**The Right Angle: Validating a standardised protocol for the use of infrared thermography of eye temperature as a welfare indicator**

Validating IRT measurement angle for eye temperature

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**Abstract**

Infrared Thermography (IRT) is a non-invasive tool for measuring eye temperature as an indicator of stress and welfare in animals. Previous studies state that images are taken from 90° but do not specify a reference point or method of standardisation. The aims of the current study were to determine whether the position of the IRT camera has an impact on recorded temperature and which camera position is optimal for indicating stress in a mammal with anterolateral eyes. IRT images were taken from 90° to the nasal plane, eye and sagittal plane on the left side of the horses' faces (N=14) at eye level before and after exposure to a novel object. Distance and angle of measurement was standardised using ground markers. Temperature at each point of measurement was compared against heart rate variability. A significant difference was found between recorded temperature at all three of the points of measurement, both before and after the novel object test, suggesting that IRT camera position has an impact on eye temperature results. There was a significant strong positive correlation between eye temperature taken from 90° to the sagittal plane and heart rate variability, but no such correlation was observed from 90° to the nasal plane or eye. This suggests that a 90° angle in relation to the sagittal plane is the optimal position for taking eye temperature measurements using IRT, whereas 90° to the eye is commonly used. This study offers a validated protocol for using IRT to measure stress and welfare in mammals with anterolateral eyes.

**Keywords:** infrared thermography; animal welfare; horse; angle of measurement; heart rate variability; eye temperature

## Introduction

A change in temperature at the eye, ear or nose is recognised as a stress response in mammals, caused by sympathetically mediated changes in blood flow to these areas in the presence of a perceived threat or novel event (Blessing 2003). Due to its association with sympathetic responses of the autonomic nervous system (ANS) and Hypothalamic Pituitary Adrenal (HPA) activation, infrared thermography (IRT) has been used to measure eye temperature in animal welfare studies concerned with arousal, stress, pain and fear (Stewart et al. 2005, McGreevy et al. 2012, Bartolomé et al. 2013, Travain et al. 2015, Fenner et al. 2016). IRT of eye temperature is widely used in equine welfare studies, for example, in determining stress in response to the Pessoa training aid (Hall et al. 2011) and to a common aversive handling procedure (Yarnell et al. 2013). IRT has been used to detect potential stress in horses at showjumping (Valera et al. 2012, Bartolomé et al. 2013) and dressage competitions (Sánchez et al. 2016).Esteves Trindade et al. (2019) suggest IRT as a potential predictor for creatine kinase activity and therefore physical fitness in horses. Johnson et al. (2011) suggest that IRT be used as a veterinary screening method for fevers. Therefore, IRT has widespread implications in equine welfare science.

Distance between the IRT camera and the target may have a significant impact on the accuracy of readings. One metre is often suggested as the optimal distance between the IRT camera and the target when measuring a small area (Al-Nakhli et al. 2012). Images taken from the other distances may suffer pixilation loss and are less precise. In all current studies using equine eye temperature as a measure of stress, where specified, the 1m distance is typically utilised for taking thermal images (e.g. Valera et al. 2012; Yarnell et al. 2013; Bartolomé et al. 2013). Critically, these studies do not specify how the distance is measured and controlled for.

Despite validation of the distance between the target and the IRT camera, very few efforts have been made to validate the angle at which the camera is positioned in relation to equine eyes. In studies concerned with human ocular surface temperature, IRT images are taken from a 1 metre distance and a 90° angle to the subject's eye (Tan et al. 2009) which, in humans, translates as directly in front of the face. Many of the studies using IRT to measure equine eye temperature do not specify the angle from which the images were taken (Johnson et al. 2011, Hall et al. 2011).

Where specified, much of the research reports taking images from 90 degrees (e.g. Valera et al. 2012, Yarnell et al. 2013, Bartolomé et al. 2013), however, there is little clarification as to whether 90° is the angle of measurement in relation to the eye or the face of the horse as no reference point is provided. Further, 90° to the head could refer to either the nasal or sagittal plane. For instance, Bartolome *et al*. (2013) report scanning the left eye of horses from a 90° angle. Trindade *et al*. (2018) took images from 90° in relation to the head, which does not translate as 90° to the eye. Further, Trindade et al. (2018) did not specify whether this was to the sagittal or nasal plane. Yarnell *et al.* (2013) report using an angle of 90° from the subject, but they do not specify whether images were taken from 90° to the eye or to the face. However, in the case of Johnson et al, (2011) images appear to have been taken at 90° to the nasal plane of the horse, as in human studies. Taken together, this evidences a wide range of definitions and applications of the guideline to take images from 90° and one metre.

A temperature measurement taken directly perpendicular to the eye may differ from a temperature measurement taken from an alternate angle. The equine eye is not placed directly on the side or the front of the horse's head but rather midway between the nasal and sagittal planes. Placing the camera at a 90° angle to the eye would capture heat radiated directly between the eye and the camera. Modifying the angle of camera placement from the eye would result in radiated heat being captured indirectly from an altered surface area and from a greater distance due to the curvature of the eye. It is therefore important that angle of camera placement and consistency of temperature readings for this species, and those with similar anterolateral eye placement, be thoroughly explored.

Of the studies which do disclose the position of the IRT camera, many do not specify how angle was standardized (e.g. Valera et al. 2012; Bartolomé et al. 2013; Soroko et al. 2016). This raises questions as to how precise recordings are across, and within, studies. Several studies have attempted to control IRT camera position (Ijichi et al. 2018b, 2018a, Squibb et al. 2018). However, it was noted during analysis that slight turning of the horse's head resulted in noticeably different temperatures in images taken seconds apart. Taken together, this indicates a lack of standardization is likely to affect results if the angle of the image is not controlled.

Recently, efforts have been made to standardize the distance and angle of IRT readings during equine studies (Ijichi et al. 2018b, 2018a, Squibb et al. 2018) but the particular method has not been validated. Further, only one angle was used within these studies which does not assess whether this particular angle was correct. Therefore, the aims of the current study were twofold: first, to discover whether equine eye temperature readings are affected by the angle from which IRT images are taken and second, to ascertain whether any of the angles tested may be appropriate for taking equine temperature readings to indicate stress. This will be used to establish a standardised protocol for collecting temperature data using IRT from equines, which can be replicated in a variety of studies within the field of equine welfare science. As such, the objectives of this work were as follows: 1) to use IRT to measure the eye temperature of horses prior to, and immediately following, exposure to a novel object; 2) to take the thermal images from three different positions and compare the temperature readings from each position to reveal whether a difference was present; 3) to compare the change in eye temperature from Pre-test to after the novel object exposure, at each camera position, with heart rate variability (von Borell et al. 2007), to determine whether a correlation exists. It was hypothesized that 1) there are significance differences in eye temperatures taken from differing angles, 2) temperature taken from a 90° angle to the sagittal plane Post-test, but not Pre-test, would correlate most closely with HRV.

## Materials and methods

### *Subjects*

A sample of 14 horses, comprising 6 mares and 8 geldings from Nottingham Trent University (NTU) Brackenhurst Equestrian Centre, were used in this project. The age of subjects ranged from 8-22 years (mean = 12.3 years ± 3.6). These subjects were experience in wearing heart rate monitor equipment and have their eye temperature taken using IRT equipment. Subjects were paired based on companion preferences to reduce the effects of isolation stress and ensure high welfare during testing (Reid et al. 2017). At the time of the study, horses were housed in either individual stables within barn, large multi-horse stables or barn-style stables that open onto small all-weather paddocks, according to individual requirements. When stabled, horses had continuous access to water, and were fed 2.5% of their body weight in pasture hay per day, from the floor of the stable. In addition to this, some horses were fed concentrates, according to body condition and workload. Data collection was carried out at Nottingham Trent University Brackenhurst Equestrian Centre on between 9am and 4pm on two consecutive days in December 2018 in an indoor arena.

### *Testing protocol*

This project was granted ethical approval by the NTU School of Animal, Rural and Environmental Sciences (ARES) Ethics Committee.

Horses were led from their stables to the indoor arena in pairs. Upon arrival at the arena, the first horse from each pair, as determined in prior within-pair randomisation, was led into the

IRT measurement area. Two jump poles were placed on the ground, parallel to each other, facing away from the novel object area. The poles were 1m apart which was wide enough for the horse to stand between them comfortably but narrow enough to aid straightness. This marked the area where the horse would stand to have thermal images taken (Fig. 1). A cavalletti (C1) was placed within the two poles to mark where the horse's head would be when standing to have images taken. Three cavalletti were placed outside of the poles, each 1m away from the first cone and at 0° (C2), 45° (C3) and 90° (C4) in relation to C1. These marked the positioning of the IRT camera when taking the thermal images. Thus C2 captured eye temperature images 90° from the nasal plane, C3 from the eye and C4 from the sagittal plane of the subject.

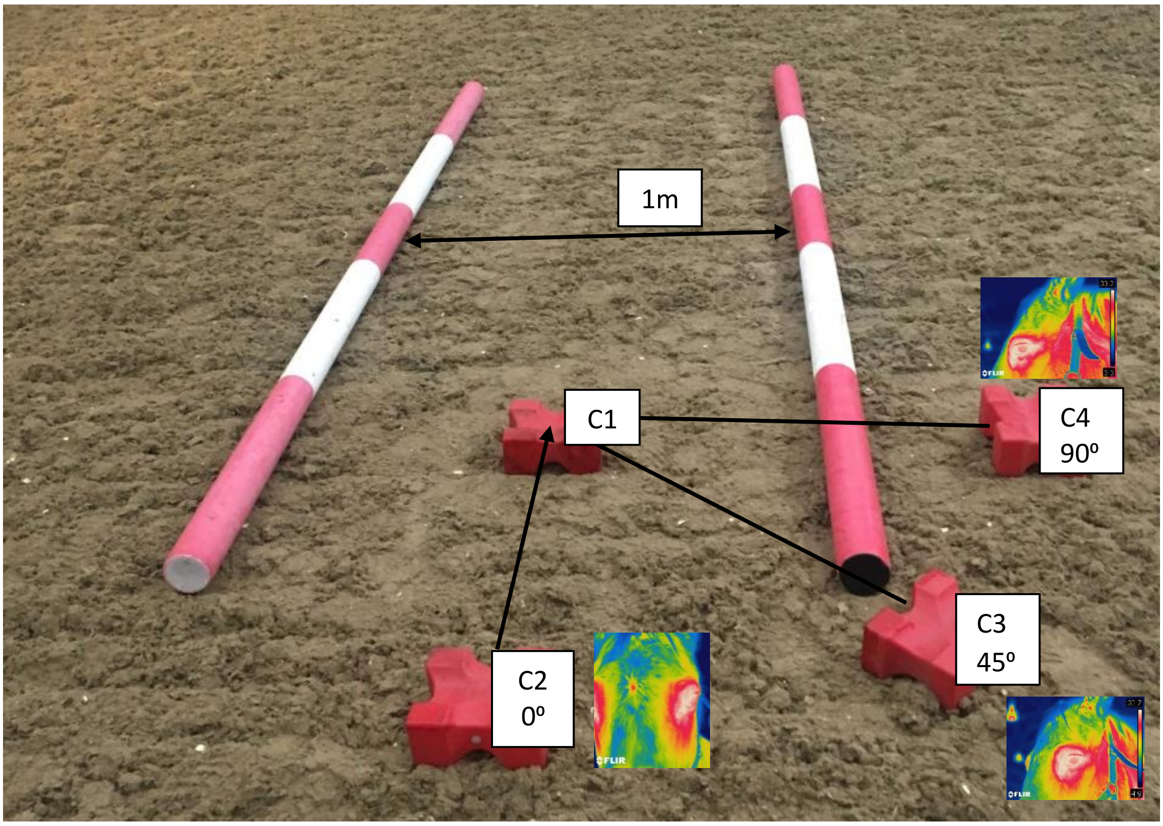


**Figure 1.** IRT measurement protocol. The horse is led between the pole and halted with their head above C1. Images are taken with the IRT camera above C2, C3 and C4 at a distance of 1m (sample images shown).

The subject was asked to stand using gentle leadrope pressure and vocal cues when C1 was directly below the horse's head. The horse was stood facing away from the novel object with their companion horse in sight. Eye temperature readings were taken using a FLIR E60 bx thermal imaging camera with a FOL 18mm lens (FLIR Systems, USA). Emissivity was set to ∑ = 0.95 (Autio et al. 2006, 2007). An image was taken from C2, C3 and C4 (Fig. 1) at the horse's eye level with approximately 15 seconds between images.

Following pre-test eye temperature readings, the horse was fitted with a Polar Equine V800 heart rate monitor (Polar Electro Oy, Kempele, Finland), by use of a surcingle around the thorax. Warm water and a sponge were used to wet the horse's skin at the thorax on their left side, where the conductive component makes contact. The electronic watch monitors were attached to the surcingle, above the conductive proponent, to maintain connectivity throughout the test. The subjects were familiar with wearing this device.

Immediately after starting the HR monitor, the horse was led into the test area by an experienced handler. The test area was cordoned off within the indoor arena using equine specific white mobile gates connected to create a 20x24m area. This allowed the horses to maintain visual and vocal contact with each other. Once inside the test are, the leadrope was unclipped from the headcollar of the horse, and the handler stayed at the shoulder of the horse for the duration of the test, allowing the horse free movement for three minutes. This allowed subjects to approach or avoid the object as they chose. The same experienced handler was present with all horses. After this time, the heart rate monitor was stopped, the lead rope re-attached and the horse was led from the experimental area.

Immediately following the novel object test, the horse was led back to the IRT measurement area to have post-test measurements taken in the same way. The Polar Equine V800 heart rate monitor was then removed from the horse, and the protocol was repeated with the second horse of the pair. Once both horses had been tested, both horses were led back to their stables and the next pair was led to the experimental setting.

***Infrared thermography analysis***

FLIR tools software (version 5.9.16284.1001, FLIR Systems Inc.) was used to analyse IRT images. The maximum temperature found between the lateral commissure and the lacrimal caruncle of the palpebral fissure (Yarnell et al. 2013) was recorded using the elliptical target function which captured no less than 1cm around the eye area. In addition to the highest absolute values taken pre and post-testing, the change in highest IRT from pre to post-testing was calculated to account for individual differences in resting temperature and any fluctuation in environmental factors that may have affected readings over the course of several days.

### Heart rate variability analysis

Kubios software (version 3.0.2 Biomedical Signal Analysis and Medical Imaging Group, Department of Applied Physics, University of Eastern Finland, Kuopio, Finland) was used to analyse heart rate data and determine HRV. Artefact correction was set to custom level 0.03, removing RR intervals varying more that 30% from the previous interval. Trend components were adjusted using the concept of smoothness priors set at 500ms, to avoid the effect of outlying intervals (Ille et al. 2014). SDNN values were recorded as these reflect long-term variability of cardiac outputs in both parasympathetic and sympathetic pathways (Stucke et al. 2015). In addition, Frequency Domain Analysis (FDA) was conducted using a fast Fourier transformation which were expresses as ratios for enhanced comparability (Stucke et al. 2015). The ratio of Low to High Frequency (LF/HF) reflects both parasympathetic and sympathetic tone as well as cardiac sympatho-vagal balance. FDAwas set at >0.01 - ≤0.07 for Low Frequency (LF) and > 0.07 - ≤0.5 for High Frequency (HF) (Stucke et al. 2015). The full recording from leaving the IRT measurement chute to returning after completing the test was selected for analysis.

### Statistical analysis

R Studio was used to analyse data (R Development Core Team 2017). Shapiro-Wilks tests were used to assess normality and determine appropriate subsequent tests as this test is suitable for smaller sample sizes (Field et al. 2012). Dependent tests of difference were used throughout as images came from the same subject. As data was largely not normally distributed, Friedman

Tests were used to determine whether there were significant differences in recorded temperature taken from the three measurement points before and after testing (Field et al. 2012). Subsequently, Wilcoxon Signed Ranks tests were performed to determine whether there were differences between pair of images taken from each angle. Spearman Ranked Correlation and Pearson correlations were used as appropriate for normality to determine whether each angle correlated with SDNN or LF/HF (Field et al. 2012).

## Results

### ***Difference Between the Angles of Measurement***

A highly significant difference was found between the three angles of measurement, both in Pre-Test eye temperature (Friedman: $\chi^2_2$ =24.327, $P < 0.0005$) and Post-Test eye temperature (Friedman: $\chi^2_2$ =20.109, $P < 0.0005$). Wilcoxon Signed Rank and Paired T tests revealed significant effects of angle on IRT readings (Table 1; Fig. 2).

TABLE 1

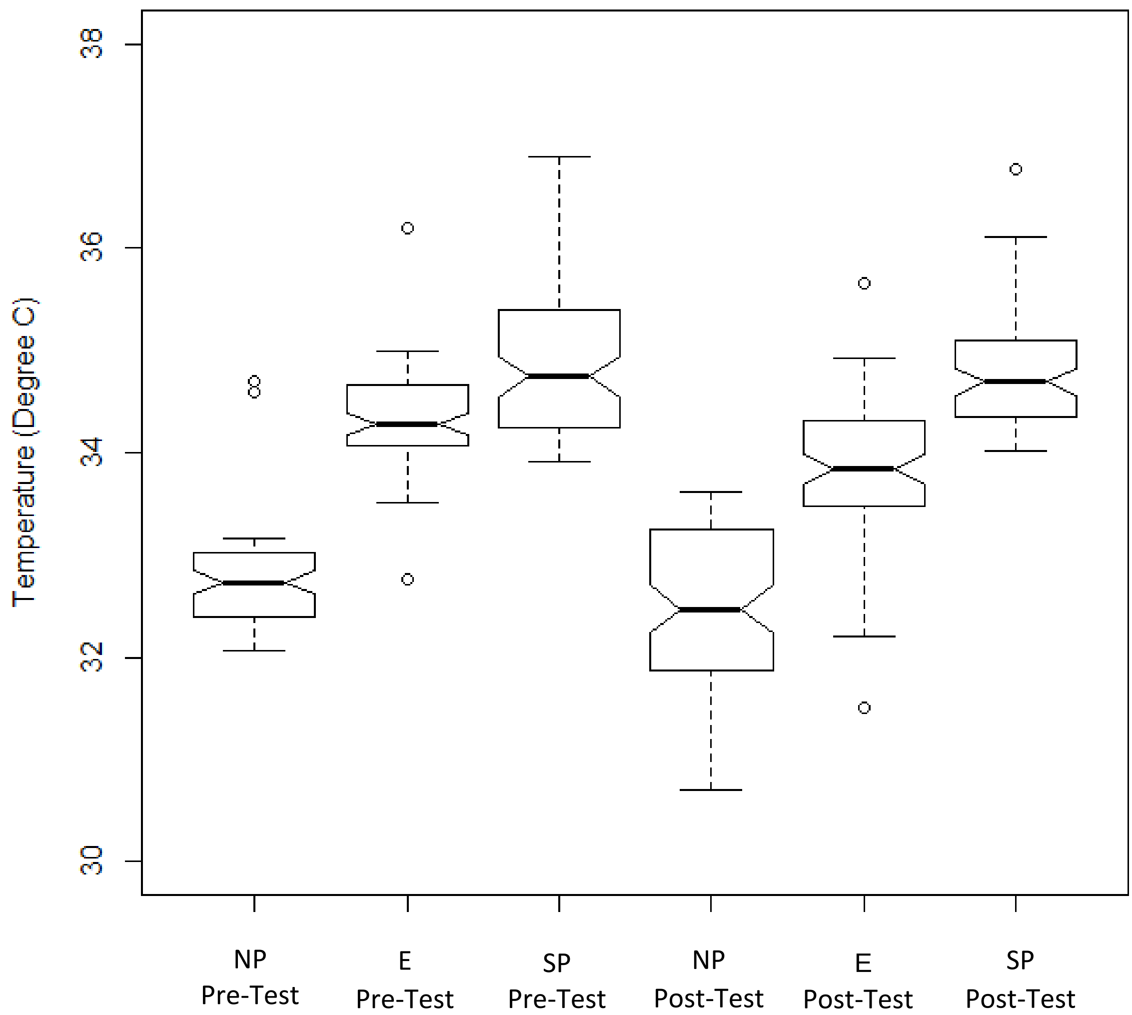


**Figure 2.** A box plot representing the Pre-Test and Post-Test eye temperature taken from 90° to the nasal plane (NP), eye (E) and sagittal plane (SP) in horses (N = 14). Where notches do not overlap this indicates a significant difference.

### *Correlation with heart rate variability*

No Pre-test IRT correlated with subsequent SDNN, while eye temperature taken from 90º to the sagittal plane correlated both from absolute Post-test readings and relative change in IRT

(Table 2). A significant positive correlation between change in eye temperature and HRV can be seen in Figure 3. No IRT measurement correlated with LF/HF measures of HRV (Table 3).

TABLE 2

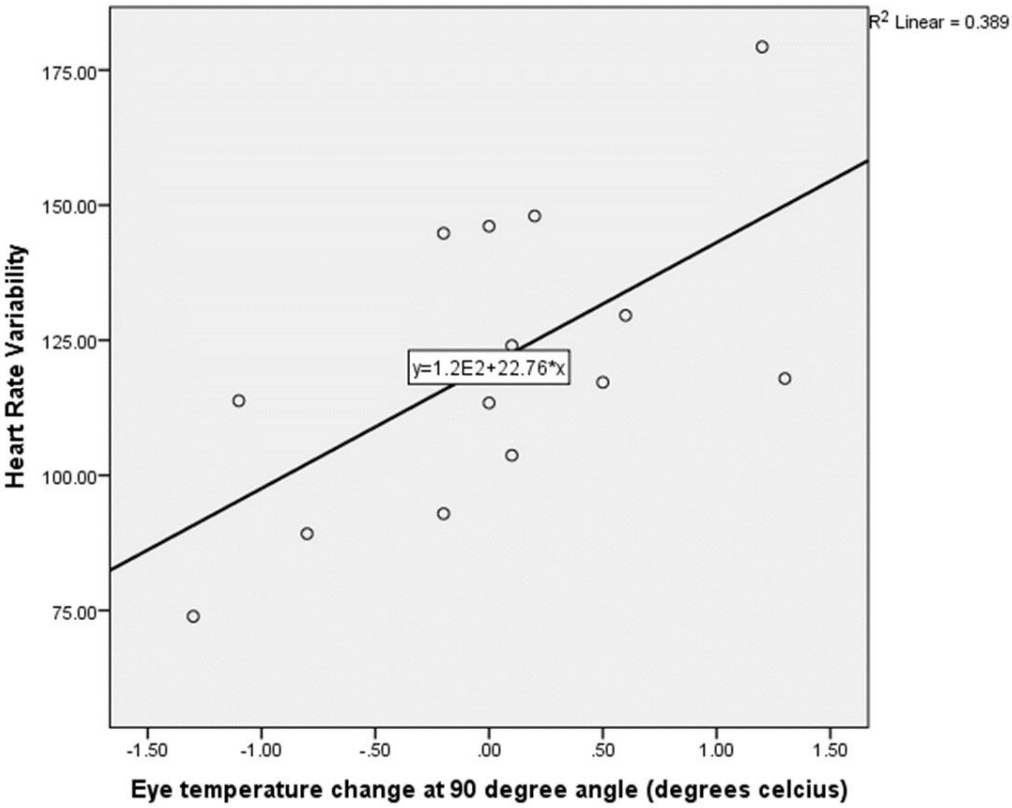


**Figure 3.** A significant strong positive correlation (Pearson: $R^2$=0.389, N = 14, P = 0.017) between the Pre-test to post-test eye temperature change taken from 90° to the sagittal plane and heart rate variability.

TABLE 3

## Discussion

The findings of the current study suggest that the angle from which IRT images of the equine eye are taken affects the temperature readings given. Statistically significant differences were found between all three of the angles tested in this study. Eye temperature was consistently higher as the camera was moved round from 90° to the nasal plane, the eye and then sagittal plane, both at rest and after an arousing experience. This suggests that inconsistency of the IRT camera positioning when taking thermal images of the equine eye may skew the results of research which uses eye temperature as a physiological measure of stress and welfare. There is a degree of ambiguity around the angle which has been used to take thermal images in existing equine IRT research (Valera et al. 2012, Yarnell et al. 2013, Bartolomé et al. 2013, Travain et al. 2015, Soroko et al. 2016). The results of the current study demonstrate that clear reporting of exact angle is important when conducting research using IRT to measure eye temperature.

In the current study, only images taken from 90° to the sagittal plane relate to SDNN. Images from this position correlate strongly with SDNN when measured immediately after, but not before, an arousing experience. This is as expected if IRT is really measuring arousal in response to a challenge. Further, the relationship between SDNN and IRT is strongest when a change in eye temperature taken from 90° to the sagittal plane is used, rather than the absolute temperature. This accounts for individual differences in resting temperature and reduces the effects of fluctuations due to environmental conditions or the subject’s experience at that time. Results indicated no significant correlation between SDNN and the change in eye temperature when images were taken from either 90° to the eye or nasal plane, suggesting that these are not the optimal positions to take IRT images from in the assessment of stress. It may have been expected that 90° to the eye would result in the highest temperature and correlation with HRV variables since that is the angle that much of the research claims to use (Valera et al. 2012, Yarnell et al. 2013, Bartolomé et al. 2013, Sánchez et al. 2016). In addition, human studies take images from directly in front of the subject’s face, which is 90° in relation to the eye (Tan et al. 2009). The difference in eye temperature readings between the three angles could be due to the surface area visible to the camera. Often in studies of free ranging animals or non-domestic species, the ability to capture clear images from a consistent angle may not always be possible. It would therefore be useful as an area of future work, to explore whether distance of the camera from the eye, can correct for temperature changes caused by limited ability to utilize optimum angles of image capture.

As SDNN increased so did IRT temperatures taken from 90° to the sagittal plane, resulting in a greater increase in temperature from resting values. This indicates that less aroused horses – as indicated by SDNN – had increased core temperature. Increased SDNN reflects a shift in balance from sympathetic towards the parasympathetic nervous system response, (Bachmann et al. 2003, von Borell et al. 2007, Schmidt et al. 2010). As such, it might be expected that as SDNN decreases, due to an increased sympathetic stress response to the novel object test, eye temperature would increase in the short term. However, eye temperature changes are not consistently reported across multiple studies (Supplementary Information Table 4), which may relate to the source of stress, insufficient stress to illicit a response or a possible rebound response after a stressor. For example, eye temperature has been recorded to both increase (Dai et al. 2015) and decrease (Ijichi et al. 2018a) in response to novel objects. Dynamic IRT measurements during challenges – rather than shortly after – may clarify this response.

Additionally, IRT readings were compared with the ratio of LF/HF . No correlations were seen between any IRT angles and frequency domain results. This may be because IRT does not correlate well with this measure or because the relatively short period of analysis was not suitable to ensure a 5 minutes sequence without arrhythmia (Stucke et al. 2015). Therefore, the relationship between IRT and HRV is not clear from the results of the current study and require further investigation using longer heart rate recordings. The absence of a correlation between any of the IRT angles and LF/HF weakens the validation of IRT as a measure of arousal. However, the highest temperature readings were consistently captured from 90° to the sagittal plane and this is the only angle which correlates with any measures from HRV. Therefore, this angle is likely to be the most appropriate to use if this technology is to be used. The protocol developed here is easily repeatable with equipment typically found on equine premises. Further, it suggests that measurement angles should be validated and standardized for species-specific protocols. The current angle may apply to other species with anterolateral eye position, such as sheep and cattle, though this should be investigated. In particular, it is worth identifying how distance and angle interact to ensure the hottest temperature is being recorded. It may also be worth exploring whether the surface area and curvature of the eye, which will vary between species, affects temperature readings. Further studies should utilise multiple images taken using a wide field of view lens as this was a limitation of the current paper.

## Animal Welfare Implications and Conclusions

It is important that animal welfare studies which use IRT to measure eye temperature to measure stress are accurate in their reporting of the position of the IRT camera. The findings of this study clearly indicate that taking images of the eye taken from 90° to the sagittal plane of the horse give higher temperature readings than images taken from 90° to the nasal plane or eye. This angle was also the only one to correlate with SDNN. The current study provides a validated, reliable methodology for obtaining equine eye temperature measurements with use of IRT. Further, the change in eye temperature from pre to post-challenge is most likely to correlate with SDNN. Attempts should now be made to validate the optimal angle for IRT image capturing in stress and welfare assessments of other species. Without accurate, standardized and validated methods of using IRT, its impact as a non-invasive animal welfare tool are clearly limited.

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