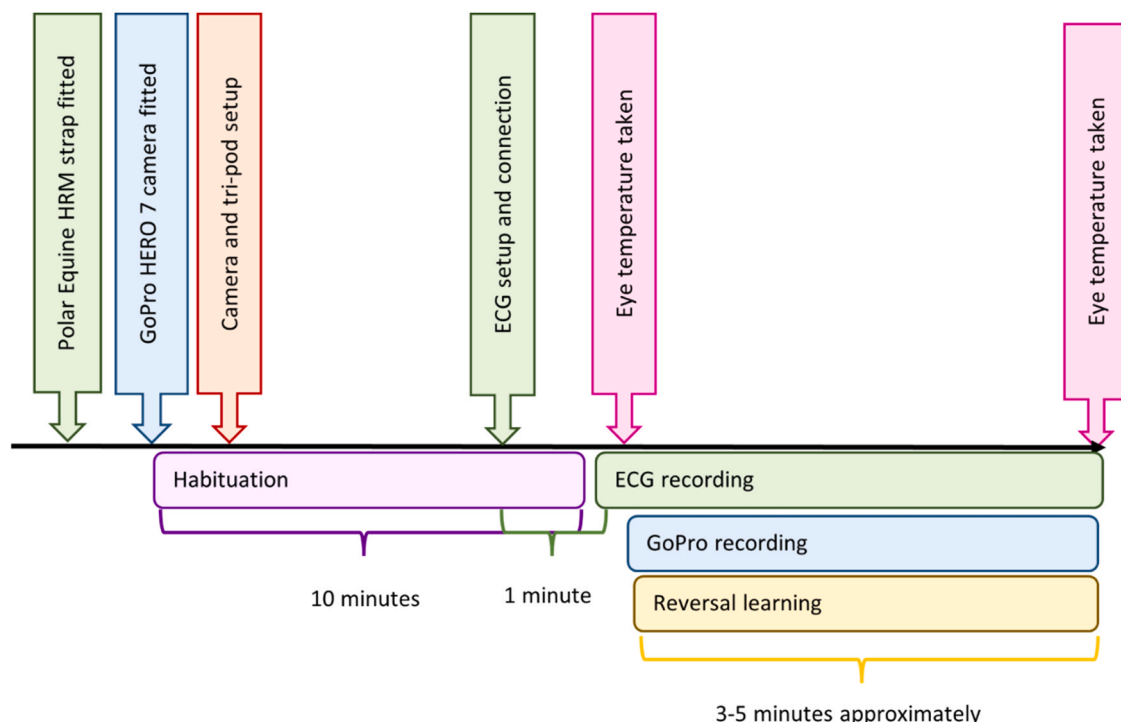


Fig. 1. Timeline of the data collection protocol. Subjects were nineteen horses (*Equus caballus*) housed and managed at Nottingham Trent University.

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 and 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 no reward was given and the next presentation was made. Between each trial the targets were removed from the subjects' sight and 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).

## 2.6. 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 =  $16.3 \pm 2.57$ ). 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.

## 2.7. Behaviour & physiology spontaneous eye blink rate

SEBR was measured at rest (referred to as Resting throughout) and again during learning trials under the same environmental conditions. Resting SEBR was measured as a potential indicator of tonic dopamine and during learning as a potential indicator of phasic dopamine (Jongkees and Colzato, 2016; Kirsty et al., 2015; McBride et al., 2017). To measure SEBR while subjects were unrestrained (to reduce disturbance), subjects' eyes were video recorded using a GoPro HERO 7 camera (GoPro Inc., California, United States) fixed to a GoPro leg strap which was attached above the noseband of a regular headcollar fitted to the horse. The GoPro camera was aimed towards the horse's eyes to capture incidences of blinking in both eyes simultaneously. For Resting SEBR, subjects wore the camera for a total of thirty minutes with the first 12 minutes used as a habituation period as determined by a pilot study which showed that both HRV and SEBR stabilise after this time (Evans et al., unpublished). The remaining 18 minutes were used to calculate control SEBR. During learning sessions, the camera was fitted, and the same 12-minute habituation period provided. The learning session then began and blink rate was recorded throughout until a maximum of 30 trials were completed and the session finished for that subject. The time to complete the session varied dependent on the subject's response latencies but was within the appropriate time-period for tolerating the camera, ranging from nine to fifteen minutes.

All blink rate videos were manually counted by the same researcher (LE). Blinks were manually coded as full blinks or half blinks. A full blink was counted when one or both eyes became momentarily fully closed (Merkies et al., 2019) for less than one second. The duration of a blink was important because subjects in a dozing state may fully close their

eyes, but this does not constitute spontaneous blinking. Half blinks were defined as any movement of the upper eyelid towards the lower eyelid which did not fully cover the eye (Merkies et al., 2019). The number of blinks during Resting recordings were averaged over 18 minutes to maximise the observed period. Blinks during learning sessions were divided by the length (min) of the recorded period, to give average blink rate per minute (referred to as blink rate).

## 2.8. Heart rate variability

Heart rate variability was measured at rest (referred to as Resting throughout) and again during learning trials under the same environmental conditions using an Actiheart ECG device (Actiheart 5 ECG: CamNTEch, Fenstanton, United Kingdom) to provide evidence of both arousal day-to-day and in response to the cognitive challenge. Root Mean Squared of Successive Differences (RMSSD) was selected as it reflects the beat-to-beat variance in heart rate which is used to estimate parasympathetic nervous system activity (Shaffer et al., 2014). Further, it is particularly suitable for estimating short term parasympathetic nervous system activity of less than five minutes (Shaffer and Ginsberg, 2017; von Borell et al., 2007), as was recorded here. The ECG was worn on a Polar Equine belt (Polar Electro Oy, Kempele, Finland), fitted around the thorax as per Mott et al., (2021), using warm water applied to the coat directly beneath the belt as a conductivity aid. Actiheart was set to record in the IBI (Inter-Beat Interval) Monitoring window on the Actiheart software.

RMSSD was measured in the subject's home environment undisturbed during Resting and all learning trials. The recording period was standardised to three minutes at baseline and 3 minutes from the beginning of each training session for all recordings to ensure that variability was not affected by the length of the recording (Eggenberger and Schwarzwald, 2017). Three minutes was selected because all subjects took at least this long to complete the task. Resting ECG recordings lasted 30 minutes with only the final 3 minutes of recording used for analysis, allowing for habituation to the equipment. 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 0.4 s as per previously validated methods for correcting IBI data (Mott et al., 2021).

## 2.9. Infrared thermography

Eye temperature (ET) was measured to indicate both arousal and potential lateralised responses to the cognitive task using a FLIR e60 bx camera with an FOL 18 mm lens (Teledyne FLIR LLC, Oregon, United States). Emissivity was set to  $\sum = 0.95$  (Autio et al., 2006, 2007). Thermal images of the left and then right eye of the horse were taken in quick succession immediately before and after each training session. Baseline images were taken immediately before the learning trial (rather than during the Resting period as for other physiological measures) to calculate changes in eye temperature as a result of the cognitive challenge. This accounts for confounding variables such as environmental temperatures and direct sunlight (Church et al., 2014) and possible individual differences not relating to cognition such as body condition. Images were taken from a 1 m distance and 90-degree angle in relation to the sagittal plane (Ijichi et al., 2020). Thermal images were analysed using FLIR Thermal Studio (Teledyne FLIR LLC, Oregon, United States) software to determine the hottest point within the palpebral fissure from the lateral commissure to the lacrimal caruncle (Elias et al., 2021; Stewart et al., 2008). Absolute temperature was recorded and the change in temperature from pre-training to post-training was calculated and recorded for both eyes.

## 2.10. 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 Learning Performance Index (RLPI) for each horse was calculated using the following formula:  $1000/MT/ME$ , where  $MT$  = number of trials to reach reversal learning criteria and  $ME$  = mean number of errors across all reversal learning trials (Fiske and Potter, 1979), meaning that higher indices indicate faster learning.

### 2.11. 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). First, 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. Next, 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 rest and during a reversal learning challenge as a predictor of learning performance. Both Resting SEBR and reversal learning SEBR were included in the statistical model to account for the possible effects of both tonic (Resting) and phasic (reversal learning) dopamine activity. Data was modelled in R using the MASS package (Venables and Ripley, 2002) to conduct a negative binomial generalised linear model (GLM). The model was carried out according to the 10-step process recommended by Zuur and Leno (2016). Data was 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, Resting blink rate, RMSSD, Resting 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 1. The response variable was the Reversal Learning Performance Index (RLPI). 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). The full R script, including all models tested with outputs, is available as Supplementary Material 2.

## 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 =  $84.44 + 55.36$ ; Fig. 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 ( $^{\circ}\text{C}$ ) 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:  $V = 167.5$ ,  $p = 0.002$ ; Fig. 3a), with a medium effect size (Cohen's  $d = -0.74$ ). Temperature ( $^{\circ}\text{C}$ ) 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:  $V = 157.5$ ,  $p = 0.05$ ; Fig. 3b), with a medium effect size (Cohen's  $d = -0.69$ ).

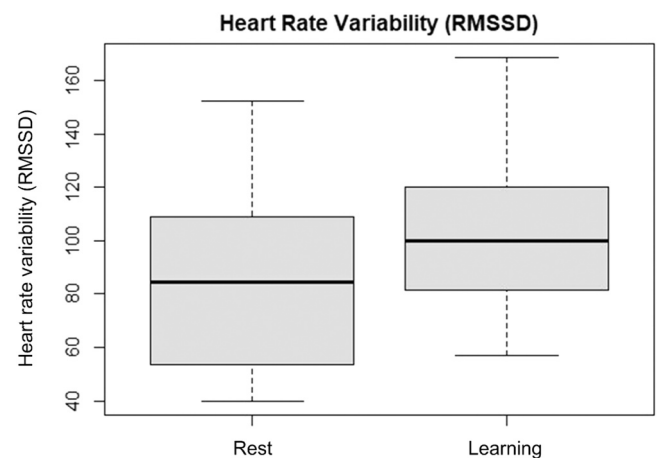
### 3.2. Physiological predictors of learning performance

The results of the negative binomial GLM of best fit are given in Table 2. Performance indices ranged from 2.22 to 49.75 (mean + SD =  $14.35 \pm 15.28$ ). Left eye temperature change was a significant positive predictor of performance ( $p < 0.0001$ ). Resting heart rate variability

**Table 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 and 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 and 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 and 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 and 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 and Dodson, 1908)

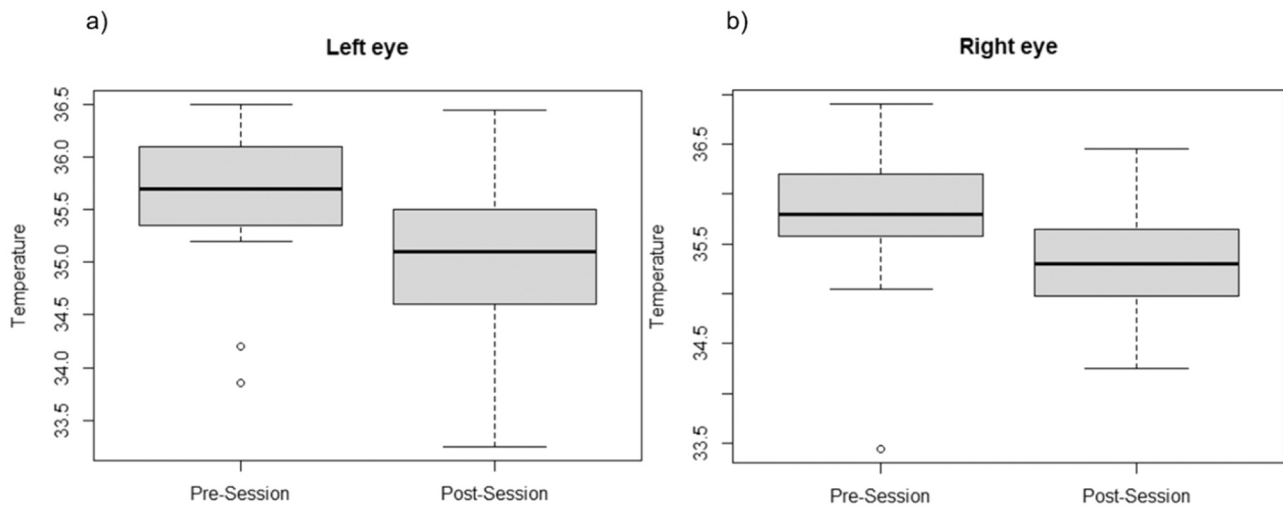


**Fig. 2.** Boxplot showing the heart rate variability as calculated by root mean square of successive differences (RMSSD) in nineteen horses at Rest and during Reversal learning for visual inspection of a possible change ( $n = 19$ ,  $V = 48$ ,  $p = 0.06$ ). The boxes represent the first to the third 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.

(RMSSD) was a significant predictor of performance ( $p = 0.009$ ). RMSSD during learning was also a significant predictor of performance ( $p = 0.002$ ).

## 4. Discussion

Average Resting blink rate did not predict performance in the



**Fig. 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.

**Table 2**

Table of results from the negative binomial generalised linear model.

Coefficients	Estimate	Std. Error	Z value	p value
Intercept	0.47	0.71	0.66	0.51
Left eye temperature change	1.23	0.18	6.75	<0.0001
Resting RMSSD	0.01	0.004	2.59	0.009
RMSSD	0.02	0.005	3.15	0.002

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](#)), we conclude 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 Resting, 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 and 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 and Chen, 2004](#); [Chen and Epps, 2014](#); [Jongkees and 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 and 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 and Paprocki, 2016](#)). [Fukuda and 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 study, would not detect such a change. [Paprocki and 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 and Paprocki, 2016](#); [Paprocki and 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 and 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 and 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 our study species (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 strategy directly aids cognitive performance (Bisazza and Dadda, 2005; Dadda and Bisazza, 2006; Rogers, 2021). In particular, the right hemisphere is responsible for sustained background detection and assessment of potential threats in the environment (Robins and Rogers, 2006; Rogers, 2002; Rogers and Andrew, 2002), while the left hemisphere deals with specific targeted responses to stimuli in the foreground (Robins and 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 and 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 and 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 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 and 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 and 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 and 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 is 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. We 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. Performance was highest in subjects that showed lower arousal, measured by HRV, both at rest and during reversal learning. To the authors' 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.

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 study 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.

## CRedit authorship contribution statement

**Heather Cameron-Whytock:** Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. **Carrie Ijichi:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. **Louise Evans:** Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing.

## Declaration of Competing Interest

The authors have no competing interests to declare.

## Acknowledgments

We would like to acknowledge the equine research technicians at the Brackenhurst Equine Centre, in particular Cath Hake and Anna Gregory, for facilitating access to participants and research equipment. We are very grateful for statistical advice in constructing our model from Dr Mark Andrews. Without funding from Nottingham Trent University Vice-Chancellors Award (2021) and The Horse Trust (Grant no. G3020), this research would not have been possible.

## Data availability

Data can be accessed via [supplementary materials](#) and by contacting the authors.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.applanim.2023.106152](https://doi.org/10.1016/j.applanim.2023.106152).

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