

AN INVESTIGATION INTO THE USE OF
INFRARED THERMOGRAPHY AS A
TOOL TO ASSESS THE
PHYSIOLOGICAL STRESS RESPONSE
IN THE HORSE

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ABSTRACT

The experiments reported in this thesis investigated the use of temperature measurement using infrared thermography (IRT) as an objective, non-invasive method to identify the physiological stress response in the horse. The primary area of investigation was the eye area within the medial posterior palpebral border of the lower eyelid and the lacrimal caruncle as in existing work in other species. The application of these findings to horse management and welfare was discussed.

Horses were exposed to potentially stressful situations that were acute (<20 seconds), short term (ten minutes) and long term or repetitive (one week) in duration. Temperature was measured using IRT in addition to measurement of salivary cortisol, faecal corticosterone and behavioural assessment, all of which are currently accepted measures of the stress response.

IRT was shown to be an unsuitable method to assess acute stress in the horse due to the species specific behavioural response of flight. Rapid evasive movement of the horse meant that recording temperature using a thermal camera was difficult. A modified experimental design may have made it possible to capture the thermal response to acute stress however restraint of the horse would have been stressful in itself and confounded results.

A significant ($p=0.005$), positive correlation was found between eye temperature and the stress hormone salivary cortisol when horses were exposed to the short term potentially stressful husbandry procedure of clipping. Behavioural assessment of the horses during clipping did not support the physiological findings.

Finally IRT was shown to be an unsuitable method to identify long term or repetitive stress associated with restrictive housing. Faecal corticosterone and

behavioural assessment were found to be more appropriate methods to monitor stress chronic duration.

These experiments provide evidence that IRT is able to identify temperature change associated with short term stress and offers an objective, non-invasive and instant physiological measure of the equine stress response. Use of IRT as a research tool will allow a better understanding of how horses perceive short term husbandry procedures and management techniques and allow alterations to be made if necessary in order to improve equine welfare and maintain well being.

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Contents

Abstract	iii
Acknowledgements	v
Contents	vii
List of Figures	xv
List of Tables	xix
Chapter 1: Introduction and review of the literature	
1.1 Introduction	1
1.2 Natural habitat of the horse and implications of domestication	3
1.2.1 Potential sources of stress in the horse	4
1.2.2 Housing	5
1.2.3 Husbandry procedures	7
1.2.4 The effect of predictability and control on equine response to stress	10
1.3 The behavioural and physiological response to stress	11
1.4 Behavioural response to stress	11
1.4.1 Assessment of activity patterns as a measure of welfare	15
1.4.2 Methods to assess the behavioural response to stress	17
1.4.3 Limitations of measuring behavioural response to stress	20
1.5 Physiological response to stress	22
1.5.1 Activation of the sympathetic nervous system in response to stress	22
1.5.2 Measurement of the sympathetic nervous system response to stress	23
1.5.3 Activation of the hypothalamic-pituitary-adrenocortical axis in response to stress	24
1.5.4 Measuring activation of the hypothalamic-pituitary-adrenocortical axis in response to stress	26
1.5.4.1 Measurement of cortisol levels in blood plasma	26

1.5.4.2	Measurement of cortisol levels in faeces	28
1.5.4.3	Measurement of cortisol levels in saliva	30
1.5.5	Limitations in measuring Activation of the hypothalamic-pituitary-adrenocortical axis in response to stress	32
1.6	The negative impact of the stress response	35
1.7	The importance of monitoring equine welfare	38
1.8	The thermal response to stress	39
1.9	Infrared thermography (IRT)	40
1.9.1	The use of infrared thermography to conduct population surveys	41
1.9.2	The use of infrared thermography to investigate thermal physiology	42
1.9.3	The use of infrared thermography in veterinary diagnostics	43
1.9.4	Potential Limitations of IRT	45
1.10	Infrared thermography as a measure of the response to stress	51
1.11	Aims and Objectives	59
Chapter 2:	Materials and methods	60
2.1	Horses and husbandry	61
2.2	Data collection	63
2.2.1	Saliva sample collection	63
2.2.1.1	Salivary cortisol assay procedure	65
2.2.1.2	Validation of ELISA for use in the horse	68
2.2.1.3	Calculation of values in salivary cortisol	69
2.2.2	Infrared thermography and temperature data collection	70
2.2.2.1	Core body temperature data collection	72
2.2.2.2	Ambient temperature data collection	73
2.2.3	Heart rate data collection	73
2.2.4	Behavioural assessment data collection	75

Chapter 3: Investigation into the potentially limiting factors of salivary cortisol analysis and thermal imaging as indicators of the stress response

3.1	Introduction	76
3.2	Aim and objectives	78
3.3	Methodology	79
3.3.1	Animals and husbandry	79
3.3.2	Infrared thermography data collection	79
3.3.3	Infrared thermography data analysis	80
3.3.4	Salivary cortisol data collection	80
3.3.5	Salivary cortisol data analysis	80
3.3.6	Core temperature data collection	81
3.3.7	Core temperature data analysis	81
3.3.8	Ambient temperature and photoperiod data collection	81
3.3.9	Correlation analysis	82
3.4	Results	83
3.4.1	Infrared thermography	83
3.4.2	Core temperature	85
3.4.3	Salivary cortisol	87
3.4.4	Correlation	89
3.5	Discussion	91
3.6	Conclusion	95

Chapter 4: Investigation into the use of IRT as a tool to assess thermal change associated with the physiological response to acute stress

4.1	Introduction	96
4.2	Aim and objectives	97
4.3	Methodology	98
4.3.1	Animals and husbandry	98
4.3.2	Test area	98
4.3.3	Test procedure	100
4.4	Data collection and analysis	101
4.4.1	Infrared thermography data collection	101
4.4.2	Infrared thermography data analysis	101
4.4.3	Core temperature data collection	102
4.4.4	Ambient temperature data collection	102
4.4.5	Salivary cortisol data collection	102
4.4.6	Salivary cortisol data analysis	102
4.4.7	Heart rate data collection	103
4.4.8	Heart rate data analysis	103
4.4.9	Behavioural assessment data collection	103
4.4.10	Correlation analysis	105
4.5	Results	105
4.5.1	Infrared Thermography	105
4.5.2	Salivary Cortisol	107
4.5.3	Heart Rate	109
4.5.4	Behavioural response	110
4.5.5	Correlations	111
4.5.6	Ambient temperature	112
4.6	Discussion	113
4.7	Conclusion	116

Chapter 5: Investigation into the use of IRT as a tool to assess thermal change associated with the physiological response to short term stress

5.1	Introduction	117
5.1.1	Preliminary study	118
5.2	Aims and objectives	118
5.3	Methodology	119
5.3.1	Animals and husbandry	119
5.3.2	Test area	119
5.3.3	Test procedure	119
5.3.4	Repeat study with no clipping	121
5.4	Data collection and analysis for sham clipping and no clipping treatments	121
5.4.1	Infrared thermography data collection	121
5.4.2	Infrared thermography data analysis	122
5.4.3	Salivary cortisol data collection	122
5.4.4	Salivary cortisol data analysis	122
5.4.5	Heart rate data collection	123
5.4.6	Heart rate data analysis	123
5.4.7	Core temperature data collection	123
5.4.8	Ambient temperature data collection	124
5.4.9	Behavioural data collection	124
5.4.10	Behavioural data analysis	125
5.4.11	Correlation analysis	126
5.5	Data analysis for comparison of sham clipping and no clipping treatments	126
5.5.1	Infrared thermography	127
5.5.2	Salivary cortisol	127
5.5.3	Heart rate	127
5.5.4	Summary of comparisons of data	127

5.6 Results	128
5.6.1 Infrared thermography sham clipping treatment	128
5.6.2 Infrared thermography no clipping treatment	132
5.6.3 Salivary cortisol sham clipping treatment	134
5.6.4 Salivary cortisol no clipping treatment	137
5.6.5 Heart rate sham clipping treatment	138
5.6.6 Heart rate no clipping treatment	139
5.6.7 Behavioural assessment sham clipping treatment	140
5.6.8 Behavioural assessment no clipping treatment	141
5.6.9 Correlations between measures for sham clipping treatment	143
5.6.10 Core and ambient temperature	144
5.7 Comparison of sham clipping treatment data and no clipping treatment data	145
5.7.1 Infrared thermography	145
5.7.2 Salivary cortisol	146
5.7.3 Heart rate	146
5.8 Discussion	148
5.9 Conclusion	152

Chapter 6: Investigation into the use of IRT as a tool to assess thermal change associated with the physiological response to long term or repetitive stress

6.1 Introduction	153
6.2 Aims and objectives	155
6.3 Methodology	156
6.3.1 Animals and husbandry	156
6.3.2 Housing treatments	156
6.3.3 Experimental design	162
6.3.4 Data collection	163

6.3.4.1	Infrared thermography data collection	163
6.3.4.2	Faecal collection	163
6.3.4.3	Core temperature data collection	164
6.3.4.4	Ambient temperature data collection	164
6.3.4.5	Behavioural activity data collection	165
6.3.4.6	Ease of handling data collection	169
6.3.5	Data analysis	170
6.3.5.1	Infrared thermography data analysis	170
6.3.5.2	Faecal corticosterone analysis	171
6.3.5.3	Core temperature data analysis	171
6.3.5.4	Ambient temperature data analysis	171
6.3.5.5	Activity pattern data analysis	172
6.3.5.6	Ease of handling data analysis	172
6.3.5.7	Correlation analysis	172
6.4	Results	173
6.4.1	Infrared thermography	173
6.4.2	Faecal corticosterone	175
6.4.3	Core temperature	176
6.4.4	Ambient temperature	178
6.4.5	Activity pattern assessment	179
6.4.6	Behavioural ease of handling	182
6.4.7	Correlation analysis	184
6.4.7.1	Temperature data	184
6.4.7.2	Faecal corticosterone and behaviour	186
6.5	Discussion	187
6.6	Conclusion	192

Chapter 7: General discussion		193
7.1	Main findings of the project	193
7.2	Temperature change in response to acute stress	194
7.3	Temperature change in response to short term stress	196
7.4	Temperature change in response to longer term stress	199
7.5	Hormonal response to stress	200
7.6	The relationship between temperature change and hormonal response to stress	202
7.7	Behavioural response to stress	203
7.8	Limitations of Infrared thermography	204
7.9	Application of IRT as a tool to assess the stress response in the horse	208
7.10	Future work	210
7.11	Conclusion	211
Appendices		230
Appendix 1	Assessment of salivary cortisol collection methods	231
Appendix 2	Validation for cortisol assays	233
Appendix 3	Standard curves of cortisol assays	236
Appendix 4	Preliminary study 1	243
Appendix 5	Preliminary study 2	249
Appendix 6	Individual physiological data for chapter 4	256
Appendix 7	Preliminary study 3	262
Appendix 8	Individual physiological data for chapter 5	270
Appendix 9	Extraction and assay protocol used by Chester Zoo	271
Appendix 10	Raw data from faecal glucocorticoid analysis	273
Appendix 11	Individual faecal corticosterone data	274
Appendix 12	Ethical approval and risk assessments	277

List of Figures

1.1	Basic time budgets for horses living in different environments from free ranging to restricted living conditions.	16
1.2	Thermal image of forelimbs of horse suffering from osteoarthritis	45
1.3(a)	Thermal image and corresponding temperature scale of zebra in direct sunlight displaying large variation in surface temperature between the black and white stripes	47
1.3(b)	Thermal image and corresponding temperature scale of zebra in shade displaying small variation in surface temperature between the black and white stripes	48
1.4	Temperature change around the human eyes after telling the truth (upper image) and telling a lie (lower image)	51
1.5	Thermal images and related temperature scale demonstrating decrease in nasal temperature of rhesus monkeys when threatened	53
1.6	Infrared image of the eye region showing temperature scale on right hand side and a circle drawn around the area of the eye where maximum temperature was extracted from (Stewart et al.,2008).	56
2.1	Salivette separated into its constituent parts of inner and outer container, lid and cotton swab.	64
2.2	Typical layout of ELISA plate with labelled (A-H, 1-12) microtiterwells with standards and control (high and low) in triplicate, and numbered equine saliva samples in duplicate	67
2.3	Semi log plot of known cortisol standard concentrations used to interpolate unknown samples.	69
2.4	Coloured thermal image illustrating right lateral profile of equine head with lateral and medial commissure labelled.	72
2.5	Horse wearing heart rate monitor. Transmitter and receiver position and position of electrodes in the region of the upper left thorax and the ventral midline are labelled.	74
3.1(a)	Mean (\pm SD) eye temperature for all study horses and ambient temperature over the duration of the study. The black and white bars show hours of daylight (white) and hours of darkness (black).	84
3.1(b)	Eye temperature for one representative horse and ambient temperature over the duration of the study. The black and white bars show hours of daylight (white) and hours of darkness (black).	84

3.2	Core temperature for one representative horse and ambient temperature over the duration of the study. The black and white bars show hours of daylight (white) and hours of darkness (black).	86
3.3(a)	Mean (\pm SD) salivary cortisol for all horses over the duration of the study. The black and white bars show hours of daylight (white) and hours of darkness (black).	88
3.3(b)	Salivary cortisol for one representative horse over the duration of the study. The black and white bars show hours of daylight (white) and hours of darkness (black).	88
3.4	Positive correlation between mean core temperature ($^{\circ}$ C) of all horses and ambient temperature over the duration of the study.	90
4.1	Plan of the test area with position of test horse, thermal and video cameras and point of presentation of aversive stimulus	99
4.2	Change in eye temperature for one horse over four second recording period. Marker (*) shows point of presentation of aversive stimulus. Frame speed was 23 frames per second.	106
4.3	Mean salivary cortisol (ng/ml)(\pm SD) of five available hormonal data sets for each sampling time point. Marker (*) indicates point of presentation of aversive stimulus	108
4.4	Mean heart rate (bpm)(\pm SD) of all horses recorded every twenty seconds from five minutes pre presentation of aversive stimulus to five minutes post presentation of aversive stimulus. Marker (*) indicates point of presentation of aversive stimulus.	109
4.5	Relationship between change in salivary cortisol (ng/ml) from pre presentation of aversive stimulus to maximum level and mean reactivity score for each horse. Pre stressor salivary cortisol level was calculated by taking the mean of the two pre presentation samples	111
4.6	Relationship between mean reactivity score and increase in heart rate from basal level to maximum level (bpm) for each horse	112
5.1	Mean (\pm SD) eye temperature ($^{\circ}$ C) of the compliant and non compliant horses over the duration of the sham clipping treatment. The arrow marker indicates when sham clipping occurred	129
5.2	Mean (\pm SD) ear temperature ($^{\circ}$ C) of the compliant and non compliant horses over the duration of the sham clipping treatment. The arrow marker indicates when sham clipping occurred	130
5.3	Mean (\pm SD) eye temperature ($^{\circ}$ C) and mean ear temperature ($^{\circ}$ C) for all horses (n=10) over the duration of the sham clipping treatment. The arrow marker indicates where sham clipping occurred	130
5.4	Mean (\pm SD) eye temperature ($^{\circ}$ C) and mean ear temperature ($^{\circ}$ C) of all horses over the duration of the no clipping treatment. The arrow marker indicates when sham clipping occurred during the sham clipping treatment	133

5.5	Mean (\pm SD) salivary cortisol of the compliant and non compliant horses over the duration of the sham clipping treatment. The arrow marker indicates when sham clipping occurred	136
5.6	Mean (\pm SD) eye temperature ($^{\circ}$ C) and mean salivary cortisol (ng/ml) of all horses over duration of the sham clipping treatment. Arrow marker indicates when sham clipping occurred	136
5.7	Mean heart rate (\pm SD) (bpm) of the compliant and non compliant horses for the duration of the sham clipping treatment. Arrow marker indicates when sham clipping occurred	139
5.8	Mean (\pm SD) activity score for the compliant and non compliant horses for a five minute segment of pre sham clipping, during sham clipping and post sham clipping footage during the sham clipping treatment.	141
5.9	Mean (\pm SD) activity score for the compliant and non compliant horses for a five minute segment of pre sham clipping, during sham clipping and post sham clipping footage during the no clipping treatment.	142
5.10	Relationship between change in eye temperature ($^{\circ}$ C) from baseline to each subsequent measure for each horse and change in ear temperature ($^{\circ}$ C) from baseline to each subsequent measure for each horse during the sham clipping treatment.	143
5.11	Relationship between change in eye temperature from baseline to maximum value and change in salivary cortisol from baseline to maximum value for each horse during the sham clipping treatment.	144
5.12	Mean (\pm SD) eye temperature ($^{\circ}$ C) of all horses during sham clipping treatment and no clipping treatment. The arrow marker indicates where sham clipping occurred during the sham clipping treatment	145
5.13	Mean (\pm SD) salivary cortisol (ng/ml) of all horses during sham Clipping treatment and no clipping treatment. The arrow marker indicates where sham clipping occurred during the clipping treatment	146
5.14(a)	Mean (\pm SD) heart rate (bpm) for the non compliant horses during the sham clipping and no clipping treatment The arrow marker indicates where sham clipping occurred during the sham clipping treatment	147
5.14(b)	Mean (\pm SD) heart rate (bpm) for the compliant horses during the sham clipping and no clipping treatment The arrow marker indicates where sham clipping occurred during the sham clipping treatment	147
6.1(a)	Paired housed full contact housing design. Image shows the barn housing each pair of horses with position of camera. The smaller image in the bottom left hand corner shows the view produced from the camera.	157
6.1(b)	Single housed semi contact design. Image shows the stables housing each horse with position of camera. The smaller image in the bottom left hand corner shows the view produced from the camera	158

6.1(c)	Single housed no contact design. Image shows the stables housing each horse with position of camera. The smaller image in the bottom left hand corner shows the view produced from the camera.	159
6.1(d)	Group housed full contact. Image shows the paddock housing each experimental group of four horses with position of camera. The smaller image in the bottom left hand corner shows the view produced from the camera.	160
6.2	The camera system at Brackenhurst Equestrian Centre	166
6.3	Mean (\pm SD) faecal corticosterone concentrations for seven horses for three sample days in each housing treatment.	175
6.4	Average time budget of ten study horses in each housing treatment.	181
6.5	Mean (\pm SD) ease of handling score for all horses during the fourth day in each housing treatment	184
6.6	Mean (\pm SD) ease of handling score for all horses in each housing treatment and mean (\pm SD) faecal corticosterone of eight horses for each housing treatment	186

List of Tables

1.1	Mean retention time (MRT) (h) of digesta in horses (1) and ponies (2) fed various diets and measured with different markers.	29
2.1	Description of each preliminary and main study carried out during the project	60
2.2	Details of all horses used throughout the project. An X marks the studies taken part in.	62
3.1	Maximum and minimum eye temperatures (°C) recorded for each horse throughout the duration of the study.	85
3.2	Results of the Wilcoxon signed rank test used to investigate any difference in mean core temperature between sampling time points. P values are given between each sampling time point. P values marked in bold indicate a significant difference between times.	86
3.3	Minimum and maximum core temperatures (°C) recorded for each horse throughout the duration of the study with times that temperature occurred in ().	87
3.4	Minimum and maximum salivary cortisol levels (ng/ml) recorded for each horse throughout the duration of the study with times recorded in ().	89
4.1	Reactivity score with scores ranging from 1 (no reaction) to 5 (large reaction). (Adapted from Anderson <i>et al.</i> , 1999 and Gorecka <i>et al.</i> , 2007).	104
4.2	Descriptive statistics for eye temperature across the ten sampling time points	107
4.3	Salivary cortisol levels (ng/ml) pre stressor, maximum salivary cortisol post stressor (magnitude of increase) and time taken to reach maximum value for the five available hormonal profiles. Pre stressor salivary cortisol was calculated by taking the mean of the two pre stressor samples.	108
4.4	Descriptive statistics for reactivity score assigned by fourteen observers for each horse	110
4.5	Reactivity score and increase in heart rate from basal level to maximum for each horse.	112
5.1	Activity score relating to avoidance or flight behaviour with scores ranging from 1 (low degree of activity) to 5 (high degree of activity). The score system was used for both sham clipping treatment and no clipping treatment.	125

5.2	Descriptive statistics for eye and ear temperature across the eight sampling points during the clipping treatment.	131
5.3	Eye temperature data for individual horses for sham clipping treatment. Data includes horse with behavioural group compliant (C) or non compliant (NC), pre stressor eye temperature, maximum eye temperature (with change in temperature), time to maximum eye temperature post onset of sham clipping and time to return to basal temperature	131
5.4	Ear temperature data for individual horses for sham clipping treatment Data includes horse with behavioural group compliant (C) or non compliant (NC), pre stressor ear temperature, minimum ear temperature (with change in temperature), time to minimum ear temperature post onset of sham clipping and time to return to basal temperatures from minimum	132
5.5	Descriptive statistics for eye and ear temperature across the eight sampling points during the no clipping treatment	134
5.6	Descriptive statistics for salivary cortisol across the six sampling points during the sham clipping treatment.	135
5.7	Cortisol data for individual horses for sham clipping treatment. Data includes horse with behavioural group complaint (C) or non complaint (NC), pre stressor salivary cortisol, maximum salivary cortisol (with change), time to maximum salivary cortisol post onset of sham clipping and lag time between peak in eye temperature(ET) and peak in salivary cortisol (SC).	137
5.8	Descriptive statistics for salivary cortisol across the six sampling points during the no clipping treatment.	138
5.9	Descriptive statistics for mean heart rate of compliant and non-compliant horses during the sham clipping treatment.	139
5.10	Descriptive statistics for mean heart rate across the eight sampling time points of the no clipping treatment.	140
6.1	Four housing designs used in the study with differing levels of social and physical contact.	161
6.2	Order of rotation of the four experimental groups through the four housing treatments	162
6.3	Ethogram of behavioural states measured for analysis of activity patterns in ten study horses with definitions and sub categories to define the behaviors Adapted from Heleski et al, (2002) and Cooper et al. (2000).	168
6.4	Ease of handling score with scores ranging from 1 (compliant to swabbing for saliva) to 5 (resistant to swabbing for saliva). Evasive movement of head is an elevated head carriage or lateral movement away from the handler.	170

6.5	Descriptive statistics for eye temperature during the four housing treatments	173
6.6	Pairwise comparison (Bonferroni) to investigate difference in eye temperature between housing treatments. Significance levels highlighted in bold indicate a significant difference in eye temperature between housing treatments.	174
6.7	Descriptive statistics for faecal corticosterone during the four housing treatments	176
6.8	Descriptive statistics for core temperature during the four housing treatments	177
6.9	Pairwise comparison (Bonferroni) to investigate difference in core temperature between housing treatments. Significance levels highlighted in bold indicate a significant difference in eye temperature between housing treatments	177
6.10	Descriptive statistics for ambient temperature during the four housing treatments	178
6.11	Pairwise comparison (Bonferroni) to investigate difference in ambient temperature between housing treatments.	179
6.12	Data for the effect of housing design on time budget (%) of ten representative horses in the four housing treatments	180
6.13	Descriptive statistics for behavioural ease of handling score during the four housing treatments	183

Chapter 1. Introduction and review of the literature

1.1 Introduction

Domestication has removed horses from their natural environment and eliminated many of the challenges faced in the wild including predation and the acquisition of food. These challenges have been replaced with new challenges associated with management practices and training procedures that some horses may perceive to be stressful. Domestication requires the horse to expend energy for the benefit of another species which conflicts with the evolutionary processes that shaped the behaviour of its predecessors (Diamond, 2002). Confinement in stables restricts movement and limits the opportunity to display natural behaviour and interaction with con-specifics. This can lead to stress related disease and the development of abnormal behaviours that may compromise welfare.

The question of how to assess equine welfare is still under debate, however a combination of behavioural and physiological measures can give an indication of how a horse perceives its environment (Bassett and Buchanan-Smith, 2007). A potential indicator of welfare is the absence of stress. There is no standard definition of stress, however an environmental stimulus that leads to an imbalance of homeostasis is often termed a "stressor" and the corresponding defence reaction of an animal "the stress response" (Mostl and Palme, 2002). The word "stress" has been used in several different contexts and can therefore be interpreted to mean more than one thing, but for the purpose of this project stress will be defined as "the experience of intrinsic or extrinsic demands that exceed an individual's resources for responding to those demands" (Dantzer, 1991).

Various methods are currently used to assess how an animal perceives the situation it is in including evaluation of the stress hormone cortisol and analysis of behavioural response. These currently accepted methods have limitations. Plasma cortisol assessment requires the invasive procedure of blood sampling which can be stressful in itself and potentially confound results. Faecal sampling for cortisol can only be carried out on an opportunistic basis and is not suitable for assessment of short term stress as it reflects an average cortisol level over time rather than the point in time sampling offered by plasma. In addition laboratory analysis for cortisol assessment is time consuming and expensive.

Behavioural assessment can be subjective and as a prey species horses may mask signs of stress as a survival mechanism. This means behavioural response is not a reliable measure of welfare however it is the only method available to everyday horse owners and handlers to assess how their horses perceive the training practices and management procedures imposed upon them. Due to these limitations there is currently no single, instant, reliable and non-invasive measure of the stress response for the horse.

Recent work into assessment of the stress response has investigated changes in thermal output as an indicator that an animal finds a situation stressful. Skin temperature directly reflects the underlying circulation and metabolism (Eddy *et al.*, 2001) therefore rapid changes in blood flow due to sympathetic activation and stimulation of the hypothalamic–pituitary–adrenocortical (HPA) axis associated with the stress response will alter the amount of radiated heat (Stewart *et al.*, 2007). The associated changes in surface temperature can be measured non-invasively using infrared thermography (IRT) and could provide an objective, more immediate indication of the stress response when compared to behavioural assessment and hormone analysis. The response of the horse to management practices and husbandry methods could then be

objectively assessed and adapted if necessary to prevent long term or repetitive activation of the stress response which could manifest as abnormal behaviour or ill health.

1.2 Natural habitat of the horse and implications of domestication

In their natural habitat horses are social animals that live in permanent family bands consisting of an adult stallion, one to three mares and their offspring (Feist and McCullough, 1975). Young or old stallions that have lost their mares will join a bachelor band of up to sixteen horses (McCort, 1984).

Horses are a free ranging species with home ranges that incorporate grazing sites and water resources that can be up to 78km² (Green and Green, 1977). Like many large grazing herbivores, group living is an important survival strategy in that it reduces the likelihood of individual predation in addition to an increased chance of predator detection. This can best be observed in large mixed groups of zebra, wildebeest and other ungulates on the African savannah (Goodwin, 1999). Wild horses also form large social groups and are therefore pre-adapted to form associations with other species and are highly social animals (McCort, 1984).

Domestic and captive species are faced with a wide range of potentially challenging situations that are often related to husbandry procedures or training techniques. Restricted movement is one of the greatest challenges for domestic and captive species (Morgan and Tromborg, 2007) and is thought to be one of the primary contributors to captivity induced stress. The impact of restricted space on 35 different species of carnivore was investigated by Clubb and Mason (2003) who found that infant mortality and stereotypic behaviour (pacing) was found to correlate positively with species home range size in the wild. This suggests that the impact of restrictive housing will be greater for animals that originate from open spaces such as the horse.

Many animals spend a large amount of their daily time budget searching for and consuming food (Herbers, 1981). The restricted space provided by a domestic or captive environment inhibits the opportunity to forage and the amount and type of food that is provided for both domestic and captive animals takes far less time to consume than in the wild. The reduced opportunities for natural foraging behaviour due to restrictive housing may be inherently stressful and can result in the emergence of stereotypic behaviour. This is due to frustration as the animal attempts to search for food in a restricted environment (Mason, 1993). Stereotypic behaviour as a result of restrictive housing has been reported in primates (Mariner and Drickamer, 1994), giraffe and okapi (Bashaw *et al.*, 2001). In stabled horses the provision of an "Equiball" (an enrichment device that requires work to extract food with the aim of extending foraging time) resulted in a reduction of stereotypic behaviour (Henderson and Waran, 2001).

1.2.1 Potential sources of stress in the horse

Everyday challenges in the wild are primarily acute (predator avoidance, social disputes) however the domestic horse is placed in potentially stressful situations for extensive periods of time (restrictive housing, transportation) which may result in prolonged stress. In addition domestic horses are subjected to potentially stressful procedures which are often repetitive in nature and from which there is no escape.

Restrictive housing is potentially detrimental to welfare especially in social and free ranging species. It is therefore important that current housing types are objectively assessed to evaluate their impact on equine welfare. It is also important to objectively assess new housing designs that allow natural behaviour to be displayed. This will allow adaptations to housing design to be made in order to reduce the impact of captivity and improve welfare.

1.2.2 Housing

In its natural environment the horse is a social animal that spends most of its time in close contact with con-specifics (Christensen *et al.*, 2002). Harem bands are typically comprised of mares and their foals, yearlings and one stallion (Rivera *et al.*, 2002) and these cohesive bands can roam vast areas of land. In contrast domestic horses are kept in a variety of housing systems which offer differing levels of physical freedom and contact with con-specifics. The predominant housing system used is individual stabling (Christensen *et al.*, 2002). The dimensions of box stalls are variable but typically they measure 9-13m² (Rivera *et al.*, 2002) and horses are often confined in these stalls for large proportions of the day. This management style is used for several reasons, including injury prevention, lack of pasture and convenience for the owner (Goodwin, 1999). To the human eye the stable appears safe and inviting and is based on an anthropomorphic belief of what the horse finds comfortable (Pedersen *et al.*, 2004; Jørgensen *et al.*, 2009) however, to a prey species such as the horse, isolation, restriction of sensory input and preventing escape if needed could be potentially stressful. In addition restriction of foraging time and reduced opportunity to express natural and social behaviour is thought to be linked to stereotypic behaviour (McGreevy *et al.*, 1995) therefore other forms of housing are being introduced where contact is possible, including group stabling and turnout. To fully understand the impact of housing design it is important to first understand the behaviour of the horse in its natural habitat and then compare that to behaviour in different housing conditions. Where this comparison shows a reduction in the horse's behavioural repertoire or a change in time budget, it is thought to be a sign of reduced welfare (Benhajali *et al.*, 2008).

A study by McGreevy *et al.* (1995) found that stable design allowing visual contact between horses was associated with a reduced risk of abnormal behaviour. Cooper *et al.* (2000) reported that increasing visual and tactile

contact between horses significantly reduced stereotypic weaving and nodding when compared to conventional stables where horses have no contact with one another. When physical or visual contact is not possible due to risk of infection, injury or cost, an alternative such as a stable mirror could be used and appears to have a similar effect to social contact (McAfee *et al.*, 2002; Mills and Davenport, 2002). A study by Waters *et al.* (2002) investigated potential factors that may influence the development of abnormal behaviour in young horses. Horses that were barn or singly housed after weaning were at significantly greater risk of developing abnormal behaviour than horses kept at pasture with con-specifics and the authors suggest housing plays a critical role in the development of stereotypical behaviour in young horses.

Rivera *et al.* (2002) aimed to investigate the effects of the domestic environment on the trainability of young horses and found group housing exerts a positive effect on behaviour. Group housed horses took less time to complete a training procedure than horses singly housed in stalls. In addition, singly housed horses showed more objectionable behaviour toward the trainer (biting and kicking) than group housed horses. It seems that housing horses in a way that reflects their natural habitat and allows social interaction has welfare benefits. This new form of housing where group housed horses are in contact with each other needs to be objectively assessed and compared to traditional box stalls to allow the design with optimal welfare benefits to be selected.

1.2.3 Husbandry procedures

How stressful an animal perceives an event to be may be associated with the characteristics of the event in relation to its evolutionary history. With regards to the horse, physical stressors including darkness, sudden or unfamiliar noise, sudden movement and isolation may evoke an innate fear response mainly due to these situations increasing the chance of predation for the horse. Many of these situations are found in the domestic environment and there are several management procedures that horses behaviourally appear to find stressful. An example of a potentially stressful management procedure is clipping.

Horses coats are clipped for a variety of reasons. By the end of October in the United Kingdom a horse will have grown a thick winter coat. This can cause excessive sweating whilst being exercised and result in the horse losing condition. Removing the hair and providing artificial warmth through the use of rugs enables owners and handlers to keep the horse clean, dry and comfortable as well as maintaining condition. Despite being totally non-invasive and pain free, some horses appear to find the procedure of clipping stressful (Gough, 1999) which could be due to a number of factors. It is thought that stress is mainly due to the noise of the clippers rather than the touch (Gough, 1997). However, often the areas that horses are particularly sensitive to having clipped, including the underbelly and flanks are the primary attack area for predators (Farmer-Dougan and Dougan, 1999).

Due to their size and innate flight behaviour horses that are behaviourally non compliant during clipping can prove dangerous. This has led to various counter measures being applied which may be stressful in themselves including severe bits to allow better control and extreme methods of restraint so that "If the horse realises it cannot escape, it may submit to the clipping" (Gough, 1997). Horses that become stressed whilst being clipped do not appear to become habituated to this procedure despite clipping being carried

out at least twice each winter and often develop reputations for being difficult to manage. Some success has been reported using behavioural conditioning in order to improve behaviour towards clipping. Ponies (n=6) were exposed to clipping on the cranial crest of the neck for five minutes and evasive head movements recorded. Ponies were then exposed to a recording of clipper noise during daily feeding for a period of fourteen days and then clipped for a second time. Results show that after behaviour modification the conditioned ponies were less reactive to the clippers and displayed less evasive head movements when compared to a control group (Gough, 1999). This is further supported by a similar study that observed number of head tosses during five minutes clipping of the neck and then subjected the seven study horses to twenty five days of behavioural modification treatment. Horses were exposed to clipper noise during feeding for ten minutes per day for twenty five days. At the conclusion of the treatment horses were clipped again and the number of evasive head movements was significantly lower than the pre treatment clip (Mackenzie *et al.*, 1987).

Behavioural conditioning may offer some improvement in equine behaviour to clipping however it is not known how long the effects last and as it was not reported how the ponies responded to clipping the following year it may be necessary to repeat conditioning which can be time consuming. Furthermore no physiological measures were taken therefore it may be the horses had simply learnt not to respond and perhaps were still physiologically compromised. In the wild, prey species have been shown to not display their suffering but conceal it as far as possible, in order not to attract predators or lose protection from their social group (Sapolsky *et al.*, 2000; Berger *et al.*, 2003). Seaman *et al.*, (2002) state that some individual animals respond to a stressful situation with no outward signs and appear to be unaffected. This type of behavioural response has been termed 'passive coping' and is the

opposite reaction to actively coping animals that deal with stressful situations by trying to escape or remove the stressful stimulus. Wechsler (1995) states that passively coping animals will stop performing behaviour during a stressful situation and wait for a change. This may result in these animals being perceived to be comfortable in a potentially stressful situation. Despite horses being flight animals Seaman *et al.*, (2002) suggests that they cannot be grouped into either passive or active copers, as horses have been categorised as overtly passive in one type of behavioural test and overtly active in another. This is supported by Wechsler (1995) who suggests that only passively coping animals are consistent in their response to stressful situations with actively coping animals adopting various strategies. These findings suggest that horses could appear unaffected during a potentially stressful situation however, the horses could be experiencing psychological stress and the subsequent physiological response and detrimental effects on health. Lack of any behavioural stress response would result in the physiological stress response going undetected by the human handler. This highlights the need for an objective measure of the physiological stress response in order to better interpret behavioural changes during a stressful situation. Husbandry procedures could then be evaluated objectively and altered if necessary in order to avoid stress related disease and improve welfare.

1.2.4 The effect of predictability and control on equine response to stress

In addition to the stress caused by restrictive housing, domestic and captive species must cope with situations they may perceive to be unpredictable or are unable to control. An unpredictable situation has been shown to contribute to stress and the predictability of an event is known to affect an animal's response to it (Weinberg and Levine, 1980) and variations in predictability have been shown to have pronounced effects on the behavioural and physiological impact of stress (Bassett and Smith, 2007). The consequences of predictability are closely related to control, which is thought to be psychologically and physiologically important to animals (Overmier *et al.*, 1980; Mineka *et al.*, 1986). An event is deemed controllable if there is a difference in the likelihood of it occurring depending on an animal's behaviour (Overmier *et al.*, 1980) therefore the inability to respond appropriately to stressful stimuli with adaptive behaviour may be stressful for the animal and result in compromised welfare (Weinberg and Levine, 1980). A trickle feeder such as the horse that cannot forage due to restrictive housing may become stressed. Even offering a small element of control such as the opportunity to move away from a stressful stimulus but not escape entirely has been found to reduce the physiological stress response (Weinberg and Levine, 1980).

The impact of stressful stimuli or stressors is also partly determined by the ability of the horse to cope with the situation (Irvine and Alexander, 1998). It is a consistent finding across species that if environmental stressors are too demanding and the individual cannot cope, its health is in danger (Koolhaas *et al.*, 1999). Coping can be defined as the behavioural and physiological effort to master the situation (Wechsler, 1995) and successful coping depends highly on the predictability and controllability of the stressor (Ursin and Olf 1993). In the domestic horse many of the potentially stressful training procedures and management practices are neither controllable nor

predictable, increasing the occurrence of stress-related disease and behavioural problems.

1.3 The behavioural and physiological response to stress

It is essential to understand the physiological changes and behavioural reaction of the horse when challenged in order to effectively measure and interpret the response of horses to potential stressors. Management and training techniques can then be assessed and altered accordingly in order to improve welfare. When an animal is confronted with environmental challenge it adapts through a range of physiological and behavioural mechanisms in order to maintain homeostasis. This response can be acute, which prepares the animal for an immediate reaction, or chronic, which involves substantial physiological adjustment and may result in long term alterations in behaviour.

1.4 Behavioural response to stress

The behaviour of an animal is its most potent interaction with the environment and largely determines survival (Goodwin, 1999). The behavioural repertoire of the horse has evolved to respond to the challenges faced by a herd dwelling social species that survives predation through flight. Domestication has removed many of the challenges faced by horses in their natural environment however the psychological need to respond may still exist even though the biological need to perform these behaviours does not (Cooper and Albentosa, 2005).

The horse's innate reaction to sources of perceived danger is one of active avoidance. This immediate behavioural response serves to remove the horse from a potentially dangerous or stressful situation and is supported by physiological mechanisms. From an evolutionary standpoint this response is in place to promote fitness in the wild; the life expectancy of an animal is increased if it can react to avoid sources of danger (Forkman *et al.*, 2007). In

the domestic horse this acute behavioural reaction can prove dangerous both for the horse and human handler. Another behavioural indicator of stress involves throwing the head in the air when startled which may serve to bring the stimulus into clearer view (Mcgreevy, 2004). Horses are primarily visual communicators and they are very sensitive to subtle changes in body posture of con-specifics (Waring, 2002). The alarm posture of the horse serves as a signal to alert con-specifics (Forkman *et al.*, 2007) and elevated head carriage displayed in domestic horses when adopting an alert stance is similar to the vigilant stance taken by wild horses when a predator is detected. Mares that were subjected to isolation in a pasture spent increased time standing immobile and in an alert stance and decreased time spent grazing when compared to time spent with conspecifics. The authors suggest these changes in behaviour could be indicative of increased anxiety or stress (Strand *et al.*, 2002).

In the wild prey animals often do not display their suffering but conceal it as far as possible in order not to attract predators or lose protection from their social group (Berger *et al.*, 2003) and lack of movement has been found to be an important behavioural indicator of stress in prey species (Erhard and Mendl, 1999). Horses that were restrained in their stables by tying with leads for one hour whilst their heads were covered by a hood spent significant periods of time immobile in an alert stance (Minero *et al.*, 2006). This response makes evolutionary sense as restraint and the inhibition of vision could potentially cause stress to a prey animal that relies heavily on sight to recognise and avoid predation. However, in the domestic environment this behavioural response could be interpreted by the human handler to suggest the horse is comfortable with the situation (Seaman *et al.*, 2002).

It has been proposed that whether a stressor has adverse affects or not depends on whether the animal can predict and control the stressor (Keeling and Jensen, 2002; Bassett and Smith, 2007). Control may be achieved by performing a coping behaviour which is why behavioural problems may emerge when horses are placed in inadequate environments (Rietmann *et al.*, 2004). It has been suggested that stereotypic behaviour in the horse may be associated with reduced welfare or stress caused by an inadequate environment (Broom, 1991; Mason, 1991; McBride and Cuddelford, 2001) or indicative of a situation in which a horse lacks a certain degree of control (McAfee *et al.*, 2002). Traditional single housing isolates the horse from conspecifics and its association with limited exercise, inability to forage and reduced social interaction appears to contribute to the development of stereotypic behaviour (Cooper and Mason, 1998; McGreevy *et al.*, 1995; Nicol, 1999).

Abnormal or stereotypic behaviour has been defined as behaviours which are repetitive and invariant with no obvious goal or function (Mason, 1991). One example of stereotypic behaviour in the horse is crib-biting, which involves the horse grasping a fixed object with its incisor teeth, contracting the neck muscles and drawing air into the cranial oesophagus (Lebelt *et al.*, 1998). Because crib-biting is an oral behaviour that involves activity of the teeth and lips as well as distension of the oesophagus, it has been suggested that it may have a regulatory function in meeting unsatisfied foraging needs (Toates, 1981). Weaving is also a common locomotor stereotypy involving an obvious lateral swaying of the head and often the neck and forequarters (McGreevy *et al.*, 1995). It has been suggested that weaving may be a response to the confinement of the stable and the frustrated motivation of horses attempting to reinstate social contact (Nicol, 1999). Weaving can cause uneven muscular development in the neck, weight loss, fatigue and lameness (Fraser and

Broom 1990; Winkskill, 1995; Cooper *et al.*, 2000). Other abnormal behaviours include box walking, wood chewing, pawing and head nodding and aside from potentially causing physical damage to the horse, they can also be destructive to the horse's stable and are considered undesirable (Cooper and McGreevy, 2002). Foals that were singly stabled post weaning were found to be at significantly higher risk of developing stereotypic behaviour than those which were pastured with con-specifics (Waters *et al.*, 2002). Paddock housed weanlings have been reported to display time budgets similar to feral horses and showed strong motivation to be near con-specifics when compared to stalled weanlings, who spent significantly more time engaged in abnormal behaviour (Heleski *et al.*, 2002). In addition to reduced incidence of stereotypic behaviour, horses managed in ways that allow natural behaviour to be expressed have shown improvements in response to training (Rivera *et al.*, 2002) and increased social behaviour (Christensen *et al.*, 2002). The functional significance of stereotypic behaviour is unclear, however it is possible that it acts to protect the animal from the physiological consequences of stress (Mason, 1991) and is a mechanism to help an animal cope with environmental change (Barnett and Hemsworth, 1990) and adapt to stressful conditions (McBride, 1980). Despite lack of clarification, these possibilities have contributed to the general hypothesis that stereotypies may be stress-coping mechanisms (Levine *et al.*, 1978). The emergence of stereotypic behaviour may be indicative of reduced welfare through poor management or a restrictive environment which could have detrimental effects on equine health. Consequently the occurrence of stereotypical behaviour should form one of the behavioural measures used when assessing the response of the horse to its environment. If reported in conjunction with physiological parameters then a more robust evaluation can be achieved.

1.4.1 Assessment of activity patterns as a measure of welfare.

The behaviour of horses in their natural state is often used to assess the welfare of domestic horses (Veasey *et al.*, 1996). The assumption is that a healthy wild horse is likely to have adequate welfare as it has the opportunity to socialise, forage and display natural behaviour and a captive horse that is restricted in its expression of certain behavioural patterns may be a welfare concern. It is worth noting that a wild environment does not always offer optimum welfare and domestication has removed many dangers faced by wild horses including predation, hunger and some diseases. A more practical approach may be to use studies of wild horses to identify those behaviours that are most important. This knowledge can then be used to modify management practices in order to allow natural behaviour to be performed. It could also be used to try and identify possible causes of behavioural abnormalities associated with human management and captivity (Winskill *et al.*, 1995).

A characteristic for the healthy unimpaired animal is repetition of daily routine (Berger *et al.*, 2003) and studies report more or less identical time patterns of behaviour from day to day in stress free horses (Mayes and Duncan, 1986). Allowing domestic horses the opportunity to display natural behaviour and managing horses in a way that reflects their natural habitat has resulted in horses displaying time budgets similar to those of wild horses. Figure 1.1 (a-d) shows the gradual decrease in similarity to natural behaviour of horses as housing design becomes more restrictive and reflects the horses natural habitat less.

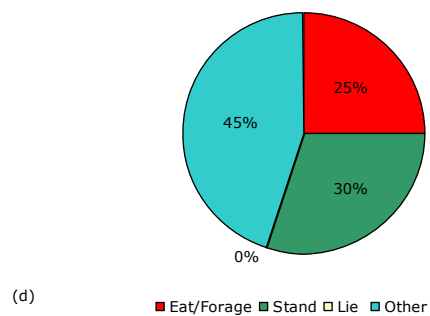
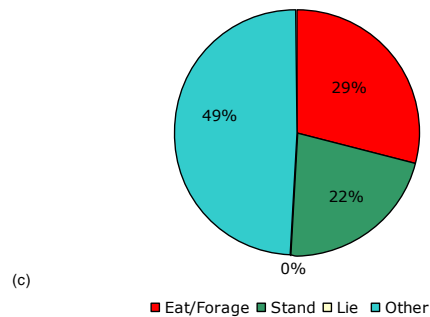
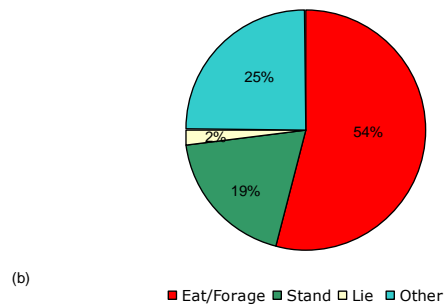
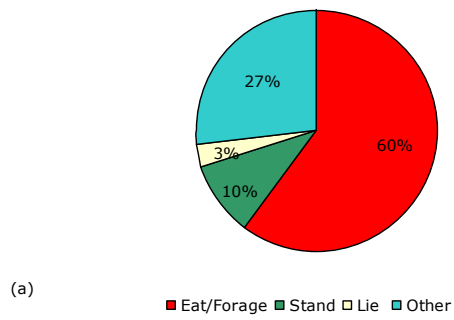


Figure 1.1 Basic time budgets for horses living in different environments from free ranging to restricted living conditions. (a) 9 adult free ranging Carmargue horses with data averaged over one year (Duncan, 1980); (b) 89 adult Przewalskis horses group housed in a large grass pasture and observed for a total of 1319 hours (Boyd, 1988); (c) 2 adult mares housed together in a pen and observed for ten hours (Haupt and Haupt, 1988); (d) 44 densely housed mares observed for six days. Data revealed restricted behavioural repertoire with missing behaviour including allogrooming and lying down (Benhajali et al., 2007).

1.4.2 Methods to assess the behavioural response to stress

Numerous experimental tests have been designed to study animal behaviour in response to potentially challenging situations and although many were originally developed for laboratory species (Hall, 1934; Archer, 1973) they have since been adapted and applied to horses. In order to reduce the frequency of potentially stressful events and to improve safety of horse and human handler, experimental tests have been designed to study equine behavioural response to potentially stressful stimuli (Forkman *et al.*, 2007; Wolff *et al.*, 2007) and preference of management methods (Houpt and Houpt 1988; Krawczel *et al.*, 2006) and can aid in the selection of horses for specific uses (Anderson *et al.*, 1999; Minero *et al.*, 2005). As behavioural testing is used to recommend appropriate management methods then consequently the welfare of the horse often depends on the reliability and validity of such tests.

Arena tests can be used to measure response to a challenging environment. They involve a single animal being placed in an open area and the amount of activity recorded and interpreted as a reflection of the response to novelty (Forkman *et al.*, 2007). This type of test has been used to measure reactivity of rats (Cowan and Barnett, 1975), cows (Kilgour, 1975) chickens (Vallortigara *et al.*, 1990) and horses (Wolff *et al.*, 2007). The arena test was originally designed for laboratory animals and species that display thigmotaxis (fear of open areas) (Forkman *et al.*, 2007) therefore there are limitations in extrapolating patterns and examples of behaviour to the horse. Horses are a free ranging species therefore an open area is unlikely to prove stressful per se and may lead to an inaccurate estimation of response to novelty. A study by Seaman *et al.* (2002) found no correlation between the behaviour of horses in an arena situation with their behaviour in a startle response test. An additional limitation of arena tests for behavioural assessment of social species is that stress due to a novel environment cannot be totally separated

from social isolation. Due to these reasons arena tests are not widely used to test the stress response in horses and more specific tests have been adapted which may be more appropriate.

Novel object tests are often performed after a habituation period to the surroundings of usually one to five minutes (Visser *et al.*, 2001; Seaman *et al.*, 2002; Gorecka *et al.*, 2007). The novel object used is often visual and introduced by a human (Gorecka *et al.*, 2007) or lowered from the ceiling (Momozawa *et al.*, 2003). Horses are usually tested alone and objects include inflated balls (Le Scolan *et al.*, 1997), a brightly coloured sledge (Seaman *et al.*, 2002) and balloons (Anderson *et al.*, 1999; Momozawa *et al.*, 2003). Behaviours recorded include locomotor activity, interest towards the novel object and exploration. Novel object testing is used to assess suitability of the horse for use in the mounted police and therapeutic riding programmes where the horse must have a calm, tolerant temperament and not be highly reactive to novel stimuli (Anderson *et al.*, 1999; Minero *et al.*, 2005). Difficulties may arise in novel object testing due to the behavioural variables measured. Exploration or latency to approach the object is one commonly used behavioural variable however a non-curious or indifferent animal and a fearful animal will both show a long latency to approach the novel object (Forkman *et al.*, 2007). Lack of exploration or willingness to approach a novel object could indicate fear or indifference in the horse and so an accurate assessment of how the horse perceives this form of stressor may be difficult to achieve.

Novelty is a particularly strong stressor when a horse is suddenly confronted with it (Grandin, 1997). Startle tests are adapted novel object tests and are used to measure reactivity of an animal to a potentially stressful situation. They involve sudden presentation of a novel object (Visser *et al.*, 2003;) or unfamiliar sound (Romeyer and Bouissou, 1992). From an evolutionary point of view, suddenness, unfamiliarity and unpredictability are the key features of

predator attack (Shelton and Wade, 1979) therefore this type of test is more appropriate to assess the response of the horse to acute stress than the tests previously discussed. The use of visual stimuli is particularly relevant to the horse as in its natural habitat it is particularly sensitive to subtle changes in stimulus motion and relies on vision as a major sensory avenue for predator detection (Christensen *et al.*, 2008).

In order to quantify the reaction of an individual horse and allow comparison with other horses, studies have assigned a predetermined score of reactivity according to the intensity of the response (Andersen *et al.*, 1999; Gorecka *et al.*, 2007). Behaviours measured as indicators of stress include vigilance (Le Scolan *et al.*, 1997; Wolff *et al.*, 1997; Seaman *et al.*, 2002), elevated head carriage (Anderson *et al.*, 1999; Visser *et al.*, 2003), pinned back ears (Kaiser *et al.*, 2006), avoidance movement or flight attempts and snorting (Anderson *et al.*, 1999; Minero *et al.*, 2006; Christensen *et al.*, 2008). Ethograms are used to record specific pre-determined behaviours and for the purpose of acute or short term observations usually involve the number of occasions the behaviour is observed or the duration for which the behaviour is performed (Martin and Bateson, 2007). A study by Reitmann *et al.*, (2004) used an ethogram to record the frequency of behaviours indicative of stress during a challenging ground training task. Behaviours observed included elevated head position, explosive or evasive behaviour, tail swishing and defaecation.

1.4.3 Limitations of measuring behavioural response to stress

Many factors can influence the equine behavioural response to a potentially stressful situation including temperament, past experience (Visser *et al.*, 2003; Minero *et al.*, 2006) and nature, predictability and severity of the stressor (Koolhaas *et al.*, 1999). What one horse perceives as stressful another may not, therefore a definitive decision on how the species as a whole perceives a specific situation or stressor cannot be made. Even stereotypic behaviour that is often used as an indicator of poor welfare does not have a direct relationship with specific stressors and the presence of stereotypies should not be used as the sole indicator of poor welfare (Mason *et al.*, 2007).

Habituation to novelty or learning not to respond to a repeated stimulus has clear evolutionary advantages. If the horse learns not to respond to every inconsequential stimulus then vital energy can be conserved. Through training the horse can memorize objects and situations that initially evoked fear, stress or a flight reaction and learn to tolerate these situations with a decrease in escape response (Gorecka *et al.*, 2007). Therefore, repeated testing using the same stimulus during a novel object or startle response test could result in a decrease in escape reaction and an inaccurate assessment of how the horse perceives the stressor. Furthermore just because a horse has learned not to respond behaviourally through training does not mean it is comfortable with the situation it is placed in. Previous exposure throughout the horses' life to potentially fearful situations and the training given to cope with such situations can offer some form of habituation or desensitisation to startle tests (Visser *et al.*, 2003, Minero *et al.*, 2006).

The experimental design of behavioural tests may also impact upon the horses reaction. It was reported by Grandin (1997) that cows and pigs approached and manipulated a piece of paper dropped on the ground but refused to approach and displayed flight behaviour when led towards it by a handler. Therefore, the paper is perceived as threatening in one situation and non-threatening in another and may confound results of such a test.

Behavioural observation is often the only means available to horse handlers to assess how horses perceive the situation they are in however behavioural assessment can be subjective and as a prey species, passive coping (showing no outward signs of stress) or masking stress can occur as a survival mechanism (Seaman *et al.*, 2002, Berger *et al.*, 2003). A horse that appears behaviourally unaffected possibly through training may still be physiologically and psychologically compromised therefore a combined use of behavioural and physiological parameters are often used for a robust and more objective evaluation of animal welfare (Anderson *et al.*, 1999; Strand *et al.*, 2002; Momozawa *et al.*, 2003; Pritchett *et al.*, 2003; Minero *et al.*, 2006).

1.5 Physiological response to stress

The physiological response to stress supports the behavioural reaction by preparing the horse for the increased physical activity involved during the flight response. The physiological response involves the immediate activation of the sympathetic nervous system (SNS) followed by stimulation of the hypothalamic–pituitary-adrenal (HPA) axis.

1.5.1 Activation of the sympathetic nervous system (SNS) in response to stress.

Within seconds of perceiving a stressor, the sympathetic nervous system (SNS) is activated. This immediate reaction is termed the “fight or flight” response because the physiological changes in cardiovascular tone and blood flow could support either behavioural reaction (Cannon, 1929). The SNS prepares the horse for the muscular action involved in the defence against external challenges by quickly mobilising energy reserves in the body. The eyes dilate, the rate and force of heart contractility increases, blood vessels constrict and blood pressure increases (Porges, 1995). Blood is diverted from areas not necessary for the flight response including the gastrointestinal tract, reproductive, and immune system and taken to the sensory organs, skeletal muscle, lungs, heart and brain. Cognitive vigilance is also increased (Sapolsky *et al.*, 2000). The cardiovascular system is directly involved in the coping mechanism of the horse. It is stimulated and facilitated by production of adrenaline, resulting in increased heart rate. This short term physiological response supports the flight reaction due to an increase in heart rate subsequently increasing circulation to allow rapid delivery of available energy to the working muscle. As a result changes in heart rate have been used as a physiological measure of reactivity during potentially stressful situations. Studies have reported changes in circulation and heart rate in the horse in

response to transportation (Stewart *et al.*, 2003), exposure to novelty (Visser *et al.*, 2002) and restraint (Momozawa *et al.*, 2003).

1.5.2 Measurement of the SNS response to stress

Transportation has been reported to cause stress and subsequent activation of the SNS in horses (Waran and Cuddeford, 1995; Stewart *et al.*, 2003,). A study that investigated a particular approach to training based on the Tellington touch equine awareness method (Tellington Jones and Bruns, 1988) aimed to determine whether training horses according to this method would decrease stress during loading in horses with a history of reluctance to load. The study used ten horses that were non-compliant with the loading process and seven horses that were compliant with the loading process as a control. Elevated heart rate in addition to an increase in the stress hormone cortisol was reported in horses that displayed behavioural stress during loading and transportation with cortisol levels elevated from seven minutes post loading. Both heart rate and cortisol level decreased when horses were subjected to a training programme and the loading protocol repeated (Shanahan, 2003). Horses that were behaviourally compliant with the procedure displayed no increases in the parameters measured.

Stress caused by handling in previously unhandled Konik ponies resulted in an increase in heart rate. In comparison the heart rates of intensively handled Konik ponies were found to be lower than the heart rates of their non handled counter parts. The treatment involved the ponies being caught and led away from their paddock, having their feet picked up and being approached by an unfamiliar handler. Intensively handled ponies also scored better on a behavioural manageability score which may be due to habituation to the treatments and positive contact with people resulting in a reduced fear response towards humans (Jeziarski *et al.*, 1999).

It is difficult to identify whether changes in heart rate are due to metabolic activity, or excitement. Heart rate also increases in response to movement and therefore has limited use in monitoring stress in ridden horses or husbandry practices that require movement by the horse. Due to these limitations the response of the hypothalamic–pituitary-adrenal axis is also used to assess reactivity to potentially stressful situations.

1.5.3 Activation of the hypothalamic–pituitary-adrenocortical (HPA) axis in response to stress

During a stressful event physiological homeostasis is disrupted. The hypothalamus in the brain releases corticotrophin releasing hormone (CRH) which travels to the anterior pituitary gland and stimulates release of adrenocorticotrophic hormone (ACTH). ACTH enters the bloodstream and stimulates the adrenal cortex (outer portion of adrenal gland situated on dorsal aspect of the kidney) to secrete glucocorticoids. The glucocorticoid produced is species specific and in equines it is primarily cortisol (Queyras and Carosi, 2004). The endocrine response takes several minutes to be fully functional (Nelson, 2005) and cortisol is responsible for several adaptive effects necessary for the flight response. Energy is rapidly mobilised from storage sites in the body in the form of fatty acids and glucose. The activated SNS and subsequent increased circulation then serves to rapidly deliver this extra energy to the working muscle. Oxygen intake is increased, sensory function is enhanced (Sapolsky *et al.*, 2000; Morgan and Tromborg, 2007) and blood flow is decreased to areas not necessary for movement (Nelson, 2005). As well as acting as a coping mechanism the stress induced rise in cortisol may also help to prepare the animal for the next stressor (Sapolsky *et al.*, 2000).

In the wild the median chase time for a zebra by a hyeana is 46 seconds with similar times for a lion chase (Kruuk, 1972). Cortisol response would not be

activated within this time frame therefore it has been suggested that it may play a role in recovering from acute stress and preparing for the next challenge and that the SNS is the primary system involved in the response to acute stress. This means that stressors must be predictable to some extent as is often the case with a social prey species. For example, dominance related aggression is often the predictable culmination of hours or days of escalating acute threats (Sapolsky *et al.*, 2000) therefore the stress hormone would be active by the time the actual conflict occurred. There are also a number of circumstances in which an individual is predictably at greater risk of a predation attempt including during parturition (Estes, 1967), individuals at the perimeter of the social group (FitzGibbons, 1993) and sick or injured animals (Kruuk, 1972). These situations and cues could result in stimulation of the HPA axis to release stress hormones in anticipation of a stressful event.

Elevated cortisol levels have been reported in response to acute and chronic stress in a variety of wild and captive species. Social isolation and confrontation have been found to alter cortisol profiles in primates (Sapolsky *et al.*, 1997) and captivity related stressors such as inadequate housing and restraint have been shown to increase species specific glucocorticoid levels in carnivores (Smith *et al.*, 1990; Carlstead *et al.*, 1993; Hennessey *et al.*, 1997). Transportation has also resulted in elevated cortisol levels in cattle (Palme *et al.*, 2000) and sheep (Lowe *et al.*, 2005). Many hormones are linked to the stress response, however cortisol is routinely used in clinical evaluations and is medically termed the stress hormone (Lane, 2006). In humans cortisol has been reported to be elevated in patients suffering from depression (Bakke *et al.*, 2004), post traumatic stress disorder (Sher, 2004) and in suicidal individuals (Westrin *et al.*, 1999). These findings suggest that animals with glucocorticoids elevated above the normal range could have compromised welfare.

1.5.4 Measuring activation of the HPA axis in response to stress

Unlike adrenaline which is another hormone associated with the stress response, cortisol has been shown to not increase in situations that could be deemed exciting or arousing rather than stressful, including light to moderate exercise (Few, 1974; Alexander and Irvine, 1991) sexual excitement (Exton *et al.*, 1999) and grooming (Hennessey *et al.*, 1998). This could be due to the action cortisol has on the body. The physiological changes that take place in response to a challenging situation are incredibly energy demanding which results in a cost the body will not pay unless absolutely necessary (Lane, 2006). Cortisol can be measured in the blood plasma, faeces and saliva of animals (Hughes and Creighton, 2007) and each method has associated advantages and limitations.

1.5.4.1 Measurement of cortisol levels in blood plasma.

In blood approximately 75% of cortisol is transported via binding proteins, principally albumin and corticosteroid binding globulin (Roshier, 1991) leaving the remaining unbound cortisol to pass through capillaries and exert a biological effect (Stabenfeldt, 1992; Beerda *et al.*, 1996). The time taken for an increase in cortisol secretion to be reflected in the plasma of horses is approximately five minutes (Ralston *et al.*, 1988). Maximum levels post stressor have been reported within thirty minutes although time to maximum levels and time to return to basal level varies significantly (Marlin and Nankervis, 2002). Elevated plasma cortisol has been reported as a result of weaning stress in foals (McCall *et al.*, 1987), transportation (Smith *et al.*, 1996) and post operative pain in horses (Pritchett *et al.*, 2003).

Since it has been proposed that stereotypic behaviour arises in response to stress (Mason, 1993), the relationship between plasma cortisol level and stereotypic behaviour has been investigated in several species, including horses. Pell and McGreevy (1999) found mean baseline and response levels of

plasma cortisol were significantly higher in crib biting horses than in non crib biting horses and stereotypic horses that were restricted from both ad libitum hay and crib biting for a twenty four hour period showed an increase in plasma cortisol levels, while no rise in plasma cortisol was detected when crib biting alone was prevented. This supports the theory that stereotypic behaviour may have a role as a coping mechanism for stress (Broom, 1991; Mason, 1991) and crib biting may serve as a function in satisfying innate foraging needs when these are prevented by stabling.

Blood sampling for plasma cortisol analysis requires handling and restraint and may be perceived as stressful in itself, which may confound results (Hopster *et al.*, 1999; Cook *et al.*, 2001; Mormede *et al.*, 2007). Furthermore the physical act of venipuncture elevates glucocorticoid levels so samples must be extracted rapidly (Broom and Johnson, 1993). This has implications for studies that require repetitive sampling.

Cortisol in the blood circulation is predominantly bound to proteins leaving approximately 25% of cortisol in the free state and biologically active (Roshier, 1991). In the bound state the hormones are unable to fit into receptors in the body and therefore will not be delivered to tissues. They are considered inactive, or non-bio available. This means that not all cortisol detected in blood plasma will have a biological effect on the body. In contrast cortisol measured in saliva is in the bio-available state (unbound) and is able to exert a biological effect. This is because the bound hormones in the blood are too large to pass through the cell membrane of the salivary glands. Only the unbound biologically active part of the hormone pass through into the saliva therefore, the cortisol measured in the saliva will be delivered to the receptors in the tissues of the body (Cook *et al.*, 1997; Irvine and Alexander, 1998).

1.5.4.2 Measurement of cortisol levels in faeces

As faecal collection can be carried out with no disturbance to the animal, analysis of faeces for corticosterone, (another glucocorticoid produced in the adrenal cortex) has been used extensively to monitor HPA activity in a number of free ranging species including African wild dogs (Monfort *et al.*, 1998), spotted hyaena (Goymann *et al.*, 1999) and leopards (Wielebnowski *et al.*, 2002).

In horses the lag time to peak concentration of glucocorticoids and their associated metabolites in faeces post stressor can vary. This is a reflection of the intestinal passage duration for the species, which can be influenced by the body weight and condition of the horse (Miraglia *et al.*, 1992), type and quality of feed (Van Weyenberg *et al.*, 2006) and exercise (Duren, 1990). Passage rate through the equine gastrointestinal tract is best described by mean retention time (MRT). MRT has been investigated using a variety of markers including coloured beads (Volter *et al.*, 1974) and the acid marker co-EDTA (Pearson *et al.*, 2001). A review by Van Weyenberg *et al.* (2006) details MRT of digesta in horses fed various diets and measured with different markers (Table 1.1) which shows that MRT can vary from 18 hours up to fifty hours.

Elevated glucocorticoids in the faeces of horses have been reported in response to potentially stressful situations including artificial insemination (Berghold *et al.*, 2007) and during post operative veterinary treatment (Merl *et al.*, 2000). Faecal sampling reflects an average glucocorticoid level over time rather than the point in time sampling offered by plasma and therefore is more suitable to assess management practices that cover a longer duration and could cause chronic or repetitive stress (Queyras and Carosi 2004; Hughes and Creighton, 2007). As the level of glucocorticoid metabolites in

faeces is the result of accumulation over a prolonged period, they may not reflect fluctuations in cortisol and corticosterone and as the ratio of faecal to plasma cortisol levels may vary by individual faecal sample, a single faecal sample will not truly represent HPA activity during a particular situation (Queyras and Carosi 2004).

Faecal collection for hormone analysis is non-invasive however collection of faeces is on an opportunistic basis therefore samples cannot be collected at pre-determined times. It has been suggested that diet may affect metabolite variability (von der Ohe and Servheen, 2002) and while this must be considered when interpreting data it is likely that as faecal glucocorticoid analysis is usually part of long term studies, a difference in lag time is unlikely to impact on the accuracy of the final data.

Table 1.1 Mean retention time (MRT) (h) of digesta in horses (1) and ponies (2) fed various diets and measured with different markers. Taken from Van Weyenberg et al. (2006).

Reference	Diet	Marker	MRT (h)
Hintz and Loy (1996)	Barley	Styrofoam particles	25
Wolter <i>et al.</i> (1974)	Meadow Hay	Coloured beads	36
Wolter <i>et al.</i> (1974)	Chopped meadow hay	Coloured beads	25
Uden <i>et al.</i> (1982)	Timothy hay	Co-EDTA	18
Pearson and Merrit (1991)	Meadow hay	Co-EDTA	31.3
Cuddeford <i>et al.</i> (1995)	Alfalfa	Co-EDTA	53.5

1.5.4.3 Measurement of cortisol levels in saliva

Cortisol is transported in the plasma of animals primarily in association with binding proteins however the fraction that remains unbound is able to cross into saliva (Beerda *et al.*, 1996). Saliva is produced by three pairs of salivary glands (parotid, submandibular and sublingual) with a small contribution from the buccal glands which line the mouth (Bayazit, 2009) Hormones enter saliva by a variety of mechanisms but for unconjugated steroids, including cortisol the route is rapid diffusion through the cells of the salivary glands and as such their concentration is independent of the rate of saliva flow (Vinning *et al.*, 1983; Eckersall, 1984; Bayazit, 2009). Salivary cortisol has been found to reflect plasma cortisol levels in the horse (Lebelt *et al.*, 1996; Van der Kolk *et al.*, 2001) and one of the main advantages of measuring cortisol in saliva is that it is non-invasive and unlikely to cause stress, particularly in domestic horses that are habituated to having their mouth handled on a daily basis for fitting of riding and training equipment, grooming and dental treatment. This method is far less stressful than blood collection and therefore less likely to confound results especially if subsequent samples are required. Studies report close correlations between free cortisol in plasma and salivary cortisol levels (Vincent and Mitchell 1992; Pell and McGreevy 1999; Lebelt *et al.*, 1996; Van der Kolk *et al.*, 2001; Le Roux *et al.*, 2002) and research suggests the time taken for salivary cortisol levels to increase post stressor is similar to that in plasma (Hughes and Creighton, 2007). Saliva sampling can also be carried out frequently and repetitively.

An increase in salivary cortisol was reported in horses in response to the routine management technique of clipping (Hughes *et al.*, 2006), with a positive correlation between behavioural stress score and difference in cortisol levels. The horses attributed with a high behavioural stress score showed a trend toward a statistically significant increase in salivary cortisol post stressor. A significant increase in salivary cortisol that correlated with increased heart rate was reported in horses that showed behavioural stress during loading and transportation (Shanahan, 2003) with cortisol levels elevated from seven minutes post loading. Significant increases in salivary cortisol have also been reported in response to abrupt weaning in foals with levels rising from 0.86ng/ml pre weaning to 70.79ng/ml post weaning (Moon *et al.*, 2004). Stewart *et al.* (2007) reported an increase in salivary cortisol levels due to isolation in cows from pre isolation levels of 4.7ng/ml to peak levels of 14.1ng/ml twenty minutes post isolation.

Salivary cortisol represents the biologically active part of the hormone. Due to its size the bound fraction is unable to cross the blood-saliva barrier (Lane, 2006). Antibodies have been produced that will only recognise and measure this free form of cortisol and have been used effectively for plasma measurement (Lewis *et al.*, 2003). These antibodies have been utilised to confirm that salivary cortisol levels are a direct reflection of the free cortisol present in plasma (Le Roux *et al.*, 2002). The non-invasive nature of sampling saliva for cortisol analysis and the ability to sample repetitively and frequently offers clear benefits when compared to other methods of cortisol analysis. The lag time that exists between presentation of a stressor and appearance of cortisol in the saliva needs to be taken into account however if only one source of stress is being investigated this can be overcome by repeated sampling (Lane, 2006).

Hormones whose secretion is regulated through the hypothalamus and pituitary gland in the brain including cortisol regulate their own secretion through negative feedback. The purpose of negative feedback is that it results in hormonal homeostasis, which is the maintenance of hormone levels within a particular appropriate physiological range. During a stressful situation cortisol levels will increase. However, as the feedback regulatory system works to reduce levels of cortisol, high cortisol levels will tend to decrease and usually last no longer than ninety minutes (Van de Kolk *et al.*, 2001; Manser, 1992) therefore plasma and salivary cortisol are most suited to assessing acute or short term stressors.

1.5.5 Limitations in measuring activation of the HPA axis in response to stress

Measuring HPA axis activity is the standard approach to the study of stress and welfare in domestic animals (Mormede *et al.*, 2007). However, care must be taken when interpreting the results of assays as a number of factors are known to influence cortisol production. Like most hormones circulating in the body, glucocorticoids are produced in a circadian manner under basal conditions (Lane, 2006). Diurnal rhythm of total plasma cortisol in horses was reported by Irvine and Alexander, (1994) with peak secretions occurring in the early morning. This circadian rhythm was fragile and could be obliterated by placing the horses in a different environment. A study by Van der Kolk *et al.* (2001) that reported diurnal rhythm of plasma cortisol did not find there to be a demonstrable diurnal rhythm of salivary cortisol. Diurnal rhythm in salivary cortisol was reported in a study by Hughes *et al.* (2006) with levels that mirrored plasma cortisol. Saliva was only sampled between 0900h and 1600h therefore the pattern of salivary cortisol over a full twenty four hour period is unknown and a more thorough assessment is needed. The diurnal rhythm of cortisol is thought to be linked to preparing the body for daily activity with a peak in the morning and subsequent decrease in the evening in

diurnal species (Moore-Ede and Sulzman, 1977; Lane, 2006). In human subjects experiments have shown that the sleep-wake cycle is a major cue for entraining circadian rhythm (Weitzman *et al.*, 1983). This is less likely to be the case in horses since sleep occupies only 12% of the 24 hour cycle (Ruckebusch, 1972). Although short term stress does not seem to impact upon the overall circadian pattern (Becker *et al.*, 1985), chronically stressful conditions have been shown to disrupt daily rhythm and the absence of a normal cortisol circadian rhythm is a reliable indicator of chronic stress and poor welfare in horses (Most and Palme, 2002).

Exercise has also been cited as a variable that may alter cortisol production (Momozawa *et al.*, 2003). As one of the main functions of glucocorticoids is the breakdown of proteins to synthesise glucose for energy, it seems logical that energy expenditure through exercise could result in elevated cortisol levels. Studies that have involved and documented exercise have only observed significant increases in cortisol at extreme levels of exercise whereas mild and moderate exercise has been reported to have no effect on plasma cortisol (McCarthy *et al.*, 1992) or salivary cortisol (Jacks *et al.*, 2002). This may be due to energy expenditure being met by fat mobilisation and carbohydrate stores, resulting in no measurable increase in glucocorticoids. However, if stores are depleted through intense activity then catabolism is required and glucocorticoids will increase (Maestu *et al.*, 2003; Ratamess *et al.*, 2005). This is supported by the fact that high intensity exercise of short duration and low intensity exercise of long duration do not cause an increase in glucocorticoids (Monnazzi *et al.*, 2002). Furthermore, extreme exercise could be seen as a stressor in itself therefore glucocorticoids could still act as stress markers.

Although it is clear that hormone analysis has a place in assessing equine welfare many of the techniques are opportunistic and/or invasive and the

required laboratory analysis is time consuming and expensive. Because salivary cortisol collection is less stressful than venipuncture and reflects plasma cortisol concentration, it has been suggested that measurement of cortisol in saliva is a suitable alternative to measurement of cortisol in plasma to assess the stress response (Beerda *et al.*, 1996; Lebelt *et al.*, 1996). Furthermore salivary cortisol collection allows frequent and repetitive sampling in a non-invasive manner. There are however practical issues associated with all biological sampling techniques including storage and refrigeration requirements and expensive and time consuming laboratory analysis meaning results are not instantaneous. Due to these limitations and the importance of monitoring welfare, there is still a demand for alternative non-invasive and instant ways to measure the physiological stress response which cannot be masked as behaviour can.

1.6 The negative impact of the stress response

When a stressor is identified the sympathetic nervous system and hypothalamic-pituitary-adrenal axis are activated to support the “fight or flight” response (Selye, 1950). While this immediate stress response can be considered adaptive, enabling horses to escape from danger, evidence suggests that stress related disease emerges predominantly due to a system that has evolved to respond to acute emergencies being activated repetitively or chronically (Sapolsky *et al.*, 2000). If the stressor persists then the horse will attempt to cope and adapt to the situation. It does this through behavioural and physiological changes including the release of glucocorticoids. If challenged with long term stress the physiological responses that allow an animal to adapt are activated for extended periods of time. Resources are therefore continuously diverted from ongoing biological activities and this can have long term adverse effects on health (Keeling and Jensen 2002). Long term activation of the stress response and subsequent elevation in glucocorticoids is unlikely to be adaptive in a free living individual as it may result in suppression of growth, metabolic exhaustion and increased susceptibility to illness (Wingfield and Ramenofsky, 1999). Occasional exposure to environmental stressors has to be accepted as part of domestication and everyday life; however relentless exposure to persistent stressors can have deleterious consequences (Morgan and Tromborg, 2007). The physiological stress response is designed to suppress processes not necessary for immediate survival therefore persistent inhibition of these systems can have detrimental effects on digestion of food (Goncalves *et al.*, 2002) immunity (Alexander and Irvine, 1998, Minero *et al.*, 2005) and reproductive status (Berghold *et al.*, 2007) all of which are detrimental to welfare. In order to reduce potential stressors and minimise their negative impact upon health we have to know what is perceived as stressful by the horse.

Cortisol is termed the stress hormone as it plays a critical role in the stress response. While excess cortisol in the system for a few hours or even days is harmless, it can be fatal if prolonged (Sapolsky *et al.*, 2000). An animal is said to be stressed when concentrations of cortisol are increased by 40% or more from basal level (Barnett and Hemsworth, 1990).

The general effect of cortisol on immunity is to inhibit synthesis of cells that mediate and promote immune and inflammatory reactions (Almawi *et al.*, 1996). In the context of survival for animals in the wild an obviously injured animal becomes a target for predators or a dominance challenge therefore masking injury through absence of inflammation is beneficial. However, repetitive or chronic suppression of the immune system which could potentially occur in the domestic environment can leave an animal with increased susceptibility to illness (McEwan *et al.*, 1997).

Corticotrophin releasing hormone (CRH) promotes cortisol release during the stress response, however CRH inhibits reproductive physiology and behaviour (Rivier *et al.*, 1986). Cortisol itself also disrupts reproduction by decreasing gonadotrophin releasing hormone and reducing gonadal responsiveness to luteinising hormone (Dubey and Plant, 1985), both hormones are associated with reproductive function. These effects have been documented when cortisol exceeds basal levels (Sapolsky 1985). Reproduction is a costly state particularly in females, therefore the anti-reproductive effects of the stress response can be rationalised as it defers an expensive physiological process during a stressor until a less challenging time.

Cortisol elevates blood glucose in part from existing stores but also through inhibition of further storage in order to divert available energy to exercising muscle. Depletion of fat occurs over the entire dose range of cortisol (Dallman *et al.*, 1993) however, cortisol has been shown to have muscle wasting effects

when present at concentrations found during stress, (Tomas *et al.*, 1979, Irvine and Alexander, 1998). The muscle wasting effects of sustained elevation of cortisol will have implications on the performance of elite sport horses as well as impacting the everyday pleasure horse. In addition to muscle wastage stress has been shown to suppress feeding behaviour in less than one hour, even in food deprived animals (Krahn *et al.*, 1986) and anorexia is a well accepted symptom of chronic stress resulting in weight loss or reduced weight gain (Harris *et al.*, 2002). This is due to CRH being a potent anorexic agent (Arase *et al.*, 1988) and high concentrations of cortisol acting as appetite suppressants (Devenport *et al.*, 1989, Irvine and Alexander, 1998). As feeding is a costly process and provides energy slowly it is obviously an expendable process during a crisis however prolonged activation of the stress response and therefore prolonged suppression of feeding will have negative consequences on the health of an animal, especially the horse, which relies on trickle feeding to maintain gut health.

Finally, numerous studies indicate that stress is an important contributing factor in gastrointestinal ulcer formation and that development of ulcers is low when animals can control or predict a stressor and high when repeated uncontrollable stressors are experienced (Koolhaas *et al.*, 1999).

In addition to detrimental effects upon equine health, the species-specific flight response to a stressful situation can prove dangerous to horse and human handler. It is for these reasons that knowing how horses perceive the management practices and training procedures they are subjected to, is very important in order to maintain optimum welfare.

Assessment of behaviour is not a conclusive means of assessing welfare in the horse. Training of horses by human handlers not to react to a given stimulus and the horse potentially masking stress as a survival method means that behavioural response cannot be relied upon as a single measure. It is therefore vital to include physiological measures of the stress response that

cannot be masked in order to provide a robust interpretation of how the horse perceives the situation it is in.

1.7 The importance of monitoring equine welfare

The importance of animal welfare is apparent within the scientific community and also in the public domain. The introduction of the most recent Animal Welfare Act 2006 aimed to improve animal welfare. Under the new act even if not compelled from an ethical point of view, owners and keepers are now under legal obligation to ensure that all the welfare needs of their horses are met, including a suitable environment, protection from suffering and the ability to exhibit normal behaviour patterns (Animal Welfare Act 2006 c.45). This increases the need for a reliable means of assessing equine welfare. Existing methods used to assess the physiological and behavioural stress response are not without limitations and there is no single reliable, non-invasive and instant measure.

Currently accepted methods used to measure the stress response can be invasive and stressful in themselves. This can confound results (Herd, 1991). Many of the available techniques are time consuming, expensive and results are not instantaneous. Existing methods to assess the stress response have been discussed however the associated limitations with existing methods emphasise the need for a reliable, non-invasive technique to assess current equine management and training practices. This will allow a review of the impact of equine husbandry upon welfare and enable management techniques to be altered if necessary.

Temperature change due to increased metabolism from cortisol production and alterations in circulation also forms part of the physiological stress response. Thermal change has been assessed in other species using infrared thermography (IRT) and validated with currently accepted measurement techniques (Levine et al., 2001; Pavlidis et al., 2001; Nakayama et al., 2004),

Cook et al., 2006; Stewart et al., 2007). Infrared thermography potentially offers an alternative and non-invasive method to assess reactivity to a potentially stressful situation however it has not been validated in the horse. The validation of IRT to measure temperature change associated with the stress response in horses would enable a more reliable, objective assessment of welfare to be carried out. IRT is non-invasive, accessible and provides near instantaneous results and could therefore be utilised in a range of situations. In addition to IRT being a potentially valuable research tool, once the impact of specific husbandry practices upon equine welfare has been established then this information can be disseminated to horse owners and handlers who do not have access to hormonal assessment and currently rely on potentially subjective behavioural assessment as a measure of welfare.

1.8 The thermal response to stress

When an animal is faced with a challenging situation the stress response is activated and the resultant increase in cortisol and metabolic activity in addition to alterations in blood flow will produce changes in heat production and heat loss (Schaefer *et al.*, 2002). As a result surface and core temperature has been measured as an indicator of stress in various species. Ear pinna temperature of sheep was found to decrease and correlate with an increase in heart rate during the potentially stressful situation of transportation (Ingram *et al.*, 2002). The authors suggest the decrease in peripheral temperature may be due to vasoconstriction and diversion of blood in response to stress-induced activation of the SNS. This initial acute response acts to redirect blood flow to areas with more urgent metabolic requirements (skeletal muscle, heart and lungs) and may also be a protective mechanism to reduce blood loss in the case of injury (Blessing, 2003; Vianna and Carrive, 2005). Stress related decrease in ear pinna temperature (measured with

temperature sensors) that correlated with an increase in stress hormones has also been reported in sheep subjected to isolation (Lowe *et al.*, 2005) and rabbits when subjected to a startle test (Yu and Blessing, 1997).

Monitoring body temperature has limitations including handling and manipulation of the animal and invasive surgical implantation of biotelemetry equipment (Parrott *et al.*, 1999) or rectal and vaginal probes (Ingram *et al.*, 2002). External sensors and remote devices used to measure surface temperature require the animal to carry bulky equipment and this can cause physiological changes in itself, regardless of how stressful the animal finds a situation (Stewart *et al.*, 2005), in addition to potentially disrupting normal behaviour patterns. External sensors can act as insulators that may confound results (Nakayama *et al.*, 2005; Stewart *et al.*, 2005) and solid probes can give false readings due to disruption by hair fibres (Cena, 1974).

It is possible to measure surface temperature without contact between handler and animal using infra-red thermography (IRT) and overcome all of these problems associated with traditional methods of thermal measurement.

1.9 Infrared thermography (IRT)

Infrared thermography (IRT) is the measurement of radiated electromagnetic energy (Stewart *et al.*, 2005). Electromagnetic energy is a stream of particles with no mass called photons. Photons travel at the speed of light in a wave like pattern. The photons with the highest energy correspond to the shortest wavelengths. The wavelengths of infrared radiation are longer than visible light and in animals 40-60% of heat loss falls within this range (Kleiber, 1975).

The infrared energy emitted from a body is proportional to its temperature. Small fluctuations in temperature result in substantial amounts of radiated energy and surface temperature changes that can be accurately detected using IRT (Stewart, 2007). Infrared energy is not visible because its

wavelength is too long to be detected by the human eye; therefore IRT is used to convert infrared energy (radiant heat) into a visible image. Specialist cameras can produce images that display variation in thermal output through coloured or grey shading. Analytical software is then used to assess patterns and changes in temperature. Operation of the camera is similar to conventional photography equipment, offering a simple and non-invasive method of measuring temperature.

Skin temperature directly reflects the underlying circulation and metabolism (Eddy *et al.*, 2001) therefore rapid changes in blood flow due to sympathetic activation and stimulation of the HPA axis will alter the amount of radiated heat (Stewart *et al.*, 2007). The associated changes in surface temperature can be measured using IRT and could provide a novel, more immediate, objective indication of the stress response when compared to hormone analysis and behavioural assessment.

Initially developed for military purposes and then utilised for industrial and medical applications (Burnay Williams and Jones, 1988) IRT is now applied to animal science and has been used to conduct population surveys (Croon *et al.*, 1968; Brooks, 1972; Cuyler *et al.*, 1992) study thermal physiology (Lancaster *et al.*, 1997, Sumbera *et al.*, 2007) and as a veterinary diagnostic tool (Turner, 1991).

1.9.1 The use of infrared thermography to conduct population surveys

A major problem with studying animals in their natural habitat is locating them. Trapping and marking techniques are time-consuming and invasive therefore some researchers may rely on visual sightings for census purposes (Boonstra *et al.*, 1994). Such surveys are constrained by camouflaged animals and the limits of human vision. Visual censusing can be enhanced

through the use of IRT that can detect dens or individual animals by their warm signal against a cooler background. Population surveys of deer (Croon *et al.*, 1968) polar bear (Brooks, 1972) whales (Cuyler *et al.*, 1992) and bats (Sabol and Hudson, 1995) have been carried out using this method. IRT is particularly useful for survey of nocturnal species as the medium measured is heat and reliance on light is not necessary (McCafferty, 2007). IRT also has the advantage of allowing remote and automated surveying of large numbers of animals. If the species being studied are warmer than their surroundings by 0.1 degree, a high specification thermal camera can detect them at distances of over 500 metres (Boonstra *et al.*, 1994). Aside from overcast days, after rains or when snow cover is present, surveys are best carried out in the early morning before the sun has heated the environment as this makes it increasingly difficult to distinguish between solar heat and radiation from animals. Insulative fur or nest material may also prevent detection of animals and a clear line of sight is also needed through thick vegetation however IRT offers ecologists a non-disruptive means of surveying a population of animals that is superior to human vision alone (Ditchkoff *et al.*, 2005).

1.9.2 The use of infrared thermography to investigate thermal physiology

IRT has been used to explore many aspects of thermal physiology in a variety of species including a comparison of wing temperature to body temperature of flying bats (Lancaster *et al.*, 1997), dorsal, ventral and lateral body surface temperature in mole rats to determine areas for dissipating heat (Sumbera *et al.*, 2007) and heat exchange by the ear pinna of the African elephant to investigate the thermoregulatory mechanisms involved in dust bathing (Rees, 2002). Elevated testicular temperature in bulls has been associated with reduced fertility (Barth and Oko, 1989) and IRT has been used to assess scrotal and testicular thermoregulation with an accuracy of 0.1°C (Purohit *et*

al., 1985; Coulter, 1988). IRT of the gluteal region was found to be a more reliable way of detecting early oestrus in cattle when compared to manual identification by an experienced herdsman in the fifty days postpartum (Hurnik *et al.*, 1985). It was believed that the African Elephant used its ear pinna as a convector and radiator of heat. This was confirmed in a study by Phillips and Heath (1992) using IRT to show that the elephant can divert blood flow to the ear pinna and dissipate or conserve heat through dilation or constriction of the blood vessels.

1.9.3 The use of infrared thermography in veterinary diagnostics

Due to its high sensitivity and instantaneous results, as well as improved safety due to absence of radiation when compared to radiography, IRT has proved useful as a veterinary diagnostic tool (Fonseca *et al.*, 2006). The dairy and beef industry has focussed on the use of IRT to detect the early stages of disease. Conditions such as mastitis which is a major welfare and economic concern for the dairy industry (Gill *et al.*, 1990), can now be detected much earlier than was previously possible by IRT of the mammary gland (Scott *et al.*, 2000; Berry *et al.*, 2003). Eye temperature change measured by IRT was found to be most effective and consistent at detecting illness in cows when compared to temperature change of other anatomical areas including the nose, ear and hooves. Increases in eye temperature were observed several days before clinical signs of bovine viral diarrhoea became apparent (Schaefer *et al.*, 2003) with an increase of less than 1°C being clinically significant. This particular study incorporated an infrared scanning station coupled with an electronic identification system into a water trough. The system could automatically identify an animal and collect an infrared image of the eye. This could then advise an owner or manager if the animal was showing early signs of disease.

Veterinary assessment of performance horses using IRT can predict joint and tendon problems up to two weeks before they become clinically apparent (Turner *et al.*, 1996). Research suggests IRT to be a rapid and efficient method for the diagnosis of lesions across the whole thoracolumbar region (Fonseca *et al.*, 2006) and it has proved particularly useful in the diagnosis, prognosis and evaluation of arthritis, laminitis, soft tissue injury and superficial orthopaedic lesions (Turner, 1991) with an asymmetry or change of 1°C or more being indicative of a problem (Turner, 1996). Figure 1.2 displays a thermal image of the forelimbs of a horse imaged during the current project. The horse was suffering from osteoarthritis and the difference in radiated heat due to inflammation is evident.

In addition to diagnosis of disease IRT has been used to evaluate topical treatments including magnetic therapy and cryotherapy (Turner *et al.*, 1989) and to detect illegal practices in the performance horse including the application of irritants to the perineal region to accentuate tail carriage (Turner and Scroggins 1989) and procedures used to obscure lameness including local injections of various pharmaceuticals (van Hoogmoed *et al.*, 2000).

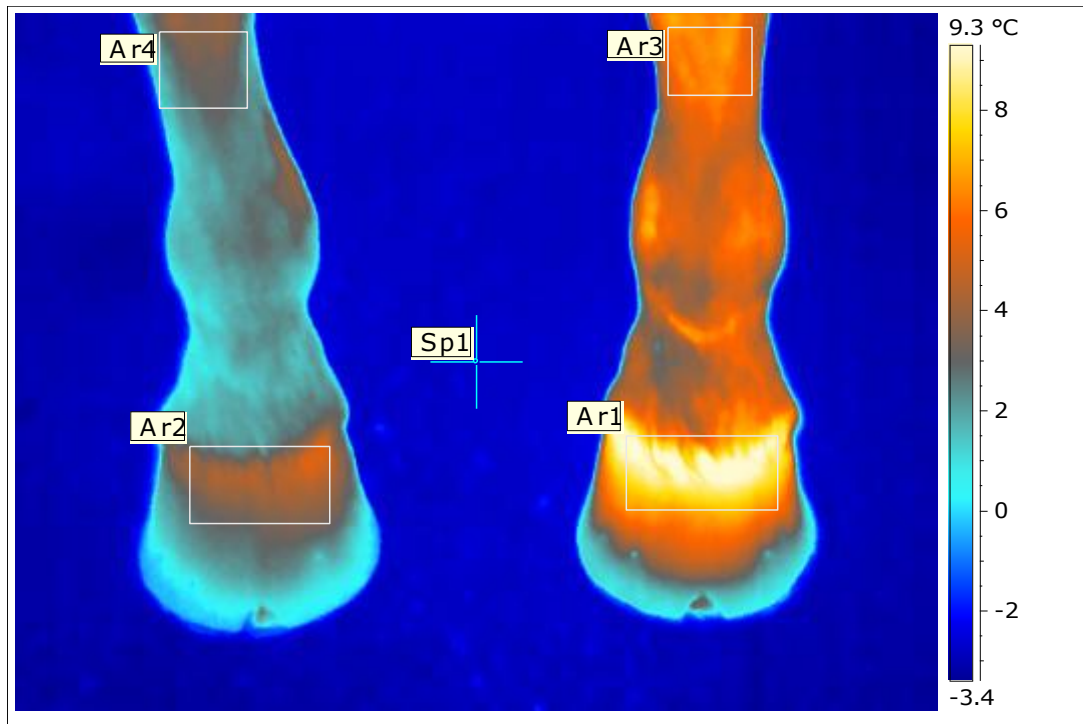


Figure 1.2. Thermal image of forelimbs of horse suffering from osteoarthritis with four areas marked using polygon analysis function and temperature scale on right side. The image shows a clear difference in radiated heat with the affected right limb being warmer due to inflammation (actual temperatures not shown).

1.9.4 Potential Limitations of IRT

There are some potential limitations cited in the literature when using IRT to measure animal thermodynamics. In many mammals hair or fur can obscure the underlying skin temperature and infrared radiation originating from the skin surface is altered due to insulative properties of the coat and the temperature gradient between the skin and the coat (Cena, 1974; McCafferty, 2007). Dirt in an animal's coat alters emissivity (ability to radiate absorbed energy) and excess moisture in the coat will increase local heat loss to the environment or to dryer areas of the coat (Palmer, 1981; Kastelic *et al.*, 1996), both of which could confound temperature results. Dirt can be removed by grooming however an acclimatisation period would then be required in order to ensure transient heat due to brushing had dissipated.

Since the variable measured is heat and not light, thermal cameras are not affected by the intensity or quantity of light in the scene and as they operate in the long wave infrared region (7-14 μ m) they are less affected by sunlight compared to shorter waves (Eddy *et al.*, 2001) Despite these findings, coat colour has been shown to affect surface temperature output from animals with areas of differing colour displaying large variation in temperature when influenced by solar heating (Cena and Clark, 1973). This is clearly demonstrated in infrared images of zebras (Figure 1.3 a and b) that show black stripes to be more than 10°C warmer than white stripes in full sun (McCafferty, 2007). This is not a reflection of underlying circulation as the temperature pattern disappears within a few minutes of standing in shade. It is therefore recommended that images be collected out of direct sunlight in a draught free area (Schaefer *et al.*, 2002). The limitations that are associated with the coat can be avoided through IRT of the eye which is the primary area assessed in existing work (Cook *et al.*, 2001, Cook *et al.*, 2006, Stewart *et al.*, 2007, Stewart *et al.*, 2008).

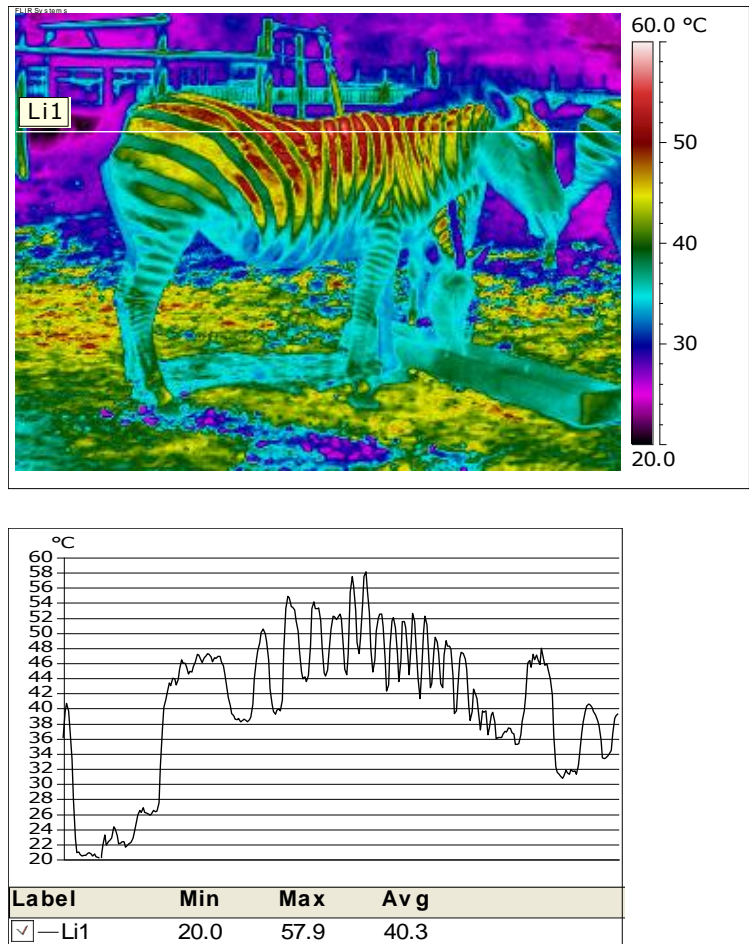


Figure 1.3 (a) Thermal image and corresponding temperature scale of zebra in direct sunlight displaying large variation in surface temperature between the black and white stripes. The corresponding chart details the temperature variation between black and white stripes at various points along the line marked on the thermal image (McCafferty, 2007).

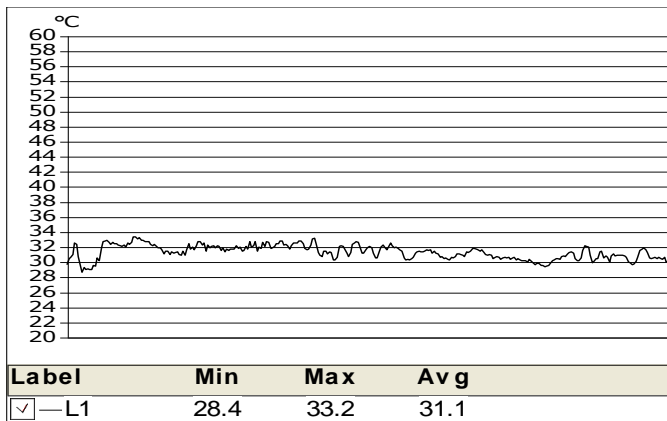
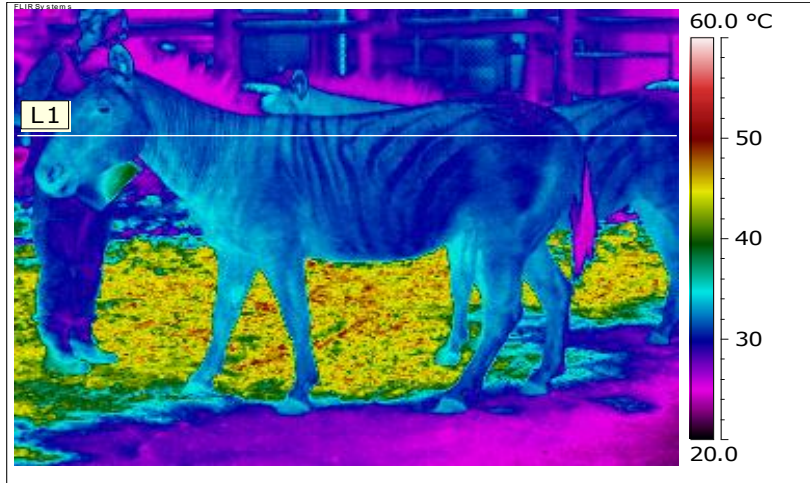


Figure 1.3 (b). Thermal image and corresponding temperature scale of zebra in shade displaying small variation in surface temperature between the black and white stripes. The corresponding chart details the temperature variation between black and white stripes at various points along the line marked on the thermal image (McCafferty, 2007).

Ambient temperature has been cited as a potential limiting factor in the use of IRT. Kastelic *et al.* (1996) found that abrupt changes in ambient temperature (using a climatic chamber) resulted in confounding results during IRT of the bovine scrotum. The authors suggest that moderate to cool temperatures of between 5-15°C are ideal to capture thermal images provided abrupt changes in temperature are avoided. Where this is not possible it is necessary to carefully monitor ambient temperature in order to rule it out as a contributing factor to thermal change (Eddy *et al.*, 2001; van Hoogmoed and Snyder 2002) and to allow for atmospheric influence when analysing temperature data (Ingram *et al.*, 2002; Nakayama *et al.*, 2005; Stewart *et al.*, 2007).

Population studies and work in free-ranging species position the thermal camera at distances of up to five hundred metres from the animal (Boonstra *et al.*, 1994) however such studies only utilise IRT to count individuals therefore only require visual identification and not detailed temperature measurement. Studies that use IRT as a diagnostic tool and as a tool to monitor stress are in agreement that the camera should be positioned or held at a ninety degree angle approximately two metres away from the subject being imaged (Nakayama *et al.*, 2005, Cook *et al.*, 2006, Stewart *et al.*, 2007). This will allow small changes in temperature to be accurately measured. The peak temperature within the eye region has been shown to be the most consistent measure giving the least variance (Cook *et al.*, 2006, Stewart *et al.*, 2007).

In order to collect accurate thermal images it is necessary to understand the thermal behaviour of the target being studied. Body temperature is amongst the physiological variables that exhibit circadian rhythm (Refinetti and Menaker, 1992) and a clear circadian rhythm of core body temperature has been reported in horses (Piccione *et al.*, 2002, Green *et al.*, 2005) with temperature starting its daily ascent at dawn, coinciding with the start of the light phase, and reaching a maximum fourteen hours later. Temperature patterns have also been reported to peak in the late afternoon or evening and trough in the morning in Kangaroos (Brown and Dawson, 1976) and various African ruminants (Bligh and Harthoorn, 1965) and could be a reflection of the storage of heat gained throughout the day.

Scott *et al.* (2000) found a distinct circadian rhythm in udder temperature when using IRT as a tool to detect early stages of mastitis however not all studies report a rhythm in temperature. Whilst using IRT to measure bovine scrotal surface temperature Kastelic *et al.* (1996) found no significant effect of diurnal rhythm on thermal output.

Studies investigating thermal rhythm in horses have used temperature probes which can be invasive (Green *et al.*, 2005) and contribute to temperature change through their insulative properties, and climatic chambers that do not offer temperature assessment in a real life situation (Morgan *et al.*, 1997, Piccione *et al.*, 2002). It is therefore important for the purpose of this project to establish if there is any circadian rhythm in eye temperature in order to better interpret any thermal changes associated with the stress response.

Veterinary diagnostic studies report that anxious patients can have significant skin temperature reductions (Eddy *et al.*, 2001), which could confound results from a diagnostic aspect. However, for the purpose of stress assessment these findings only serve to reinforce the ability of IRT to detect physiological changes associated with potentially stressful situations.

1.10 Infrared thermography as a measure of the response to stress

An instant rush of blood to the eye area is a phenomenon that has been linked to telling lies and the subsequent stress in humans and can cause eye temperature to rise by several degrees (Levine *et al.*, 2001). An IRT system developed to screen passengers as a security measure at airports was able to detect liars with a comparable accuracy to polygraph equipment. In 20 tests performed at the United States department of defence, a thermal camera capable of detecting temperature change of 0.025°C identified 75% of candidates who had lied and 90% who had told the truth based on blood flow changes to the eye and surrounding area (Figure1.4) (Levine *et al.*, 2001).



Figure 1.4 Temperature change around the human eyes after telling the truth (upper image) and telling a lie (lower image) Levine et al.,(2001)

Humans that were subjected to an acoustic startle stimulus displayed warming of the periorbital area associated with increased blood flow and associated cooling of the cheeks (Pavlidis *et al.*, 2001). Both studies into the thermal response of the human eye area to stress suggest that the results make physiological and evolutionary sense as they could represent a mechanism to facilitate rapid eye movements during preparedness for flight and escape.

IRT can be used to non-invasively identify these changes in temperature (McCafferty, 2007) and the focus of recent research has been the use of IRT to detect the response of animals to stressful situations. Temperature change of various species specific anatomical areas in response to stress has been recorded and validated against currently accepted measures (behavioural assessment and cortisol analysis) to assess whether IRT can be used reliably to measure the stress response in a number of species including monkeys (Nakayama *et al.*, 2004), elk (Cook *et al.*, 2006) and cows (Stewart *et al.*, 2007). Studies have used a number of species specific body sites alone or in combination to assess the impact of potentially stressful events.

When confronted by a threatening handler the nasal temperature of rhesus monkeys (n=4) measured using IRT decreased significantly (mean of 0.2 degrees) within ten seconds and continued to decrease throughout the stimulation period of three minutes (Nakayama *et al.*, 2004). This was associated with an increase in temperature on the eyelids and adjacent area (Figure 1.5). Lip temperature also decreased however nasal temperature was shown to be the most consistent measure for this species. The authors report that the decrease in temperature originated from an area in the uppermost portion of the nasal region and then spread to the lower nasal regions. Temperature started its descent between 10 and 110 seconds post onset of confrontation in all monkeys and had returned to pre stressor levels within

four minutes post removal of confrontation. The change in temperature was supported by the species specific behavioural response to a challenging situation of bared-teeth and lip smacking, suggesting that the monkeys found the treatment to be stressful.

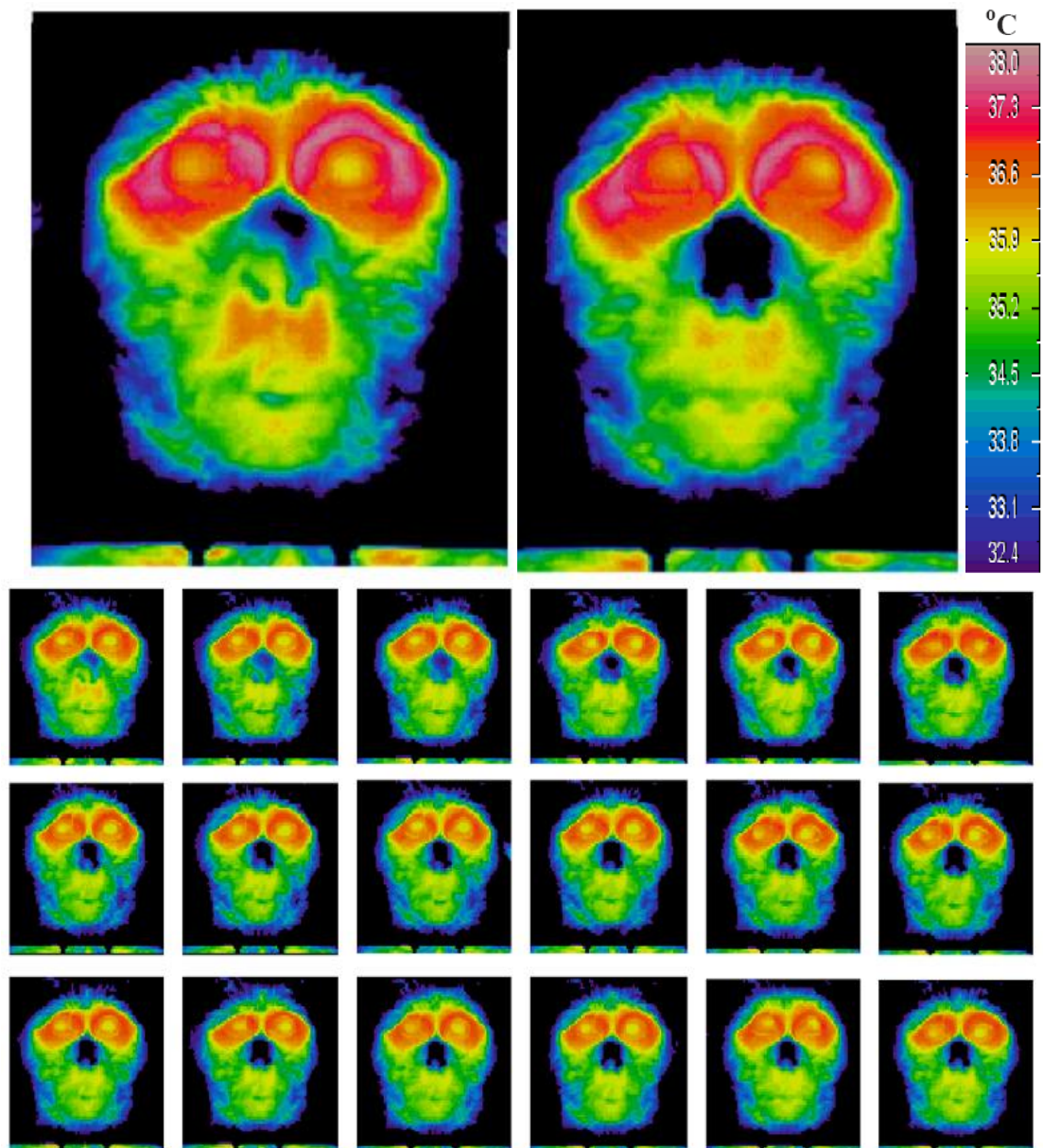


Figure 1.5. Thermal images and related temperature scale demonstrating decrease in nasal temperature of rhesus monkeys when threatened. The two larger images depict thermal pattern pre and post stimulation while smaller images detail successive cooling of nasal region over three minute stimulation period (Nakayama et al., 2006).

The possibility that changes in eye temperature were due to activation of the HPA axis during the stress response was investigated using IRT in cattle (Stewart *et al.*, 2007). The HPA axis was artificially stimulated by administration of ACTH hormone. Blood was extracted via jugular catheter for plasma cortisol analysis and thermal images of the eye were captured at a distance of 0.5-1.0m at a ninety degree angle every two minutes throughout the treatment period. The maximum eye temperature was then determined within an oval area traced around the eye including the eyeball and approximately 1cm surrounding the eye. There were two treatment sessions over a two week period and catheters were removed after the first treatment before being reinserted for the second treatment. An increase in plasma cortisol post ACTH injection confirmed the HPA axis was stimulated however the authors found no evidence to support their hypothesis that eye temperature increases in response to artificial stimulation of the HPA axis. Eye temperature and cortisol concentration did not increase after catheterisation in week one however both parameters were higher after catheterisation when the procedure was repeated in week two. The authors suggest that this may be due to anticipation of the procedure, i.e., there was a perceived stressor as well as the artificially induced physiological response. Therefore it may be that a psychological component is required to evoke the thermal response to a challenging situation.

A further study by Stewart *et al.* (2008) also investigated the possibility that stress in calves caused by disbudding can be detected from temperature changes in the eye area and heart rate. Eye temperature was chosen as the authors felt it was easily measured without the interference of fur or hair and had been shown to be a more consistent measure of temperature change when compared to other anatomical areas. The maximum temperature within the area of the medial posterior palpebral border of the lower eyelid and the

lacrimal caruncle was extracted (Figure 1.6). Calves were randomly assigned to four treatments of sham disbudded (control 1), local anaesthetic and sham disbudded (control 2), sham anaesthetic and disbudded and local anaesthetic and disbudded. The authors reported a rapid drop and then subsequent rise above basal levels in eye temperature in the calves disbudded without local anaesthetic. Eye temperature increased from five minutes post treatment in both disbudded groups by 0.6 and 0.66 degrees Celsius respectively and was significantly higher than in the two control groups. One week later the disbudded cows were split into two groups and given an ACTH hormone injection to artificially stimulate the HPA axis or saline injection (control). There were no significant differences in eye temperature before or after administration of either ACTH or saline, suggesting eye temperature change is not a direct consequence of changes in HPA activity.

The authors suggest that the rapid drop in eye temperature following disbudding may have been caused by redirection of blood via sympathetically mediated vasoconstriction of the capillaries surrounding the eye. The magnitude of the change in eye temperature is consistent with that found following fright in cattle (Schaefer *et al.*, 2006) and also with the decrease in nasal temperature in monkeys when confronted by a threatening handler (Nakayama *et al.*, 2004).

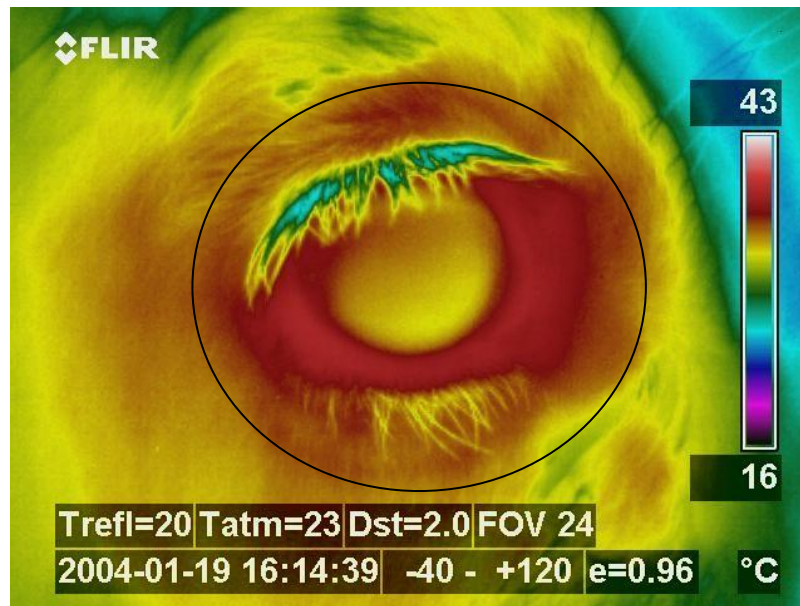


Figure 1.6 Infrared image of the eye region showing temperature scale on right hand side and a circle drawn around the area of the eye where maximum temperature was extracted from (Stewart et al.,2008).

Although Stewart et al., (2007) report no association with activation of the HPA axis and eye temperature change measured using IRT in cattle, Cook et al. (2001) did report a relationship in horses. Matched blood and saliva samples as well as IRT images of the eye were collected before and after artificial stimulation of the HPA axis through injection of ACTH. Temperature was extracted from each thermal image and minimum, maximum and mode temperatures calculated. Results showed a significant correlation between eye temperature and both plasma and salivary cortisol. Salivary cortisol demonstrated a maximum increase of 314% one hour post injection with the relationship between salivary cortisol and eye temperature being the strongest. All measures of infrared temperature demonstrated an increase during the sampling period however the measure most closely associated with time was maximum eye temperature when compared to mean and minimum temperature. The authors conclude that these findings demonstrate that salivary cortisol is a more responsive marker for HPA activity than plasma cortisol and that measures of HPA activity and infrared thermography of the eye are positively correlated. This study artificially induced the stress

response in the horse and it is likely that physiological changes occurred in response to the ACTH administration and blood sampling procedure rather than to the ACTH itself in comparison to the work carried out by Stewart *et al.* (2008) that investigated the stress response in a real life situation.

Existing work is in agreement that images should be collected at a distance of between 0.5-2.0 metres from the test subject at a ninety degree angle (Cook *et al.*, 2001; Levine *et al.*, 2001; Nakayama *et al.*, 2004; Stewart *et al.*, 2007; Cook *et al.*, 2006; Stewart *et al.*, 2008) when using IRT as a measure of the stress response. So far only thermal response to short term (<3 minutes) stress has been investigated. The study by Cook *et al.* (2001) artificially induced the stress response in the horse rather than investigating any physiological response to an actual potentially stressful husbandry procedure and offered limited results. No further study into the thermal response of the horse to stress has been carried out to date.

Monitoring behavioural response is an important factor when assessing reactivity to management procedures and training techniques however as a prey species horses do not always flee from danger and can mask stress. As a result, additional physiological measures are required in order to accurately interpret behavioural response. IRT may offer a non-invasive and instant physiological assessment.

Horses that are comfortable in their environment and with the management and training techniques they are subjected to as a consequence of domestication are safer to handle and are less likely to suffer illness and it is the duty of responsible horse owners to ensure that welfare needs are met. In order to do this an objective assessment of how horses perceive their environment is required.

The limitations in current methods to assess welfare and the delay in obtaining results from salivary cortisol analysis indicate that there is no single,

immediate method capable of measuring response to potential stressors and there is a need for a reliable non-invasive and instant measure of stress in the horse (Reitmann *et al.*, 2004).

IRT is a novel, non-invasive tool that could potentially measure the stress response in horses. If temperature change occurs in response to stressful situations and corresponds with the currently accepted physiological measures then IRT could be validated and has the potential to provide an alternative, immediate, objective measure. IRT could then be used to assess management techniques when the horse may not necessarily exhibit a behavioural response, but is still physiologically and psychologically compromised.

A search of the literature shows there are a number of anatomical areas that have been studied alone or in combination to monitor the impact of a range of potential adverse events using IRT. However, many of the limitations associated with IRT can be overcome through imaging of the eye, which is free from hair and dirt and has been reported to provide a consistent measure of temperature change associated with the stress response (Cook *et al.*, 2001; Pavlidis *et al.*, 2002; Cook *et al.*, 2006).

IRT is an excellent, original candidate as a research tool and could refine existing research methods. Developments in technology mean that thermal imaging devices are now the size of conventional video cameras making it easy to capture and store high resolution images. Image analysis is rapid and most models allow instantaneous temperature to be read straight from the camera. Despite these benefits and positive results in other species little is known regarding temperature change in response to stress in the horse and currently no investigation into the thermal response to longer term stress has been carried out.

Domestication of horses has removed them from their natural environment and placed them into situations they may potentially find stressful. As a result stress related disease and abnormal behaviour may emerge and compromise welfare. It is therefore important that management practices are objectively assessed and altered if necessary. Existing methods of assessing welfare have limitations. The currently accepted measure of hormonal assessment is time consuming, potentially invasive and expensive and behavioural evaluation can be subjective. There is a need for a reliable and non-invasive, objective measure of the physiological response to stress. IRT potentially offers a non-invasive, instant measure of the stress response and is able to measure physiological changes that cannot be masked as behaviour can. It can offer a more reliable assessment of how domestic horses perceive management practices and training procedures and allow us to alter them if necessary in order to improve welfare. Validation of IRT using currently accepted methods as a tool to measure the stress response is now needed.

1.11 Aims and Objectives

Project Aims:

- To investigate the use of infrared thermography (IRT) as a measure of the stress response in the horse and compare thermal changes with currently accepted stress measurement parameters (behavioural and hormonal assessment).
- Application of IRT as a non-invasive measure of acute, short term and long term stress in the horse during management procedures.

Project Objectives:

- Establish any diurnal and seasonal rhythm present in thermal output and hormone levels in the horse in order to improve interpretation of physiological data.
- Investigate the thermal response of the horse to a potential acute stressor and compare against hormonal and behavioural response.
- Investigate the thermal response of the horse to a potentially stressful short term husbandry procedure (clipping) and compare against hormonal and behavioural response.
- Investigate the thermal response of the horse to various housing designs that could potentially cause long term or repetitive stress and compare against hormonal and behavioural response.

Chapter 2. Materials and Methods

The following methods were used to assess the use of infrared thermography as a tool to measure the physiological stress response in horses. All of the studies were carried out at the Brackenhurst Equestrian Centre, Nottingham Trent University, between October 2007 and January 2010. Table 2.1 details each study. Thermal images were collected and temperature extracted using the same method for each study. Saliva was also collected and assayed for cortisol using the same method for each study. Both methods will be detailed in this chapter. Timings of the physiological samples, statistical analysis of data and method of behavioural assessment differed for each study and will be discussed in each relevant data chapter. Risk assessments and ethical approval forms can be found in Appendix 12.

Table 2.1 Description of each preliminary and main study carried out during the project.

Study	Description
Preliminary study 1	Investigation into the effect of distance on the accuracy of temperature measurement using infrared thermography
Preliminary study 2	Investigation into eye and ear temperature output of horses in their usual environment measured using IRT
Preliminary study 3	Investigation into the thermal response of a horse to a short term routine husbandry procedure measured using IRT.
Main study 1	Investigation into the potentially limiting factors of thermal imaging and salivary cortisol analysis as indicators of the stress response
Main study 2	Investigation into the use of IRT as a tool to assess thermal change associated with the physiological response to acute stress
Main study 3	Investigation into the use of IRT as a tool to assess thermal change associated with the physiological response to short term stress
Main study 4	Investigation into the use of IRT as a tool to assess thermal change associated with the physiological response to long term stress

2.1 Horses and husbandry

All horses involved in this project were provided by the Equestrian Centre and were regularly ridden by University students for up to two hours per day, six days per week during the academic terms. During the course of each study horses were removed from all ridden and practical lessons unless otherwise stated in the relevant chapter. Table 2.2 provides details of all horses used during the project. During the summer holidays the horses were out at grass, not ridden and brought into the stables when required for testing. Horses were fed a mixture of forage and concentrate feed during term time and forage only when brought into the stable during the summer holidays. All horses had constant access to water.

Table 2.2 Details of all horses used throughout the project. An X marks the studies taken part in.

NAME	YEAR OF BIRTH	SEX	BREED	PRELIM STUDY			MAIN STUDY			
				1	2	3	1	2	3	4
Libby	1997	M	IDxTB		X			X		X
Sparky	1995	G	IDxTB	X	X					
Oliver	2000	G	TB		X					
Robbie	2001	G	cob		X					
Angus	1998	G	SWBxTB				X		X	X
Beau	1995	G	IDxTB				X		X	X
Conan	1997	G	IDxTB				X	X	X	X
Kitkat	2005	G	WCxTB				X		X	X
Desmond	1995	G	IDxTB				X		X	
Tosca	1995	M	cob			X	X		X	X
Brock	2005	G	BWB					X		
Leisle	1995	M	BWB					X		X
Impy	1995	M	TB					X		
Blackberry	1995	M	IDxTB					X		X
Dice	1999	G	ID	X				X		
Palmer	2001	G	IDxTB	X				X		
Nola	1990	M	TB					X		
Annie	1998	M	SWBxTB						X	
Harriet	1995	M	IDxTB						X	
Visi	1990	G	TB						X	X
Pie	1990	M	cob						X	
Woody	1994	G	BWBxTB	X						X
Del	1995	G	Shire x	X						X
Herbert	1998	G	TB	X						X
Ernie	1995	G	TB	X						X
Ellie	1996	M	ISP	X						X
Fanta	1990	M	cob	X						X
Puzzle	1990	M	cob	X						X

Key	
Breed	Sex
TB	Thoroughbred
DWBxTB	Dutch warmblood cross TB
IDxTB	Irish draft cross TB
ISP	Irish sport horse
BWB	British warmblood
DWB	Dutch warmblood
WCxTB	Welsh cob cross TB
WCxTB	Welsh cob cross TB
BWBxTB	British warmblood cross TB
M	Mare
G	Gelding

2.2 Data collection

2.2.1 Saliva sample collection.

An initial pilot study was conducted to determine the most suitable method to collect saliva from horses (Appendix 1).

Salivary cortisol was used as a physiological marker to indicate that activation of the hypothalamic-pituitary-adrenal (HPA) axis had occurred. Salivary cortisol was selected as it offers a non-invasive method of hormone sampling and research suggests it reflects plasma cortisol levels (Van der Kolk *et al.*, 2001, Creighton and Hughes 2007). Saliva is easy to collect and sampling can be performed frequently and non-invasively which was a requirement for this project.

Saliva was collected using the commercially available salivette[®] (Sartedt, UK). This collection device is 97mm in length and provides a hygienic and effective means of obtaining saliva. The salivette consists of two plastic cylindrical tubes, one inside the other. The inner tube holds a cotton swab and has a small outlet in the base. The swab is placed in the mouth and chewed to stimulate production of saliva. The swab is then placed inside the salivette and centrifuged and the saliva collects at the base of the outer container (Figure 2.1). As the salivette was originally designed for human use it was necessary to modify them for use in horses. A cotton thread was stitched down the centre of the swab and could be held by the human handler as the horse chewed to prevent it from being swallowed. Each salivette was labelled on the outer container with the horse's name, date and time of collection using a permanent marker pen.

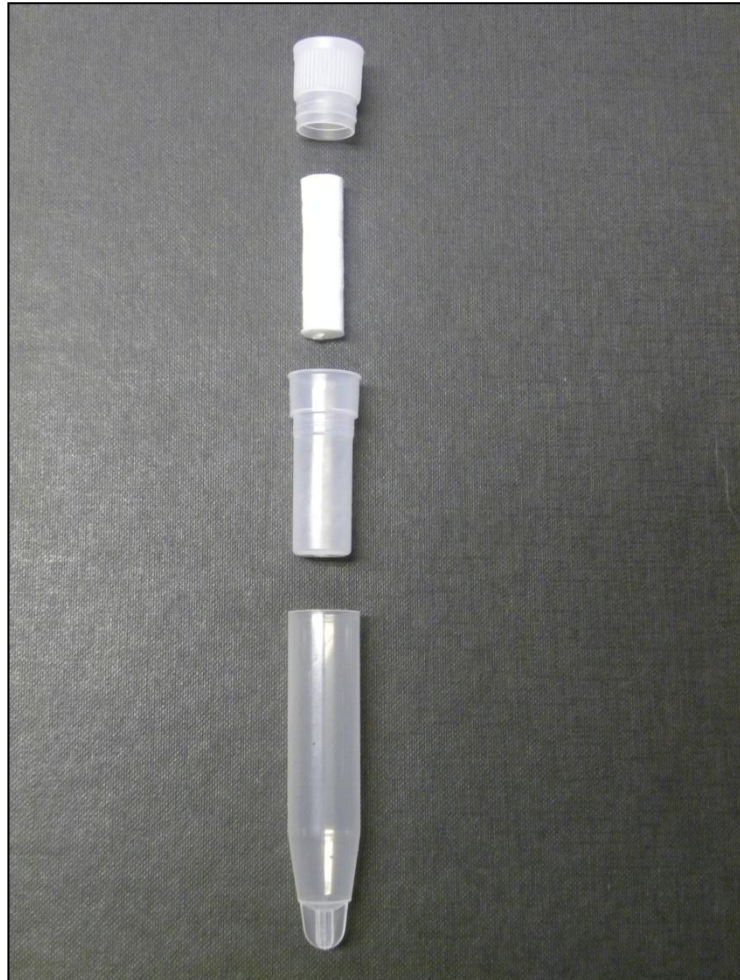


Figure 2.1. Salivette separated into its constituent parts of inner and outer container, lid and cotton swab. The salivette was modified by stitching thread through the cotton swab. This allowed the human handler to hold the thread, preventing the horse from swallowing the swab.

Swabs were placed in the horses' oral cavity at the height of the third premolar in the maxilla. The sampling period was approximately thirty seconds to prevent destruction of the swab through mastication. Salivettes were refrigerated at 4°C for no longer than two hours before they were transferred to a freezer and frozen at -20°C until analysis. All horses involved in this project were familiar with having their mouths handled for fitting of equipment and grooming. To avoid the horses associating the salivette and handling of the mouth with impending stress, all horses involved in the project had their mouths swabbed with a salivette at least twice per week. This was carried out in their stable and was not followed by any kind of stressful procedure.

2.2.1.1 Salivary cortisol assay procedure.

Salivettes were thawed and centrifuged (Rotina 380, DJB Labcare) at 1000x *g'* for ten minutes. The collected saliva was then retrieved and transferred to 1.5ml microtubes via pipette and assayed immediately.

The saliva was analysed for cortisol using a commercially available Enzyme-linked immunosorbent assay (ELISA) (DRG Diagnostics). The assay is based on the competition principle. An unknown amount of cortisol present in the sample and a fixed amount of cortisol conjugated with horseradish peroxidase compete for the binding sites of mouse monoclonal cortisol antiserum coated onto the wells. After one hour incubation the microplate is washed to stop the competition reaction. After addition of a substrate solution (Tetramethylbenzidine) the concentration of cortisol is inversely proportional to the optical density measured.

The assay kit included Microtitrewells in twelve by eight well break apart coated strips (ninety six wells) that allowed the desired number of strips to be placed in the frame holder or plate. Seven cortisol standards of 0, 2, 5, 10, 20, 40 and 80 ng/ml cortisol were used along with a high and low control.

Amounts of the high and low control were specific to each quality control datasheet included with each kit and contained 0.003% proclin 300 as a preservative. Figure 2.2 displays the layout of a typical plate with positioning of the standards and controls in triplicate and numbered equine saliva samples in duplicate.

A total of eleven salivary cortisol assays were carried out. The % coefficient of variation of means for high control was 3.9% and the % coefficient of variation of means for low control was 15%. The mean inter-assay coefficient of variation was 9.5%.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0ng/ml	0ng/ml	0ng/ml	1	1	9	9	17	17	26	26	33
B	2ng/ml	2ng/ml	2ng/ml	2	2	10	10	18	18	27	27	33
C	5ng/ml	5ng/ml	5ng/ml	3	3	11	11	19	19	28	28	34
D	10ng/ml	10ng/ml	10ng/ml	4	4	12	12	20	20	29	29	34
E	20ng/ml	20ng/ml	20ng/ml	5	5	13	13	21	21	30	30	35
F	40ng/ml	40ng/ml	40ng/ml	6	6	14	14	22	22	31	31	35
G	80ng/ml	80ng/ml	80ng/ml	7	7	15	15	23	24	32	32	spare
H	High	High	High	8	8	16	16	25	25	low	low	low





-  Standards containing known amounts of cortisol from 0ng/ml through to 80ng/ml in triplicate
-  High control in triplicate
-  Low control in triplicate
-  Samples of equine saliva numbered 1-35 in this example in duplicate

Figure 2.2. Typical layout of ELISA plate with labelled (A-H, 1-12) microtiterwells with standards and control (high and low) in triplicate, and numbered equine saliva samples in duplicate

Once the desired numbers of strips were secured in the plate then 100µl of each cortisol standard and control were dispensed into the appropriate wells in addition to 100µl of each saliva sample. Enzyme conjugate was then added (200µl) into each sample, standard and control well and then the plate was mixed thoroughly for thirty seconds (Boekel Microjive Microplate Shaker). The plate was incubated at room temperature for sixty minutes after which the contents were briskly shaken out by hand. The plate was rinsed three times with diluted wash solution (30ml provided with kit diluted in 1200ml deionised H₂O) and then residual droplets removed by striking the inverted wells on absorbent paper. Substrate solution (200µl Tetramethylbenzidine) was dispensed into each well and the plate incubated at room temperature for thirty minutes. The reaction was stopped by adding 100µl of Sulphuric acid (14mL, 0.5M) to each well and then the absorbance of each well determined (Lt 400 absorbance microplate reader) at 450nm. Data was then exported to Excel and formatted to correspond to the plate layout.

2.2.1.2 Validation of ELISA for use in the horse

A validation test was carried out on the kit used for the analysis of equine saliva for cortisol and the test used by Chester Zoo to measure corticosterone in equine faeces. Faecal corticosterone was measured during main study 4. This was to ensure that the assays were measuring cortisol and corticosterone accurately and there was no cross reaction with other components in the saliva or faeces. A pooled sample of saliva was assayed neat (10ng/ml) and diluted to 8ng/ml, 6ng/ml, 4ng/ml, 2ng/ml and 1ng/ml alongside the standards provided with the kit. Once the results were plotted if the standard curves of both the sample and the standards run parallel to each other this indicates that the kit is measuring what it is designed to measure (Mostl and Palme, 2002). The validation test for salivary cortisol assessment was carried out at Nottingham Trent University and the validation of the assay for faecal

corticosterone was carried out by Chester Zoo. Appendix 2 displays the results of both validation tests which show analysis for cortisol in saliva and corticosterone in faeces were the most appropriate methods.

2.2.1.3 Calculation of values in salivary cortisol

The average absorbance values for each set of standards, controls and samples (unknown concentration of cortisol) were calculated using excel and a standard curve produced by plotting the mean absorbance from each standard against the log of its concentration (Figure 2.3). Unknowns were interpolated from a semi-log plot of known standard concentrations. The same method was used to estimate the concentration of high and low controls provided with the kit; if these were within the range given on the data sheet then the assay was valid. Once the concentration of cortisol (ng/ml) in the controls was calculated then it was entered into an Excel spreadsheet. This allowed cortisol concentration over time to be plotted to investigate the position and dispersion of the data.

The standard curve and semi log plots for each study plot can be found in Appendix 3.

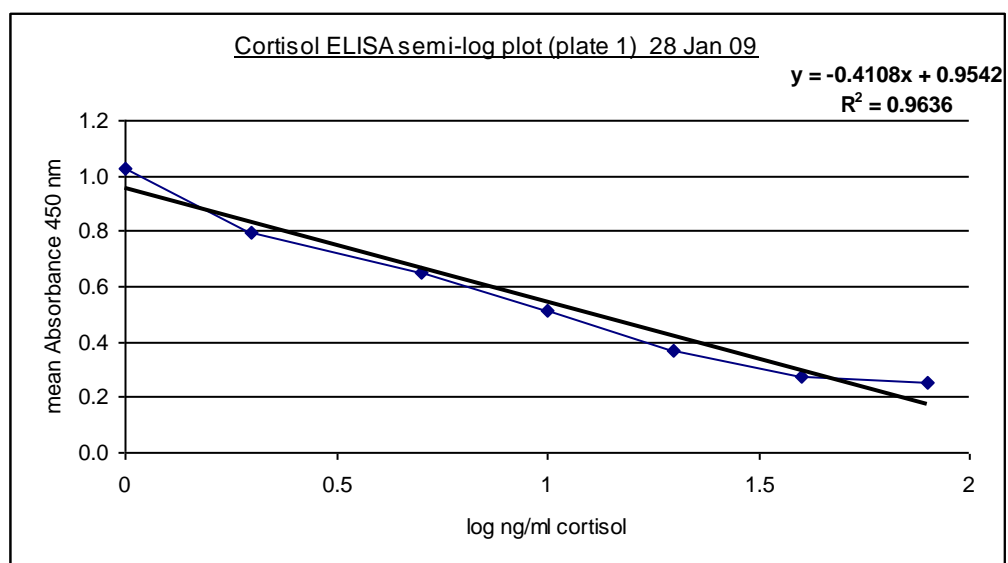


Figure 2.3. Semi log plot of known cortisol standard concentrations used to interpolate unknown samples.

2.2.2 Infrared Thermography and temperature data collection

Three types of thermal camera were used during this project depending on whether continuous or static thermal images were required. These were a Mobir® GuidIR M4 static thermal image camera, a Flir ThermaCAM SC640 and a FLIR ThermoVision A40M which all self-calibrated at regular intervals. The Mobir® GuidIR thermal camera has a thermal sensitivity of $\leq 0.1^{\circ}\text{C}$ and can detect temperature at a range of -20°C to 250°C . It has the capacity to store up to 600 thermal images until they are uploaded via USB 1.1 connection to a PC. The Guide IR analytical software includes a polygon analysis function; this allows a polygon to be manually placed over a desired area of the thermal image. Once the polygon is in place then a range of temperature analyses can be carried out within the selected area (Figure 2.4).

The Flir ThermaCAM SC640 has a thermal sensitivity of 0.6°C with a range of temperature detection between -40°C and 1500°C . It can also store thermal images, the amount depending on the size of memory card used and transfers data to analytical software (FLIR Quickreport) using either Firewire, USB 2.0 or SD card interfaces.

The study into acute fright (chapter 4) required a camera capable of video recording therefore a FLIR ThermoVision A40M thermal camera was used. This camera has a thermal sensitivity of 0.08°C at 30°C with a range of -40°C to 500°C and captures thermal images in sequence at 30 frames per second. Sequences of thermal images were captured to disk as the startle response test was carried out and uploaded to a PC for analysis using the FLIR analytical software package mentioned previously. Static thermal images were taken at a distance of 1 metre \pm 50cm from the horse at a 90 degree angle in accordance with recommendations from existing research (Nakayama *et al.*, 2005, Cook *et al.*, 2006, Stewart *et al.*, 2007). The camera was aimed by previewing the image with the inbuilt display and images of the right and left lateral aspect of the horse were taken at each sampling time point for all

static images throughout the project. The acute nature of the startle response study required continuous thermal monitoring of the horse and as only one camera was available images were taken from the left lateral aspect only.

Once the images were uploaded to the thermal analysis software, extraction of the temperature for each specific region was then performed. Initially a coloured or greyscale image without the temperature information is displayed. The human user's task was to define the region to be analysed using a mouse interface. The software then scanned this region in order to find the peak temperature. Eye temperature analysis recorded maximum temperature within the medial posterior palpebral border of the lower eyelid and the lacrimal caruncle as in Stewart *et al.*, (2008) (Figure 2.4). For each time point a temperature for both left and right eyes was captured. Temperatures were entered into an Excel spreadsheet and a mean temperature calculated from left and right eye for each time point.

Ear temperature analysis recorded maximum temperature of the ear pinna. The polygon analysis tool was used to highlight the area on the back of the ear from the base to the tip (Figure 2.4). For each time point a temperature for both left and right ears was captured. Temperatures were entered into an Excel spreadsheet and a mean temperature calculated from left and right ear for each time point. This allowed temperature over time to be plotted to investigate the position and dispersion of the data.

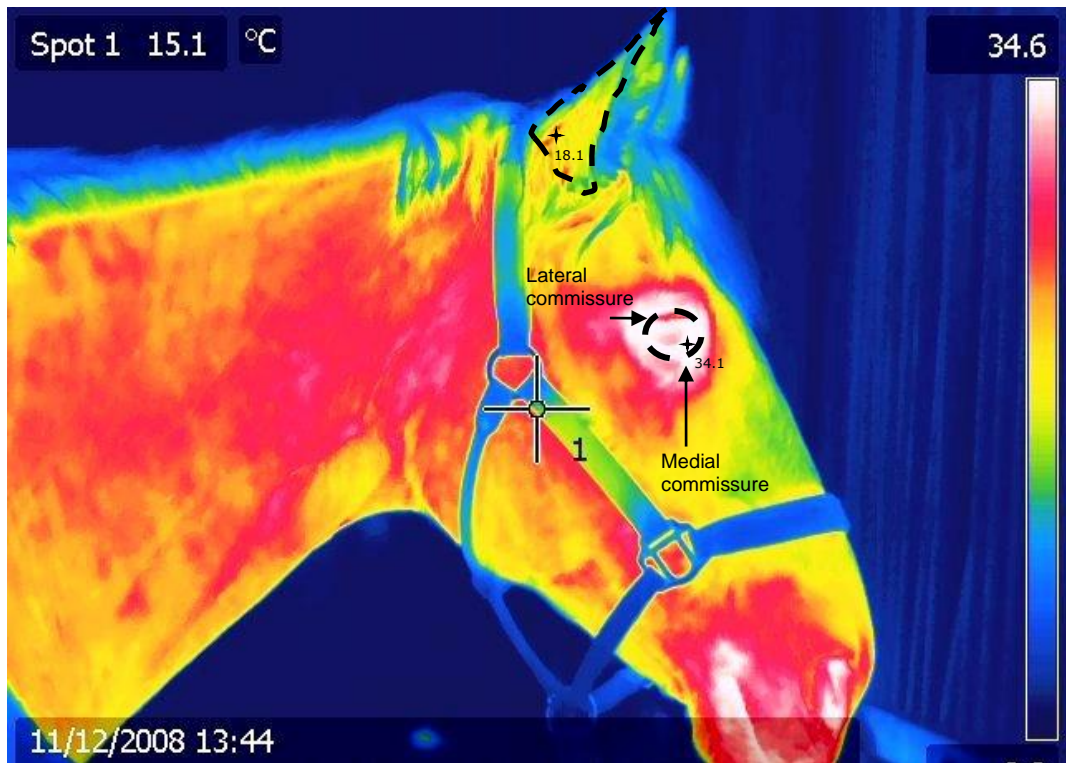


Figure 2.4 Coloured thermal image illustrating right lateral profile of equine head with lateral and medial commissure labelled. Spot analysis function (1) and corresponding temperature in top left corner. Polygon function has been utilised to highlight eye and ear pinna regions. Markers (+) show area of maximum temperature within each polygon. Temperature range is displayed down the right side.

2.2.2.1 Core body temperature data collection

To allow a more robust interpretation of thermal change, core temperature was measured in order to investigate any potential contribution of change in core temperature to change in eye temperature. core temperature was measured using a digital thermometer (Boots, UK) inserted 2cm into the rectum. The thermometer had an in built alarm that indicated when core temperature had been reached. Temperatures were recorded manually on record sheets and then transferred to an Excel spreadsheet that also contained the corresponding eye and ear temperatures for each specific horse.

2.2.2.2 Ambient temperature data collection

Ambient temperature was recorded throughout each study and compared to changes in equine thermal output in order to monitor the potential effect of microclimate as a contributing factor to thermal change. Study B (chapter 4) used a wet bulb thermometer to record ambient temperature. All other studies used a small and portable temperature data logger (Lascar EL-USB-2). The data logger is capable of continuously monitoring ambient temperature for up to one year if set to record at five minute intervals. The temperature logger is inserted into the USB hub of a PC and synchronises with Lascar configuration and analytical software. This enables required sampling rate to be set along with a starting date and time. Once data collection is complete the logger is inserted into the PC to enable data to be downloaded. A histogram is then produced showing temperature over time. Specific times can be selected with their corresponding temperature.

2.2.3 Heart rate data collection

A Polar Equine RS800G3 heart rate monitor was used for measurement of heart rate. It consists of a wireless textile transmitter in the form of an elasticated surcingle with two interwoven electrodes. The electrodes are made from conductive elastic fibres enabling them to adapt to the horse's movement and ensure permanent contact with the skin. The surcingle was secured on the horse and the interwoven electrodes were positioned in the region of the upper left thorax and the ventral midline (Figure 2.5). Electrode gel (Signa gel, UK) was applied to the electrodes on the surcingle and their associated points on the horse to improve contact and enhance electrical conductivity. A detachable transmitter was clipped onto the surcingle which communicates with the electrodes and transmits heart rate data to a receiver that has the capacity to store up to ninety nine hours of heart rate data. The receiver can be worn by the human user as a wristwatch or in this case it was

attached to the surcingle. This allowed the horses to be left unattended if required.

Data was continuously collected and stored in the receiver. It was later uploaded to PC based analytical software (Polar pro-trainer equine edition) using an infra-red interface. The analytical software allowed heart rate at specific times to be selected.



Figure 2.5 Horse wearing heart rate monitor. Transmitter and receiver position and position of electrodes in the region of the upper left thorax and the ventral midline are labelled.

2.2.4 Behavioural data collection.

To provide a more robust assessment of the horses response to aversive stimuli, physiological measures were used in combination with behavioural analysis. Each study involved a behavioural assessment suitable for the length and type of potentially stressful procedure being investigated. A detailed description of behavioural analysis will be provided in each relevant chapter.

The studies into behavioural and physiological response to acute aversive stimuli and behavioural and physiological response to a short term potentially aversive husbandry practice were video recorded using a hand held camera (Hitachi DVD/HD digital video camera) mounted on a tripod. The study into behavioural and physiological response to long term or repetitive stress utilised the camera system installed throughout the equestrian centre. The method used for behavioural assessment was specific to each study and will be discussed in each relevant chapter.

Analysis of data is specific to each study and will be discussed in the relevant chapter. SPSS v 15.0 for windows was used for all statistical data analysis throughout this project. The significance level for each null hypothesis was set at $p < 0.05$.

Chapter 3 Study A; Investigation into the potentially limiting factors of thermal imaging and salivary cortisol analysis as indicators of the stress response

3.1 Introduction

The insulative properties of the coat in addition to coat colour have been shown to affect temperature readings taken using IRT (McCarthy, 2007). These limitations can be overcome by thermal imaging of the eye area within the medial posterior palpebral border of the lower eyelid and the lacrimal caruncle (Figure 2.4). However, body temperature is amongst the physiological variables that exhibit circadian rhythm (Refinetti and Menaker, 1992) and a clear circadian rhythm of core body temperature has been reported in horses (Piccione *et al.*, 2002, Green *et al.*, 2005) with temperature starting its daily ascent at dawn, coinciding with the start of the light phase, and reaching a maximum fourteen hours later.

Investigation into diurnal rhythm of surface temperature measured using IRT is limited. Scott *et al.* (2000) found a distinct circadian rhythm in udder temperature when using IRT as a tool to detect early stages of mastitis however whilst using IRT to measure bovine scrotal surface temperature Kastelic *et al.* (1996) found no significant effect of diurnal rhythm on thermal output.

As findings of existing work into diurnal rhythm of surface temperature measured using IRT are limited (Morgan *et al.*, 1997, Piccione *et al.*, 2002), contradictory and carried out in species other than the horse (Kastelic *et al.*, 1996; Scott *et al.*, 2000), it is important to investigate whether the equine eye exhibits any daily pattern in temperature output in order to better interpret any thermal changes in subsequent studies during this project.

It has also been suggested that changes in ambient temperature may confound results of IRT (Eddy *et al.*, 2001; van Hoogmoed and Snyder 2002)

therefore the extent to which ambient temperature affects surface temperature of the eye needs to be explored.

Like most hormones circulating in the body, glucocorticoids are produced in a circadian manner under basal conditions (Lane, 2006). Daily rhythm in plasma cortisol concentration has been reported in horses with levels peaking between the hours of 0600h and 0900h and decreasing to their lowest between 1900h and 2300h (Irvine and Alexander, 1994; Van der Kolk, 2001). Daily rhythm in salivary cortisol has been reported in one study (Hughes *et al.*, 2006), other studies have been unable to demonstrate a diurnal rhythm (Van der Kolk *et al.*, 2001 Harewood and McGowan, 2005). In addition to these conflicting results, lack of diurnal rhythm in plasma cortisol has been linked to long term stress (Harewood *et al.*, 2005) therefore before any investigation into long term potentially aversive situations is carried out, presence or absence of diurnal rhythm in salivary cortisol needs to be investigated in the study horses. This will allow a more robust interpretation of physiological data. This study allowed the investigation of the presence of circadian rhythm in both eye and surface temperature measured using IRT in addition to salivary cortisol.

A review of the literature into the use of IRT as a measure of the stress response shows that the distance from the subject from which the thermal image is taken is standardised at between 1-1.5 metres (Nakayama *et al.*, 2005, Cook *et al.*, 2006, Stewart *et al.*, 2007). A preliminary study carried out during this project revealed that eye temperature decreased significantly ($p=0.001$) when thermal images were taken at a greater distance from the horse (Appendix 4). This highlighted the importance of a standardised distance when capturing thermal images during this project.

A second preliminary study provided an opportunity to investigate the functionality of the University thermal camera and assess whether it was able

to meet the requirements of the project in addition to providing an initial investigation into the most suitable anatomical areas to capture thermal images (Appendix 5). Preliminary results showed that eye temperature fluctuates less over time when compared to ear temperature. This is in agreement with existing work that has used eye temperature as a measure of temperature change associated with the stress response in other species (Cook *et al.*, 2001; Pavlidis *et al.*, 2002 Cook *et al.*, 2006; Stewart *et al.*, 2008T). A significant ($p=0.006$) effect of time on eye temperature was found however a limited number of samples (3 per day) were available. Therefore, a larger and more detailed investigation into the daily pattern of eye temperature was now needed, in addition to salivary cortisol measurements, in order to investigate diurnal rhythm. This would allow better interpretation of the thermal and hormonal data collected as a measure of stress.

3.2 Aim and objectives

Aim;

- Assess the impact of potentially limiting factors of thermal imaging and salivary cortisol analysis as indicators of the stress response

Objectives;

- Establish whether there is a daily rhythm in eye temperature measured using IRT and compare to ambient and core temperature in the study horses.
- Establish whether there is a daily rhythm in salivary cortisol in the study horses.

3.3 Methodology

3.3.1 Animals and husbandry

Horses (n=6) were a mix of sex, age and breed representative of horses found in a riding school. Horses were managed as previously discussed in chapter 2.0 with details of the individual animals shown in table 2.1. The horses were housed in single box stalls and remained there over four consecutive days. This was to standardise feeding and exercise. Horses were placed on a horse walker (Monarch equestrian) for one hour between 0900h and 1000h after physiological samples had been taken and for a further hour at 1500h. Horses had access to water at all times and were fed 5kg hay twice daily.

The study took place over four consecutive days during spring (April). Sunrise was at approximately 0630h and sunset at approximately 2000h

3.3.2 Infrared thermography data collection

The Mobir GuideIR thermal camera was used to capture a static thermal image of the head (left and right lateral aspect) every three hours for each horse, starting at 1500h on a Monday and continuing for 72 hours until 1500h on the Thursday. Images were processed and eye temperatures extracted as previously discussed in chapter 2.2

Temperatures were plotted over time using Excel to descriptively analyse any daily pattern in thermal output. Initially this was done for each individual horse and then a mean eye temperature for all horses for each sampling time point was also plotted against time.

3.3.3 Infrared thermography data analysis

Distribution of data varied significantly from normal (Kolmogorov-Smirnov Test, $p=0.001$). A Friedman test was conducted to investigate any difference in eye temperature between each day and a second Friedman test was carried out to investigate any difference in eye temperature between each sampling time point. A Wilcoxon signed rank test was used to investigate between which time points there was a significant difference in eye temperature

3.3.4 Salivary cortisol data collection

Saliva was sampled every three hours for cortisol analysis for each horse, starting at 1500h on a Monday and continuing for 72 hours until 1500h on the Thursday. Saliva was collected, analysed and processed using the method previously discussed in chapter 2.2. saliva was sampled immediately after each thermal image was captured.

Salivary cortisol for each time point was plotted against time using Excel to investigate any daily pattern. Initially this was done for each individual horse and then a mean salivary cortisol for all horses for each sampling time point was also plotted against time.

3.3.5 Salivary cortisol data analysis

Distribution of data varied significantly from normal (Kolmogorov-Smirnov Test $p=0.02$). A Friedman test was conducted to investigate any difference in salivary cortisol between each day and a second Friedman test was carried out to investigate any difference in salivary cortisol between each sampling time point.

3.3.6 Core temperature data collection

Core temperature was measured every three hours for each horse starting at 1500h on a Monday and continuing for 72 hours until 1500h on the Thursday. Temperature was measured using the method previously discussed in chapter 2.0. Temperatures were recorded by hand onto the horse's data sheet and transferred to an Excel spreadsheet at a later date.

Core temperature for each sampling time point was plotted against time. This allowed any daily pattern and any relationship between eye temperature and ambient temperature to be investigated. Core temperature was initially plotted for each individual horse and then a mean core temperature for all horses for each sampling time point was calculated

3.3.7 Core temperature data analysis

Distribution of data varied significantly from normal (Kolmogorow-Smirnov Test). A Friedman test was conducted to examine the effect of time on core temperature. Separate tests were carried out to investigate any difference in core temperature between each day and also any difference in core temperature between each sampling time point. A Wilcoxon signed rank test was carried out to investigate between which time points there was a significant difference in core temperature

3.3.8 Ambient temperature and photoperiod data collection

Ambient temperature was monitored for the duration of the study using a temperature logger (Lascar EL-USB-2). The temperature logger was set to record at three hourly intervals that corresponded with the physiological sampling times. Data were uploaded and processed using the methods previously discussed in chapter 2.2. The photoperiod (hours of daylight) for the days the study was carried out was obtained from the British Meteorological Office website (www.metoffice.gov.uk) in addition to sunrise

and sunset timings. Ambient temperature for each sampling time point was entered into an Excel spreadsheet and plotted with eye and core temperature against time. This allowed any daily pattern and any relationship between the parameters to be investigated. Hours of daylight and darkness were also indicated on the charts generated by Excel to investigate any relationship between the physiological parameters and photoperiod.

3.3.9 Correlation analysis

A Spearman rank order correlation was used to investigate any relationship between

1. Mean eye temperature of all horses and ambient temperature over the duration of the study.
2. Mean eye temperature of all horses and mean core temperature of all horses over the duration of the study
3. Mean core temperature of all horses and ambient temperature over the duration of the study.

3.4 Results

3.4.1 Infrared thermography

The results of a Friedman test indicated that there was no significant difference in eye temperature between day 1, 2 and 3, $X^2 (2, n = 6) = 4.0$, $p = 0.135$. A second Friedman test indicated a significant difference in eye temperature between sampling times $X^2 (7, n = 6) = 19.485$, $p = 0.007$. A Wilcoxon signed rank test revealed that this difference was between 1800h and 0900h $z = -2.214$, $p = 0.02$, with a large effect size ($r = 0.63$). The median temperature decreased from 33.2°C to 32.2°C so eye temperature was higher in the evening when compared to the morning.

Figure 3.1(a) displays mean eye temperature for all horses throughout the duration of the study (with ambient temperature) and Figure 3.1 (b) displays eye temperature of one representative study horse (with ambient temperature). Eye temperature ranged from 28.3°C to 37.5°C in all study horses. Table 3.1 displays maximum and minimum eye temperature for each horse throughout the duration of the study. The thermal images from the 1500h sample on the second day of the study showed that all horses had very low eye temperatures compared to the rest of the week. This has not been included in the minimum and maximum data as it is not representative of temperatures during the rest of the project.

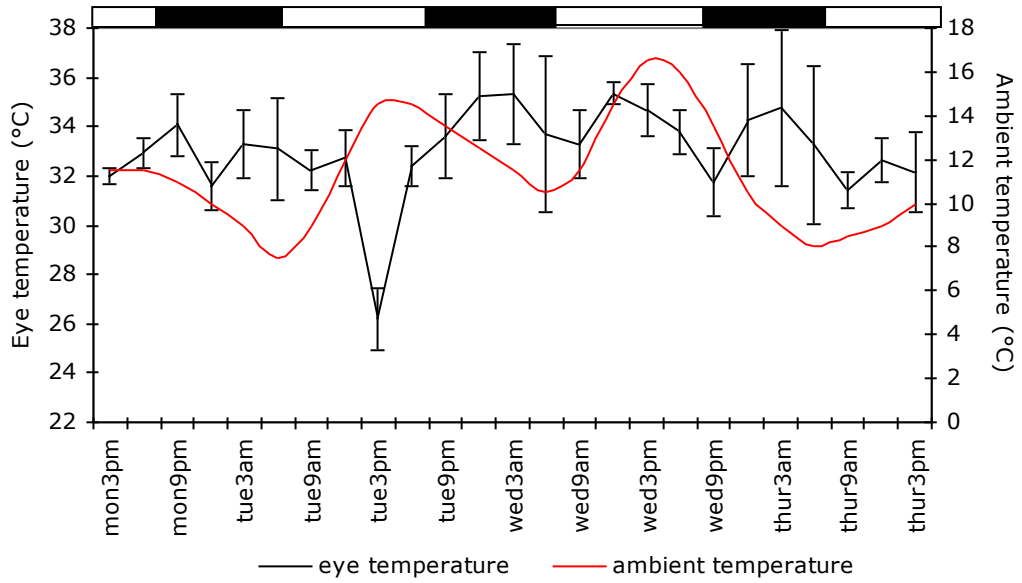


Figure 3.1(a) Mean (\pm SD) eye temperature for all study horses and ambient temperature over the duration of the study. The black and white bars show hours of daylight (white) and hours of darkness (black).

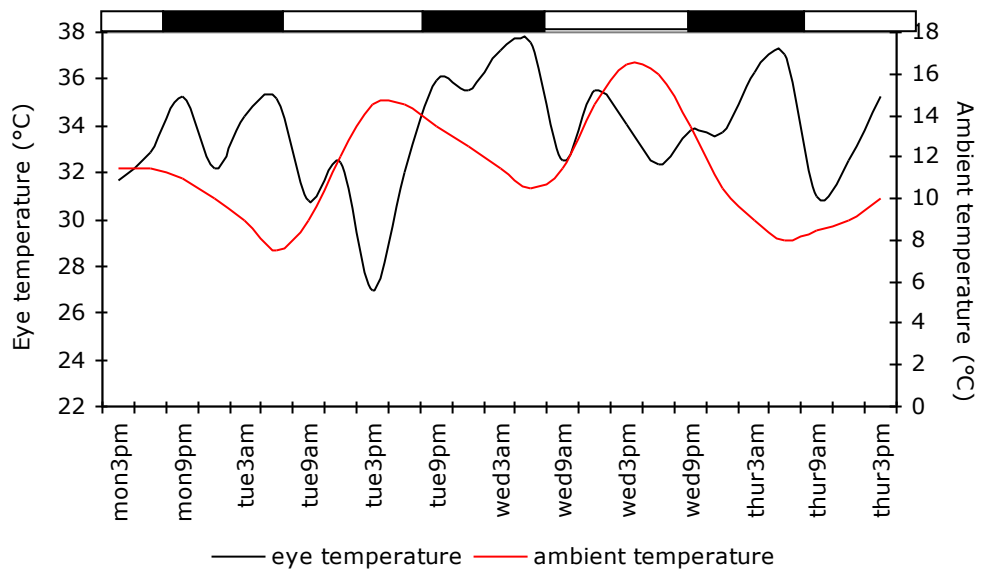


Figure 3.1(b) Eye temperature for one representative horse and ambient temperature over the duration of the study. The black and white bars show hours of daylight (white) and hours of darkness (black).

Table 3.1 Maximum and minimum eye temperatures (°C) recorded for each horse throughout the duration of the study.

Horse	Maximum eye temperature (°C)	Minimum eye temperature (°C)	Range (°C)
Angus	36.8	28.3	8.5
Beau	35.7	31.1	4.6
Conan	37.2	30	7.2
Desmond	37.5	31.4	6.1
Tosca	36.4	31	5.4
Kitkat	37.6	30.8	6.8

3.4.2 Core temperature

The results of a Friedman test indicated that there was no significant difference in core temperature between day 1, 2 and 3, $X^2 (2, n = 6) = 1.238$, $p = 0.538$. A second Friedman test indicated a significant difference in core temperature between sampling times $X^2 (7, n = 6) = 30.722$, $p = 0.000$. A Wilcoxon signed rank test was conducted to investigate between which time points the significant difference in mean core temperature occurred (Table 3.2).

Core temperature followed a similar trend to ambient temperature in all horses. Figure 3.2 shows core and ambient temperature for one representative horse throughout the duration of the study. There was a trend for core temperature to reach maximum levels at 1800h before descending to a minimum at 0600h. Core temperature ranged from 35.4°C to 37.9°C in all study horses. Table 3.3 displays maximum and minimum core temperature for each horse throughout the duration of the study.

Table 3.2 Results of the Wilcoxon signed rank test used to investigate any difference in mean core temperature between sampling time points. P values are given between each sampling time point. P values marked in **bold** indicate a significant difference between times.

	1500h	1800h	2100h	0000h	0300h	0600h	0900h	1200h
1500h		0.027	0.336	0.104	0.104	0.026	0.071	0.705
1800h	0.027		0.023	0.026	0.026	0.027	0.026	0.039
2100h	0.336	0.023		0.027	0.024	0.027	0.024	0.357
0000h	0.104	0.026	0.027		0.180	0.026	0.891	0.167
0300h	0.104	0.026	0.024	0.180		0.039	0.461	0.140
0600h	0.026	0.027	0.027	0.026	0.039		0.068	0.042
0900h	0.071	0.026	0.024	0.891	0.461	0.068		0.167
1200h	0.705	0.039	0.357	0.167	0.140	0.042	0.167	

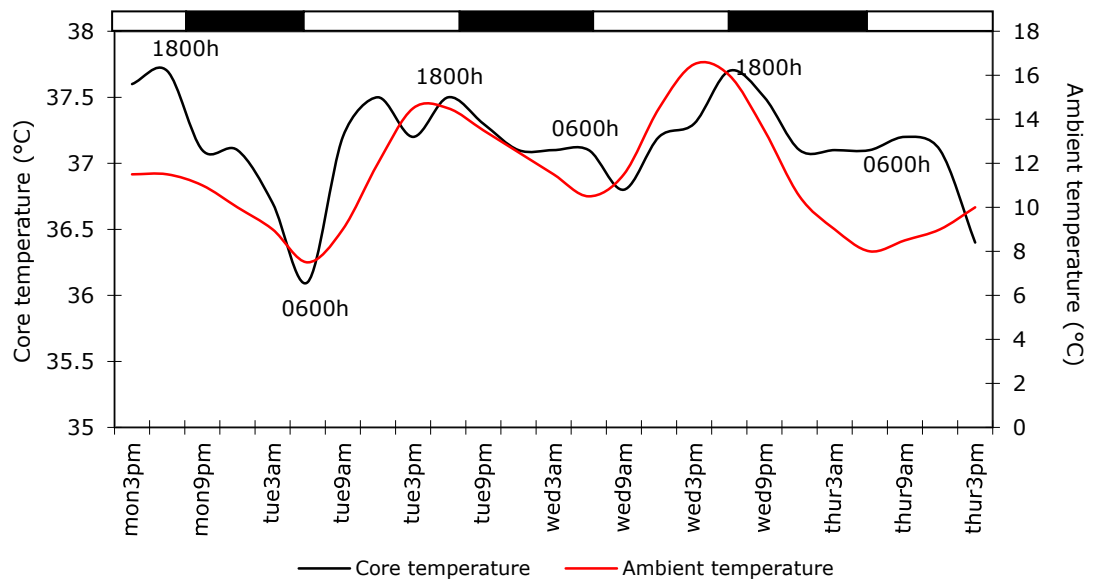


Figure 3.2 Core temperature for one representative horse and ambient temperature over the duration of the study. The black and white bars show hours of daylight (white) and hours of darkness (black).

Table 3.3 Minimum and maximum core temperatures (°C) recorded for each horse throughout the duration of the study with times that temperature occurred in ().

Horse	Maximum core temperature (°C)	Minimum core temperature (°C)	Range (°C)
1	37.7 (1800h)	36.7 (1500h)	1
2	37.9 (0900h)	36.1 (0600h)	1.8
3	37.9 (1800h)	37 (1200h)	0.9
4	37.4 (1800h)	35.4 (0300h)	2
5	37.8 (1800h)	36.7 (0600h)	1
6	37.7 (1800h)	36.1 (0300h)	1.6

3.4.3 Salivary cortisol

The results of a Friedman test indicated that there was no significant difference in salivary cortisol between day 1, 2 and 3, $X^2 (2, n = 6) = 3.739$, $p = 0.154$. A second Friedman test indicated there was no significant difference in salivary cortisol between sampling time points $X^2 (7, n = 6) = 5.796$, $p = 0.564$.

Figure 3.3(a) displays mean salivary cortisol of all horses throughout the duration of the study and Figure 3.3(b) displays salivary cortisol of one representative horse throughout the duration of the study.

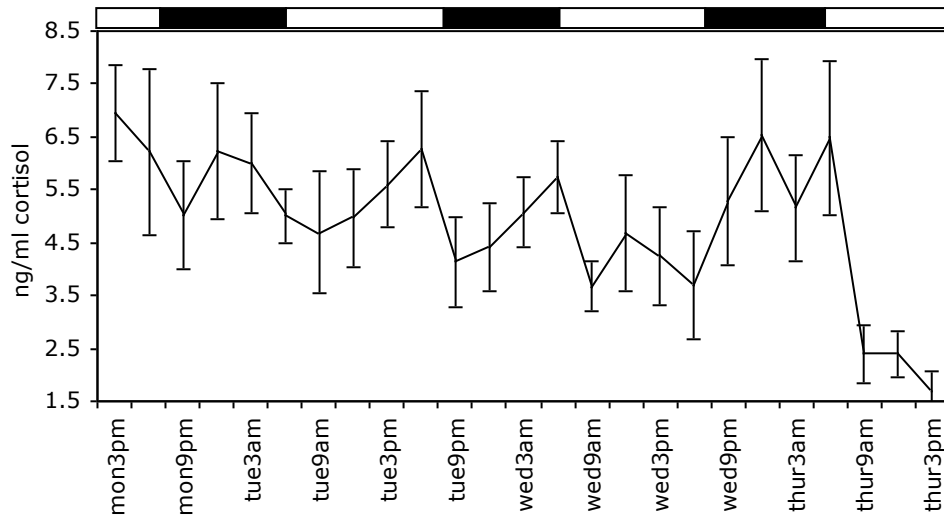


Figure 3.3(a) Mean (\pm SD) salivary cortisol for all horses over the duration of the study. The black and white bars show hours of daylight (white) and hours of darkness (black).

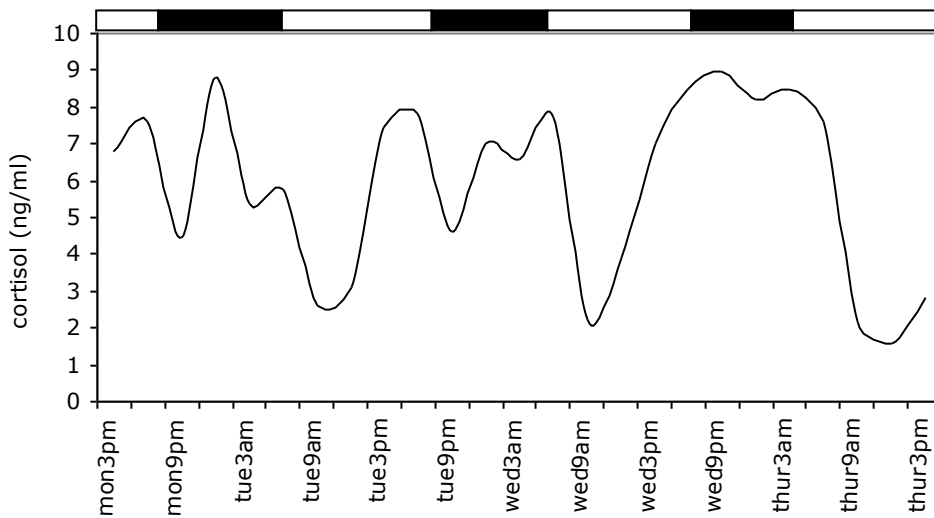


Figure 3.3(b) salivary cortisol for one representative horse over the duration of the study. The black and white bars show hours of daylight (white) and hours of darkness (black).

Mean salivary cortisol ranged from 1.7ng/ml to 7ng/ml throughout the week. Table 3.4 details the minimum and maximum cortisol levels (ng/ml) for each of the horses involved.

Table 3.4 Minimum and maximum salivary cortisol levels (ng/ml) recorded for each horse throughout the duration of the study with times recorded in ().

Horse	Maximum salivary cortisol (ng/ml)	Minimum salivary cortisol (ng/ml)	Range (ng/ml)
1	9.1 (1500h)	1.0 (0900h)	8.1
2	9.7 (0600h)	1.2 (1500h)	8.5
3	9.1 (1500h)	1.1 (1500h)	8
4	12.6 (1800h)	1.0 (1500h)	11.6
5	9.0 (2100h)	1.6 (1200h)	7.4
6	10.3 (0900h)	1.2 (1500h)	9.1

3.4.4 Correlation

A Spearman rank order correlation test revealed that there was no relationship between mean eye temperature and ambient temperature ($r=0.21$, $n=25$, $p=0.35$).

A Spearman rank order correlation test also revealed there to be no relationship between mean eye temperature and mean core temperature ($r=0.235$, $n=25$, $p=0.259$).

There was a positive correlation between mean core temperature and ambient temperature ($r=0.543$, $n=25$, $p=0.005$); as ambient temperature increased, core temperature also increased (Figure 3.4)

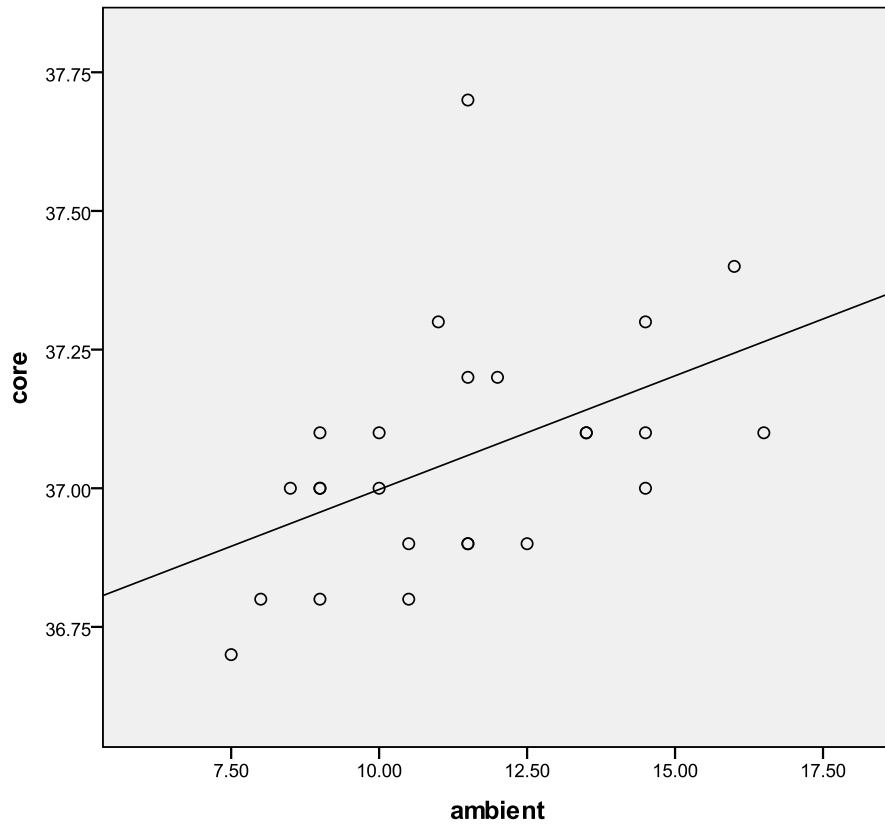


Figure 3.4 Positive correlation between mean core temperature ($^{\circ}\text{C}$) of all horses and ambient temperature over the duration of the study.

3.5 Discussion

The primary aim of this study was to investigate potential factors that may affect reliability of eye temperature measured using IRT and salivary cortisol analysis.

The results of the first preliminary study into the effect of distance on accuracy of temperature measurement using IRT indicate the importance of standardising distance when capturing thermal images. A finding of the second preliminary study suggests that sudden potentially aversive stimuli may result in warming of the eye area.

There was no significant effect of day on eye temperature however there was a significant ($p= 0.007$) effect of time on eye temperature between two of the sampling times (1800h and 0900h). Mean eye temperature decreased by 1°C and may be a reflection of the drop in ambient temperature between the two times. The average ambient temperature at 1800h was 14°C and the average temperature at 0900h was 9.5°C. Eye temperature ranged from 28.3°C to 37.5°C however at the fifth sampling point (1500h Tuesday) eye temperature decreased in all horses below this range. There was no observable association between ambient temperature change and eye temperature change at this point or throughout the duration of the study. No correlation between mean eye temperature and ambient temperature was found. In addition to this, at the time point that a decrease in eye temperature was recorded, ambient temperature was in fact increasing. The cause of the decrease in eye temperature in all horses is unclear but may be due to camera malfunction. It is possible that it was a result of a compensatory thermoregulatory mechanism in order to maintain thermal homeostasis due to the increase in ambient temperature, however no other decreases of this magnitude were recorded at any other point during the study and at no other point did an increase in ambient temperature result in a decrease in eye temperature. Core temperature also remained within normal range for all horses at this

time point and there was no correlation between mean eye temperature and mean core temperature.

There was a significant ($p= 0.000$) effect of time on core temperature. Core temperature started its daily ascent at dawn (0600h), coinciding with the start of the light phase, and reached a maximum twelve hours later (1800h) in all study horses. This association of core temperature with the start and end of light phase is in agreement with existing work for the horse (Piccione *et al.*, 2002, Green *et al.*, 2005) and other species (Brown and Dawson, 1976 in kangaroos; Bligh and Harthoorn, 1965 in African ruminants) and could possibly be a reflection of the storage of heat gained throughout the warmer daylight hours. Core temperature did follow the same daily pattern as ambient temperature, with warmer temperatures recorded during daylight hours and there was a positive correlation between core and ambient temperature. As ambient temperature increased, core temperature also increased. The range in core temperature from minimum to maximum was minimal for each horse (0.9-1.8°C). This is to be expected due to the thermoregulatory mechanisms in place to maintain homeostasis of internal temperature (Cymbaluk and Christison, 1990). As there was no correlation between eye and ambient temperature this suggests eye temperature may be a better stress index than core temperature.

There was no effect of day or time on salivary cortisol. This could have been due to a number of reasons. Seasonal changes have been reported in plasma cortisol levels, with higher levels and a more pronounced daily rhythm present during the autumn months (Donaldson *et al.*, 2005). The circadian system provides animals with the ability to anticipate periods of activity and to time their behaviour and physiology in ways that will optimise survival (Murphy, 2009), therefore the increased cortisol levels reported during autumn could be

related to anticipation of winter and the associated challenge of decreased forage (Donaldson *et al.*, 2005). This study was carried out during the spring therefore it is possible that daily rhythm in salivary cortisol levels was less pronounced.

No investigation into seasonal effects on salivary cortisol was carried out during this project. The purpose of the project was to validate IRT as a non invasive measure of stress. No study spanned more than one season and no study was carried out during the autumn season therefore such an investigation was beyond the remit of the work.

Other non photic cues that could potentially disrupt daily rhythm of salivary cortisol include exercise (Alexander and Irvine, 1991) and changes in diet (Donaldson *et al.*, 2005). It is unlikely that exercise disrupted daily rhythm in this study as maximal or prolonged exercise is required in order to increase cortisol (Alexander and Irvine, 1991) and the horses were not subjected to this. Horses were fed a diet of hay and were conditioned to this diet prior to the study commencing therefore it is also unlikely that changes in diet disrupted daily rhythm.

Irvine and Alexander (1993) suggest that as light is the primary environmental time cue serving to entrain circadian changes (Pittendrigh and Minis, 1964) it is possible that lack of circadian rhythm in plasma cortisol during their study was attributable to the dim night lighting in the area where the horses were housed. The horses in this study were stabled under artificial light that was present after daylight hours resulting in an artificially lengthened photoperiod. In addition lights were turned on during the overnight sampling which potentially could have further confounded results. Despite this, core temperature displayed a clear daily rhythm but it could be that this was associated more with ambient temperature change rather than

photic cues. It is also possible that the disruption to the horses during the overnight samples may have interfered with sleep patterns, which have been shown to alter cortisol secretion in humans (Weitzman *et al.*, 1983).

Chronic stress has been shown to disrupt daily rhythm in physiological variables and alter behavioural routine, making any daily pattern unclear. Irvine and Alexander (1998) report that moving horses from an open social environment into an enclosed housing design resulted in disruption of daily rhythm in plasma cortisol. Horses involved in this investigation had constant turnout in grass paddocks with their familiar companions prior to the study commencing however in order to standardise food intake and exercise they were placed in isolated stables with no turnout and no contact with conspecifics for a four day period. This situation could potentially have been stressful to the horses and resulted in altered daily rhythm of salivary cortisol. Despite absence of a clear daily rhythm, cortisol levels did fluctuate throughout the course of the day, highlighting the importance of baseline or pre stressor measures in order for each horse to act as its own control during the course of the project.

Finally existing work that has reported a daily pattern in cortisol measured the hormone in blood plasma (Irvine and Alexander, 1994; Lane, 2006). In the blood 75% of cortisol is bound to proteins (Roshier, 1991) and biologically inactive in the body. The bound form of the hormone is unable to cross into saliva due to the large size of the protein molecule. As cortisol was measured in saliva during this study and was therefore free and biologically active, it may be that the unbound form of the hormone lacks daily rhythm. This supports the findings of Van der Kolk *et al.* (2001) who reported diurnal rhythm of plasma cortisol but did not find there to be a demonstrable diurnal rhythm of salivary cortisol.

3.6 Conclusion

The results of this study indicate that the unbound fraction of cortisol present in saliva does not demonstrate the same daily rhythm as plasma cortisol. Alternatively it may be that it is potentially difficult to identify daily rhythm of salivary cortisol in a working yard environment due to potential disturbance from artificial lighting and changes in routine related to necessary experimental control of diet, exercise and housing. Eye temperature did fluctuate between each sampling time point although the only significant ($p=0.007$) effect of time on eye temperature was between two sample points. This may have been due the decrease in ambient temperature between the evening and early morning measure. Despite no significant effect of day and time on salivary cortisol, levels also fluctuated throughout the study period. This makes it important to sample salivary cortisol and eye temperature pre stressor in each horse to establish baseline levels and compare the changes in both physiological parameters during potentially stressful events to the baseline values recorded.

In conclusion, although there was a significant difference in eye temperature between two of the sample times no other significant differences were found and no clear daily rhythm in eye temperature or salivary cortisol was observed. It is still important to record pre stress measures of both parameters and repetitively sample at regular intervals as fluctuations (increase and decrease) between times were seen. Now that the potential limiting factors of distance, ambient temperature and time of day have been investigated and are better understood it is now possible to investigate the temperature response of the eye to an aversive situation and compare this to the currently accepted stress measure of salivary cortisol.

Chapter 4 Study B; Investigation into the use of IRT as a tool to assess thermal change associated with the physiological response to acute stress

4.1 Introduction

Everyday challenges for wild or free ranging horses are primarily acute (social disputes, predator avoidance) and the innate species specific response is to flee. Domesticated horses will also display flight behaviour during acutely aversive circumstances which is indicative that the horse finds the situation stressful. Previous work in other species has investigated and reported changes in eye temperature that correlate with elevated cortisol in response to acute and short term aversive stimuli (Cook *et al.*, 2006, Schaefer *et al.*, 2006, Stewart *et al.*, 2008). As work in other species has primarily examined the thermal response of the eye to acute aversive stimuli using IRT and found changes in temperature, it was logical to begin by investigating whether the response was mirrored in the horse. Although domestication has brought with it new challenges of longer duration for horses it was important to first establish whether an increase in eye temperature in response to acute aversive stimuli was observable as this had already been established in other species. If eye temperature change in response to acute stress was observed then further investigation into eye temperature response to longer term stress during situations found in domestication could be carried out.

The purpose of applying the startle response test during this study was not to assess response to the specific stressor (umbrella) but rather to elicit an stress response in order to measure the associated physiological changes in an attempt to replicate findings of existing work. A startle test was appropriate to assess the response of a prey species to acute aversive stimuli as from an evolutionary point of view suddenness, unfamiliarity and unpredictability are the key features of predator attack (Shelton and Wade, 1979). It is reasonable to suggest that there will be some degree of

behavioural reaction during a startle response test as the horse's species-specific reaction is to flee when faced with a challenging situation. The use of visual stimuli is particularly relevant to the horse as in its natural habitat it is particularly sensitive to movement and relies on vision as a major sensory avenue for predator detection (Christensen *et al.*, 2008). The behavioural response of flight in response to the startle test would indicate that the horses found the situation stressful and support the suggestion that any changes in eye temperature and cortisol were stress related.

A preliminary study carried out during this project suggested that sudden potentially aversive stimuli may result in warming of the eye area (Appendix 5). If it was possible to reproduce the results obtained in other species and capture a thermal change of the eye in the horse in response to an aversive stimulus, then use of IRT as an indicator of stress could be validated using currently accepted behavioural and physiological measures.

4.2 Aim and objectives;

Aim

- Establish whether changes in surface temperature in response to an acutely stressful situation occurs in horses as it does in other species,

Objectives

- Investigate the thermal response of the horse to an acute aversive stimulus.
- Compare any thermal response with the currently accepted indicators of stress (salivary cortisol and behavioural assessment).

4.3 Methodology

4.3.1 Animals and husbandry

Horses (n=10) included mares and geldings were supplied by Brackenhurst Equestrian Centre and were managed as previously discussed in chapter 2.0. Details of the individual horses can be found in table 2.1.

4.3.2 Test area

The test area was a covered barn measuring 10 metres long by 9 metres wide. The end of the yard facing the horse during the test procedure was totally enclosed by a stone wall to roof height and the sides were enclosed with stone walls measuring 1.5 metres high. Over one side of the wall a row of traditional box stables with half doors ran adjacent to the barn. The test horse was able to see horses housed in these stables. Over the opposite wall was a grass area with fields beyond. The end of the yard to the rear of the horse was a 2 metre wire fence and another sand covered area lay beyond which was empty for the duration of the testing. Horses were often housed in the barn and also took part in horse husbandry lessons in the same area therefore all were familiar with the area and were unlikely to associate it with aversive procedures or situations.

The horses were held by the same familiar handler during the testing to ensure handling consistency. They were allowed to express natural behaviour and move away from the aversive stimulus with no restrictions. A point was marked in the sand 1 metre from the side wall and five metres from the end wall to indicate where the horse was to stand. Another point was marked in the sand 2 metres back from this to indicate where the novel object was to be presented. See Figure 4.1 for a plan of the test area.

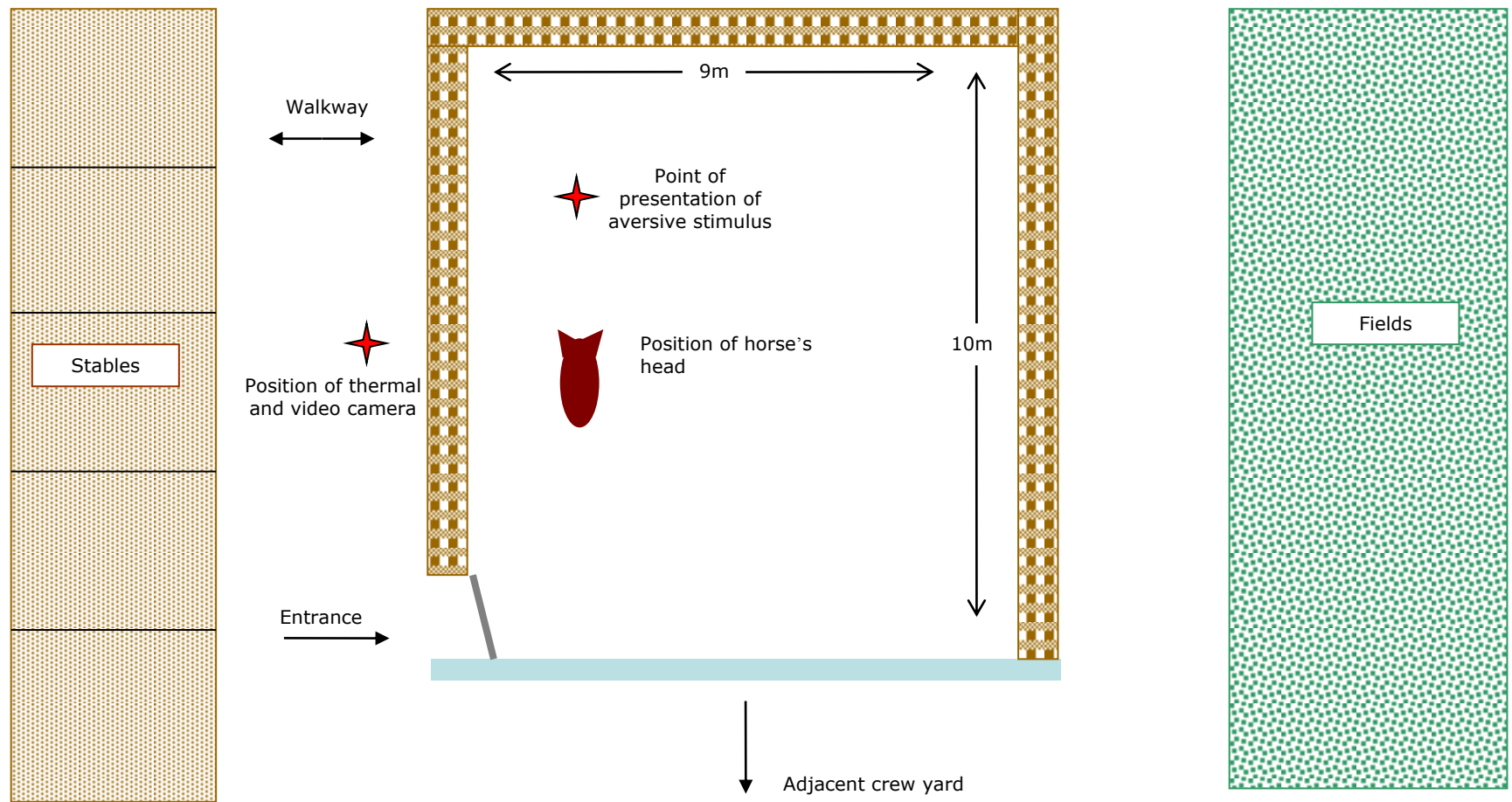


Figure 4.1 Plan of the test area with position of test horse, thermal and video cameras and point of presentation of aversive stimulus. Figure not to scale.

4.3.3 Test procedure

The test began with the horse in the familiar environment of its stable. A static thermal image was captured of the left and right eye, saliva was sampled for cortisol analysis and a heart rate monitor (Polar Equine RS800G3) was attached as described in chapter 2. Recording of heart rate began immediately. After five minutes in the stable a second static thermal image and saliva sample was taken and the horse walked the short distance to the barn.

Horses involved in the study were assessed individually and were not able to see the procedure being carried out on any of the other horses. Each horse was allowed five minutes to reach the crew yard and acclimatise to the new environment. Once in the crew yard a thermal recording (FLIR ThermoVision A40M thermal camera) of the left eye began five minutes pre presentation of aversive stimulus and lasted until five minutes post presentation of aversive stimulus.

The aversive stimulus used was a yellow and blue umbrella and was chosen as it is an unfamiliar object to a horse and offers sudden motion and noise. In addition to this yellow and blue were amongst the colours that caused the greatest number of adverse reactions in horses (n=16) when encountered for the first time in the form of ground mats (Hall and Cassaday, 2005). The umbrella was opened directly in front of the horse by the same person and then held out and rotated at waist level for ten seconds. The umbrella did not come into contact with the horse however the horse was free to approach and investigate it. The handler did not pull, touch or talk to the horse and if the horse wished to move away from the umbrella it was able to. Subsequent saliva samples and static thermal images were taken every five minutes from five minutes post presentation of aversive stimulus up to thirty minutes post presentation of aversive stimulus. Each individual test was videotaped for

behavioural analysis post procedure. When all samples had been taken the horse was returned to its stable.

4.4 Data collection and analysis

Data record sheets were produced for each horse and used to hand log when physiological measures had been taken. This ensured all necessary samples were collected for each horse. Data were then transferred from the record sheets into Excel spreadsheets at a later date.

4.4.1 Infrared thermography data collection

Both continuous and static thermal images were captured and temperature extracted as previously discussed in chapter 2.0. Thermal response of the left eye was continuously monitored from five minutes pre presentation of aversive stimulus until five minutes post presentation of aversive stimulus. The thermal recording camera was supplied and operated by a PhD student from the School of Computing and Informatics at Nottingham Trent University who also extracted and supplied the temperature data. Details of the camera can be found in chapter 2.2.2. Static thermal images were captured every five minutes beginning five minutes pre presentation of aversive stimulus until thirty minutes post presentation of aversive stimulus.

4.4.2 Infrared thermography data analysis

Data from only one horse was available from the continuous video thermal recording. The sudden flight response of the horse's upon presentation of the stimulus caused them to move out of the screen shot of the thermal camera therefore a thermal image could not be captured. One horse appeared less behaviourally responsive and did not step out of the range of the camera. A thermal profile for this horse was plotted against time using Excel software to investigate any temperature fluctuations (Figure 4.2). Once temperatures had

been extracted and processed from the static images as previously discussed, mean eye temperature of left and right eye for each horse for each time point was calculated. Distribution of data was normal (Kolmogorov-Smirnov Test, $p=0.2$) therefore a one way repeated measures ANOVA was used to investigate effect of time on mean eye temperature of all horses.

4.4.3 Core temperature data collection

The acute aversive nature of the study and the anticipated flight response of the horses prohibited core temperature being taken at the times it was most relevant. For the purpose of handler safety core temperature was not taken during this study.

4.4.4 Ambient temperature data collection

Ambient temperature was monitored in the barn for the duration of the study using a wet bulb thermometer. Temperature was recorded on a data sheet at five minute intervals beginning five minutes pre presentation of aversive stimulus until thirty minutes post presentation of aversive stimulus.

4.4.5 Salivary cortisol data collection

Saliva was sampled every five minutes, beginning five minutes pre presentation of aversive stimulus, until thirty minutes post presentation of aversive stimulus. Saliva was collected, analysed and processed as discussed in chapter 2.2.1

4.4.6 Salivary cortisol data analysis

Equipment malfunction resulted in only five complete and one partial (data up to 15 minutes post stressor) hormonal profiles being assayed. Salivary cortisol for each horse for each time point was calculated as previously discussed in chapter 2.2.2. This was carried out for the five complete

hormonal profiles and plotted against time using Excel software to investigate any fluctuations in cortisol over the study period. Distribution of data varied significantly from normal (Kolmogorov-Smirnov Test, $p=0.04$) therefore a Friedman Test was conducted to examine the effect of time on cortisol levels and the time point with the greatest effect on cortisol was also investigated (Wilcoxon Signed Rank Test).

4.4.7 Heart rate data collection

Heart rate was logged every twenty seconds from the time the heart rate monitor was attached to the horse in its stable pre procedure until five minutes post presentation of the aversive stimulus. Data were recorded, stored and uploaded to analysis software as discussed in chapter 2.2.3

4.4.8 Heart rate data analysis

Mean heart rates for all horses were plotted against time to investigate any fluctuations during the study period. Distribution of data varied significantly from normal (Kolmogorov-Smirnov Test, $p=0.001$) therefore a Friedman Test was used to investigate effect of time on heart rate.

For comparison to salivary cortisol and behavioural reactivity the magnitude of increase in heart rate (bpm) was calculated from the pre stress measures to maximum value (bpm) post stressor for each horse.

4.4.9 Behavioural assessment data collection

Behaviour was recorded using a hand held video camera mounted on a tripod placed adjacent to the thermal camera (Figure 4.1). Recording the study allowed objective behavioural analysis to be carried out post data collection. Footage was uploaded to a PC using a USB interface and played back for assessment (Windows media player for Windows Xp) at a later date. The reactivity of each horse was ranked to assess the degree of behavioural

response to the acute aversive stimulus. Scores were first attributed to a behavioural definition according to degree of reactivity (Table 4.1.). Fourteen BSc Equine Science undergraduate students were separately shown the video footage of each horse at the point of presentation of the aversive stimulus and a score awarded. A mean score from the fourteen respondents was calculated for each horse to allow degree of reactivity to be compared to any change in salivary cortisol and thermal output.

Table 4.1 Reactivity score with scores ranging from 1 (no reaction) to 5 (large reaction). (Adapted from Anderson et al., 1999 and Gorecka et al., 2007).

<u>Score</u>	<u>Behavioural definition</u>
1	No reaction to novel object
2	Minimal reaction, no foot movement, eyes widening, ear movement, head elevated/flinch and looking at object
3	Foot movement 1-4 steps away from object and any of the reactions in #2
4	Foot movement 5-10 steps or small jump where more than one foot leaves the ground at the same time and any of the reactions in #2
5	Any jump where more than one foot leaves the ground or more than 10 steps/rearing and any of the reactions in #2

4.4.10 Correlation analysis

The relationship between behavioural reactivity score of each horse and increase in heart rate (bpm) from baseline rate to maximum rate post presentation of aversive stimuli was investigated (Spearman's Rank Order Correlation). In addition, the relationship between behavioural reactivity score and increase in salivary cortisol from baseline to maximum value (ng/ml) for each horse was investigated (Spearman's Rank Order Correlation).

4.5 Results

4.5.1 Infrared Thermography

The one available continuous thermal recording revealed eye temperature immediately decreased by 1.8°C from 34.4°C to 32.6°C within one second post presentation of aversive stimulus and then increased by 1.1°C to 33.7°C (Figure 4.2). The behavioural response of this one horse was atypical as it displayed limited evasive movement when compared with the other study horses. Eye temperature returned to basal level within four seconds post presentation of the aversive stimulus and remained at basal level for the duration of the recording.

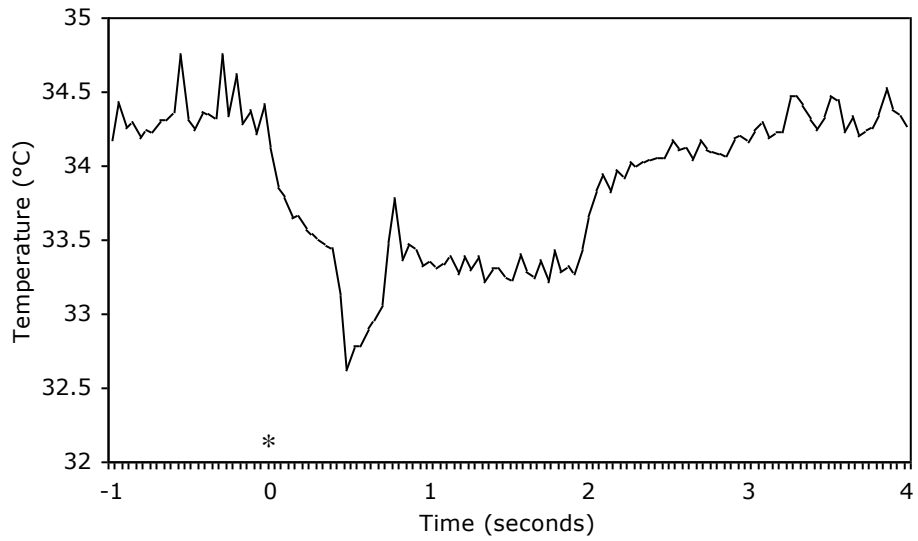


Figure 4.2 Change in eye temperature for one horse over four second recording period. Marker () shows point of presentation of aversive stimulus. Frame speed was 23 frames per second.*

The results of the one way repeated measures ANOVA revealed no significant effect of time on eye temperature over the ten sampling points for the static thermal images (Wilks' Lambda = 0.01, $F(9,1) = 9.5$, $p = 0.247$). The means and standard deviations are presented in Table 4.2.

Table 4.2 Descriptive statistics for eye temperature across the ten sampling time points for ten horses

Time	Mean Temperature (°C)	Standard Deviation
1	35.8	1.89
2	35.2	1.69
3	35.5	1.04
4	35	0.96
5	34.9	0.93
6	34.8	1.08
7	34.8	1.05
8	34.8	1.15
9	34.3	1.36
10	34.4	1.51

4.5.2 Salivary Cortisol

The results of the Friedman Test indicated that there was a statistically significant effect of time on salivary cortisol across the nine sampling time points, $X^2(8, n=5) = 15.907, p=0.04$. Pre and post stressor cortisol levels for each horse are presented in Table 4.3. A Wilcoxon Signed Rank test revealed that the greatest effect of time on salivary cortisol levels was between five and ten minutes post presentation of the aversive stimulus although this was not significant ($p= 0.138$). Figure 4.3 shows the mean salivary cortisol from the five available data sets for each sampling time point. See Appendix 6 for individual data which also details the large difference in basal levels and magnitude of increase in salivary cortisol.

Table 4.3 Salivary cortisol levels (ng/ml) pre stressor, maximum salivary cortisol post stressor (magnitude of increase) and time taken to reach maximum value for the five available hormonal profiles. Pre stressor salivary cortisol was calculated by taking the mean of the two pre stressor samples.

Horse	Pre stressor salivary cortisol (ng/ml)	Maximum post stressor salivary cortisol (ng/ml)	Time to maximum post stressor salivary cortisol (mins)
1	5	8 (3)	15
2	12.1	21 (8.9)	20
3	2	14.3 (12.3)	15
4	11.9	81.5 (69.6)	30
5	4.5	17.1 (12.6)	20

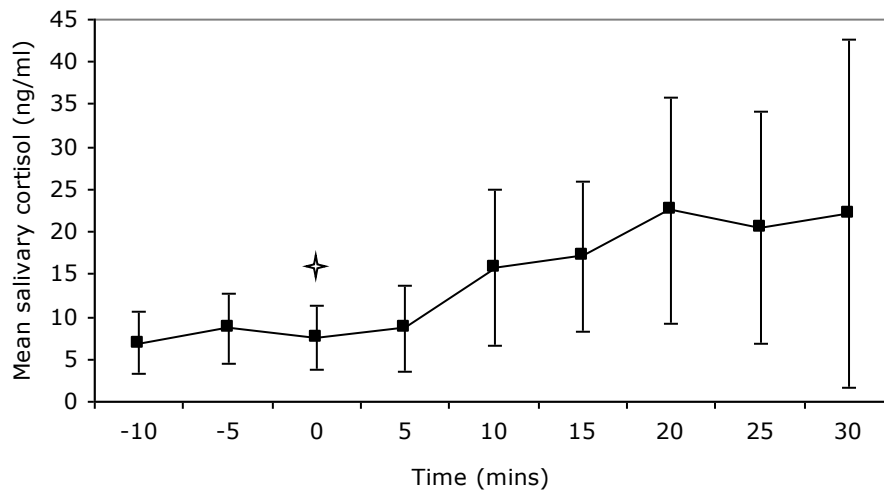


Figure 4.3 Mean salivary cortisol (ng/ml)(\pm SD) of five available hormonal data sets for each sampling time point. Marker (⚡) indicates point of presentation of aversive stimulus.

4.5.3 Heart Rate

The results of the Friedman Test indicate that there was a statistically significant effect of time on heart rate $X^2(9, n=31) = 237.9$ ($p < .0001$). Mean heart rate of all horses was plotted against time. Figure 4.4 clearly shows the increase in heart rate (bpm) occurs immediately post presentation of the aversive stimulus. Appendix 6 details the individual heart rate profiles for each horse and highlights the variation in baseline measures.

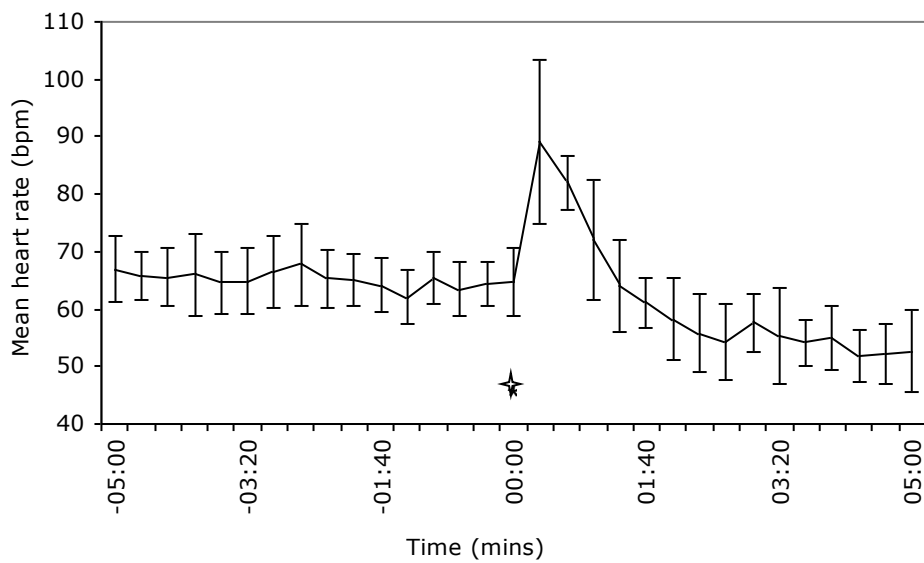


Figure 4.4 Mean heart rate (bpm)(\pm SD) of all horses recorded every twenty seconds from five minutes pre presentation of aversive stimulus to five minutes post presentation of aversive stimulus. Marker (\star) indicates point of presentation of aversive stimulus.

4.5.4 Behavioural response

A mean reactivity score was calculated for each horse from the fourteen scores assigned by Equine BSc undergraduate students. Table 4.4 details the descriptive statistics for the behavioural scores attributed to each horse. Horse number one is the animal that displayed minimal flight behaviour and a continuous thermal recording was able to be captured. This horse is known to be compliant with handling and has been described as having a calm temperament. Horse number four displayed the greatest behavioural reaction to the aversive stimulus. This horse has a nervous temperament and can often be difficult to handle. All horses showed some behavioural response and movement away from the aversive stimulus apart from horse number one who displayed elevated head carriage but did not move away.

Table 4.4 Descriptive statistics for reactivity score assigned by fourteen observers for each horse. Higher scores relate to increased activity level

Horse	Mean Score	Standard Deviation
1	1.3	0.5
2	4	0
3	2.9	0.4
4	5	0
5	3.1	0.3
6	2.9	0.3
7	2.1	0.3
8	4.1	0.3
9	4	0
10	3	0

4.5.5 Correlations.

The relationship between change in salivary cortisol from pre presentation of aversive stimulus to maximum level and mean reactivity score for each horse (n=6) was investigated using Spearman's Rank Order Correlation. There was a strong positive correlation (Figure 4.5) between the two variables, ($r=.812$, $p=.05$) with high levels of salivary cortisol associated with high behavioural reactivity.

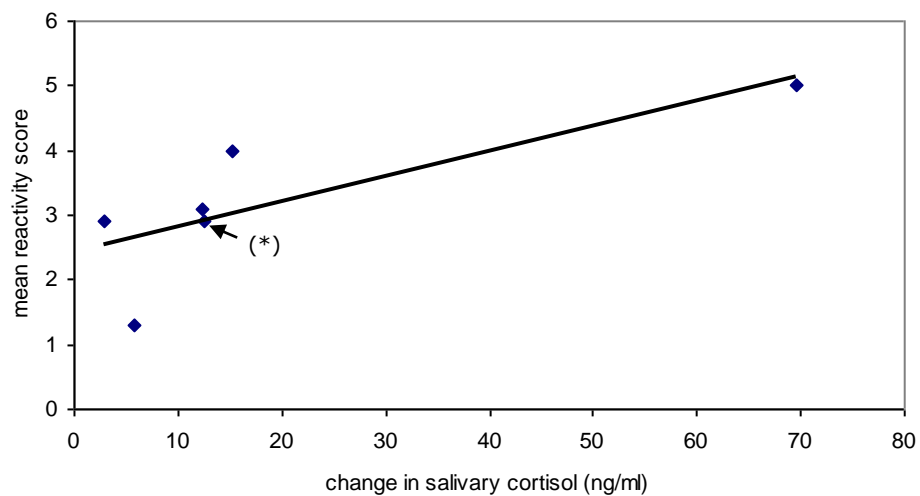


Figure 4.5. Relationship between change in salivary cortisol (ng/ml) from pre presentation of aversive stimulus to maximum level and mean reactivity score for each horse. Pre stressor salivary cortisol level was calculated by taking the mean of the two pre presentation samples. Data is shown from five horses with complete hormonal profiles and one horse with hormonal data available up to 15 minutes post stressor () (Increase in salivary cortisol 12.6ng/ml, reactivity score, 2.9).*

The relationship between mean reactivity score and increase in heart rate from basal level to maximum level post stressor (bpm) for each horse (n=10) was investigated using Spearman's Rank Order Correlation. There was a strong positive correlation (Figure 4.6) between the two variables, $r=0.694$, $p=0.038$ with a high reactivity score associated with a large increase in heart rate. Table 4.5 presents the reactivity score for each horse and the corresponding increase in heart rate from basal levels.

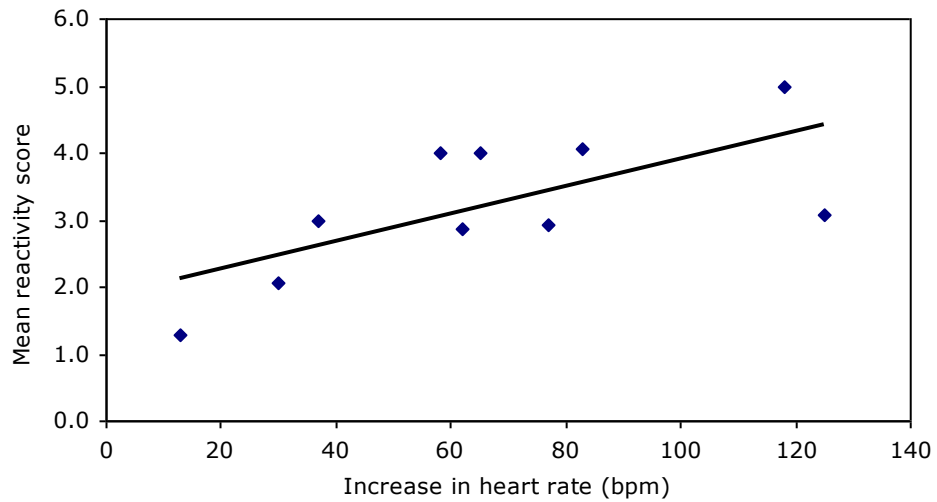


Figure 4.6 Relationship between mean reactivity score and increase in heart rate from basal level to maximum level (bpm) for each horse

Table 4.5 Reactivity score and increase in heart rate from basal level to maximum for each horse.

Horse	Mean reactivity Score	Increase in heart rate from basal level (bpm)
1	1.3	13
2	4	65
3	2.9	62
4	5	118
5	3.1	125
6	2.9	77
7	2.1	30
8	4.1	83
9	4	58
10	3	37

4.5.6 Ambient temperature

Ambient temperature fluctuated from 11°C to 23°C throughout the course of the day. Temperature changed a minimum of 1°C and a maximum of 8°C during the course of each horse’s treatment.

4.6 Discussion

Due to the flight behaviour of the horses upon presentation of the aversive stimulus and the fixed position of the thermal video camera it was only possible to capture acute thermal response of the eye to the aversive stimulus in one horse. Restraining the horses to prevent movement would have caused stress in itself and assessment of behavioural reactivity would have been prevented. A decrease in eye temperature of 1.8°C was recorded for this one horse which is consistent with the drop in eye temperature found following acute fright (Schaefer *et al.*, 2006) and disbudding (Stewart *et al.*, 2008) in cattle. The magnitude of the decrease in eye temperature was also consistent with the drop in nasal temperature in monkeys that were threatened by a handler (Nakayama *et al.*, 2004). Eye temperature of the one study horse then increased by 1.1°C back to basal level however it did not mirror the increase above basal levels reported in the disbudded cows (Stewart *et al.*, 2008). Data were from one study horse that was assigned the lowest reactivity score. Its behaviour upon presentation of the aversive stimulus was atypical for the horse and the magnitude of increase in salivary cortisol was also the lowest. This may suggest that eye temperature is a more sensitive measure of the stress response. The reduced physiological response and behavioural reaction may indicate that the horse did not perceive the procedure to be as stressful as the other study horses and this could account for the absence of any subsequent rise in eye temperature. The static thermal images for this study did not reveal any change in eye temperature over time. This, in addition to absence of any increase above basal levels in the continuous recording, may be due to the acute nature and immediate removal of the stressor being interpreted by the horse as the passing of danger rather than the disbudding procedure which is longer in duration and indicated pain not fright, therefore the stress response was no longer stimulated. Nakayama

et al. (2004) reports that nasal temperature started its descent between 10 and 110 seconds post onset of confrontation in all monkeys and had returned to pre stressor levels within four minutes post removal of confrontation therefore at the time of the first post stress static thermal image for this study any changes in eye temperature had possibly dissipated. This theory is supported by Stewart *et al.* (2007) who state that it is possible that studies which have only reported increases in temperature in response to acute stress, may have failed to detect an initial decrease in eye temperature due to its instantaneous nature. It is likely that thermal sampling was too infrequent or not constant and therefore could not capture such an immediate temperature change.

Ambient temperature was recorded in order to better interpret any thermal changes of the eye, however static thermal images revealed no change in eye temperature across the ten sampling time points. The decrease in eye temperature in the one continuous recording available was of an acute nature (one second) and measurement of ambient temperature was limited to every five minutes. It is therefore hard to attribute any change in eye temperature to change in ambient temperature and a more accurate method of recording ambient temperature must be used for future studies.

Salivary cortisol was significantly ($p=0.04$) affected by time with levels peaking between fifteen and twenty minutes post stressor in all but one horse, which displayed maximum cortisol levels thirty minutes post stressor. The time to maximum cortisol level in this study is consistent with previous work that reports a peak in cortisol between ten and thirty minutes post stressor (Colborn *et al.*, 1991, Shanahan, 2003, Stewart *et al.*, 2007). The highest cortisol level displayed was 81.5ng/ml which was an increase of 69.6ng/ml in horse number 4 and is similar to the increase in salivary cortisol reported by Moon *et al.* (2004) in abruptly weaned foals. The remaining cortisol levels and magnitudes of increase were similar to the change in

cortisol reported in cows during disbudding (Stewart *et al.*, 2007). Two horses displayed an increase in cortisol prior to the presentation of the aversive stimulus (Horse 2 and 5, Appendix 6) which may be due to anticipatory stress. In both horses cortisol increased post stressor.

The resting heart rate of a horse is between 38 to 42 beats per minute (Hayes, 2002) and although presentation of the aversive stimulus evoked an immediate and statistically significant ($p < .0001$) cardiac response in all horses, mean pre stressor heart rate was greater than normal resting levels at 70 beats per minute. This is likely to be due to a combination of exercise from the horses walking a short distance to reach the barn and anxiety of certain horses due to unfamiliar equipment, a change in routine and the entrance of the person into the barn carrying the aversive stimulus.

It was clear from the behavioural reactivity score that all horses displayed some degree of evasive behaviour and therefore found the procedure aversive. Individual differences between horses in reactivity may be associated with temperament; breeding and past experience (Visser *et al.*, 2003).

There was a positive relationship between increase in salivary cortisol and reactivity of the study horses. The positive relationship between increase in salivary cortisol and reactivity score supports the findings of Hughes *et al.* (2006), who also reported that horses with a high behavioural score showed a trend toward a statistically significant increase in salivary cortisol post stressor.

4.7 Conclusion

The physiological and behavioural response of the study horses indicates they found the test procedure to be stressful, however due to the species specific flight behaviour it was only possible to capture thermal change of the eye in all one horse. Data from the one available thermal recording revealed the immediate decrease and then increase back to basal level in eye temperature as a response to acute aversive stimuli, however there was no increase above basal levels as reported in existing work in other species.

The timing and magnitude of the change in salivary cortisol were similar to those reported in past work. All horses displayed an increase in salivary cortisol post presentation of the acute aversive stimulus although the magnitude of the rise in cortisol levels varied between horses.

Static thermal images showed no effect of time over the duration of the study, however it is possible that the acute nature of the stressor resulted in any thermal response of the eye having dissipated before thermal monitoring began. It may be that the immediate sympathetic response that prepares the horse to flee from a stressful situation quickly subsides whereas the slower activation of the HPA axis to further support the flight response is also slower to dissipate. Although investigation of the acute response of the equine eye to aversive stimuli was necessary to see whether results of work in other species were mirrored in the horse, it seems that the use of IRT to monitor acute stress is not appropriate for a flight species. Furthermore, situations that present the domestic horse with an acute challenge are limited and the potentially stressful situations that domestication has exposed the horse to and that owners and trainers can influence are of longer duration than ten seconds. If IRT is to be validated as a tool to monitor how horses perceive these procedures it is necessary to investigate thermal response to potentially stressful situations, some of which have been discussed in chapter 1., that are of longer duration and are more specific to the horse.

Chapter 5 Study C; Investigation into the use of IRT as a tool to assess thermal change associated with the physiological response to short term stress

5.1 Introduction

The startle response study revealed that IRT is an inappropriate measure to monitor acute stress in a flight species. Data collection was difficult due to the evasive behavioural response of the horse and the application of monitoring acute stress is limited although data collected from one horse suggests the response of eye temperature to acute stress is similar to that of other species. The management practices and training procedures that may potentially be stressful to the horse and require objective assessment are of longer duration. In order to validate IRT as a non-invasive measure of stress and apply this to equine welfare it was logical to investigate any potential thermal response to a stressor of longer duration and in a situation more relevant to how domestic horses are managed.

Clipping was used as a potentially stressful husbandry procedure in order to investigate the thermal response of the equine eye to a short term stressor and compare this against currently accepted measures. Clipping was chosen as it would allow an immediate, objective and non-invasive way of assessing how aversive the horses found the procedure, particularly in trained horses who may not show an overt behavioural response.

Clipping is a routine husbandry practice necessary for the welfare of ridden horses. In the UK by the end of October a horse will have grown a considerably longer and denser winter coat. A horse carrying this thick coat will quickly overheat when exercised and as a mechanism of thermoregulation it will sweat to dissipate this excess heat. This can lead to discomfort whilst being exercised which in turn can lead to problems maintaining fitness. Keeping the horse clean and free from parasites is also difficult with a long winter coat and so it is removed and artificial warmth is provided through

specialised rugs. Although this procedure is totally non-invasive and causes no physical harm to the horse, the noise and feel of the clippers seem to cause stress to some horses whilst others appear unaffected (Gough, 1999).

5.1.1 Preliminary study

A preliminary study was carried out to investigate the thermal and hormonal response of a horse to a short term potentially aversive procedure (Appendix 7). This small preliminary study revealed an increase in eye temperature and salivary cortisol in response to sham clipping. The increase in both eye temperature and salivary cortisol warranted further investigation using a larger study into thermal and hormonal response of the eye to a short term husbandry practice.

5.2 Aims and objectives

Aim

- Determine whether there is a thermal response to short term stress in the horse

Objectives

- Investigate the thermal response of the horse to the short term potentially stressful husbandry procedure of clipping
- Compare any thermal response with the currently accepted indicators of stress (salivary cortisol and behavioural assessment).

5.3 Methodology

Data record sheets were produced for each horse and used to hand log when physiological measures had been taken. This ensured all necessary samples were carried out for each horse. Data was then transferred from the record sheets into Excel spreadsheets at a later date.

5.3.1 Animals and husbandry

Horses (n=10) were a mix of sex, age and breed representative of horses found in a riding school. Horses were chosen by the manager of Brackenhurst equestrian centre and included five who were consistently behaviourally compliant with the clipping procedure and five known to consistently show behavioural signs of stress during clipping. Horses were managed as previously discussed and details of the individual horses can be found in section 2.2.

5.3.2 Test area.

Clipping was carried out in an enclosed barn familiar to all of the horses but not previously associated with the clipping procedure. The layout of the barn has been previously described in section 4.2.2

5.3.3 Test procedure

Each horse was led the short distance from its stable by the same familiar handler and tied up using a conventional head collar and lead rope in the barn. The horses were tied so they could not escape the barn but were still able to move around in the immediate area as is usual during clipping. As it is possible that exercise and anticipation may have contributed to pre stressor changes in physiological measures during the startle response study, each horse was allowed to acclimatise to the new environment for ten minutes and the first physiological measure (ten minutes pre sham clipping) did not start

until after this period. The horse was then exposed to ten minutes of sham clipping using guarded electric clippers (Lister, UK) with the blades removed. Clippers were placed on the cranial crest of the neck (two minutes each side), flanks (two minute each side) and each front leg (one minute per leg). The horses could feel and hear the clippers but no hair was removed. Both the handler and the operator remained silent for the duration of the sham clipping and at no time verbally or physically comforted, coaxed or rewarded the horse.

The timing for taking samples was altered to reflect the time taken for physiological measures to peak and return to basal level observed so far during the investigation into the use of IRT as a tool to assess thermal change associated with the physiological response to acute stress and the associated pilot work. Thermal images were taken every five minutes from ten minutes pre onset of clipping until thirty minutes post onset of clipping. The primary anatomical area under investigation was the eye as this had been reported to be a more consistent measure of thermal change (Cook *et al.*, 2006, Stewart *et al.*, 2008) and had indeed increased in response to a potentially distressing procedure during pilot work. Eye temperature also avoided the confounding variables of hair and dirt reported in past work (Cook *et al.*, 2001). The investigation into thermal change of ear temperature in response to a distressing situation was also included as there is evidence that ear pinna temperature does alter in stressful situations in other species (Ingram *et al.*, 2002) and the results of the pilot study involving one horse was not sufficient to rule this out as an option. Saliva was sampled for cortisol analysis at ten and five minutes pre clip and then every ten minutes until forty minutes post onset of clipping. The larger time difference between saliva samples compared to five minute intervals for thermal images was necessary due to time and human resources available at the time of the study. These timing parameters were considered appropriate as work to date had shown the response time of

salivary cortisol post stressor to be ten minutes and the maximum time to return to basal levels thirty minutes. However, the extended saliva sampling time was chosen to allow for any potential changes in cortisol response times especially as times reported so far were as a result of an acute (ten second) stressor and the stressor in this study would remain for a longer duration.

Horses involved in the study were individually sham clipped for ten minutes duration and were not able to see or hear the procedure being carried out on any of the other horses.

5.3.4 Repeat study with no clipping

A repeat study was carried out on a separate day using the same horses and same order of testing and involved the same experimental design. However, the potentially stressful stimulus (presence of the clippers) was not included. This second study was carried out to ensure that any physiological changes and behavioural response observed during the sham clipping treatment was due to the presence of the clippers.

5.4 Data collection and analysis for sham clipping and no clipping treatments

5.4.1 Infrared thermography data collection

Static thermal images were captured using the method previously discussed in chapter 2.2.2. Images were captured every five minutes from five minutes pre onset of sham clipping until thirty minutes post onset of sham clipping. Sham clipping began immediately following the second pre clip sample.

5.4.2 Infrared Thermography data analysis

Temperature was extracted from the thermal images as previously discussed. Mean temperature of left and right eye and left and right ear were calculated for each horse for each time point. Initially eye and ear temperature for each horse for each time point was plotted against time using Excel software to investigate any fluctuations in temperature over the study period. Distribution of data for both eye and ear temperature varied significantly from normal (Kolmogorov-Smirnov Test). A Friedman test was conducted to examine the effect of time on both eye and ear temperature over the duration of the study and the time point with the greatest effect on eye and ear temperature was also identified (Wilcoxon Signed Rank Test). A Mann Whitney-U Test was also carried out to determine any differences in eye and ear temperature between the compliant and non compliant groups. Thermal data for the no clipping treatment was managed in the same way.

5.4.3 Salivary cortisol data collection

Saliva was sampled for cortisol analysis ten minutes and five minutes pre sham clipping and then every ten minutes thereafter until thirty minutes post onset of sham clipping. Saliva was collected, analysed and processed using the method previously discussed in chapter 2.2.1.

5.4.4 Salivary cortisol data analysis

Initially salivary cortisol for each horse for each time point was plotted against time using Excel software to investigate any fluctuations in cortisol over the study period. Distribution of data varied significantly from normal (Kolmogorov-Smirnov Test). A Friedman test was conducted to examine the effect of time on salivary cortisol over the duration of the study and the time where salivary cortisol peaked was also investigated (Wilcoxon Signed Rank

Test). A Mann Whitney-U Test was carried out to determine any differences in salivary cortisol between the compliant and non compliant groups. Hormonal data for the no clipping treatment was managed in the same way.

5.4.5 Heart rate data collection

Heart rate was logged every five minutes from five minutes pre onset of sham clipping until thirty minutes post onset of sham clipping using a heart rate monitor (Polar Equine RS800G3). Data was recorded, stored and uploaded to analysis software as previously discussed in chapter 2.

5.4.6 Heart rate data analysis

Mean heart rate for each sampling time point for the compliant and non compliant groups was calculated using Excel. Distribution of heart rate data during the sham clipping treatment was normal (Kolmogorov-Smirnov, $p=0.250$ non compliant horses, $p=0.246$ compliant horses) therefore an independent samples t-test was conducted to compare the mean heart rates of the compliant and non compliant horses. Distribution of heart rate data for the no clipping treatment varied significantly from normal (Kolmogorov-Smirnov, $p=0.001$ non compliant horses, $p=0.008$ compliant horses) Therefore a Mann-Whitney U Test was conducted to compare the mean heart rates of the compliant and non compliant horses.

5.4.7 Core temperature data collection

Core temperature was taken every five minutes from five minutes pre onset of sham clipping until thirty minutes post onset of sham clipping using the method previously discussed in chapter 2. Data was recorded by hand on the horses log sheet and then transferred to an Excel spreadsheet post study.

5.4.8 Ambient temperature data collection

Ambient temperature was monitored for the duration of the study (Lascar EL-USB-2). The temperature logger was set to record at five minute intervals that corresponded with the sampling time of the thermal images. This was checked and altered if necessary between each horse through the use of a laptop computer in the test area. Data was uploaded and processed using the methods previously discussed in chapter 2.2.2.2

5.4.9 Behavioural data collection

Behaviour was recorded using a hand held video camera mounted on a tripod and placed adjacent to the horse. Recording the study allowed objective behavioural analysis to be carried out post data collection. Footage was uploaded to a PC using a USB interface and played back for assessment (Windows media player for Windows Xp) at a later date.

The video recording for each horse was divided into three stages which were pre sham clipping, during sham clipping and post sham clipping (the no clipping study video footage was also split into stages that mirrored the timings of the sham clipping session). A five minute sample from each stage for each horse was extracted from the footage. The pre sham clipping footage was from the first five minutes of the recording which started as soon as the horse was tied in the barn, the during sham clipping footage was from the first five minutes of sham clipping and the post sham clipping footage was from the first five minutes after sham clipping had ceased. Five minutes was chosen for the duration of the clip to avoid interference from saliva samples and thermal images being taken.

For each stage, each horse was assigned an activity score. Scores were first attributed to a behavioural definition according to degree of avoidance or flight behaviour (Table 5.1) The video was shown to twenty BSc equine science students in a blind random order of horses, stage of the session and

treatment (sham clip or no clip) and a score assigned to each horse for each stage. A mean activity score for the compliant and non-compliant groups was calculated for pre-clip, during clip and post clip for the sham clipping session and the no clipping session.

Table 5.1 Activity score relating to avoidance or flight behaviour with scores ranging from 1 (low degree of activity) to 5 (high degree of activity). The score system was used for both sham clipping treatment and no clipping treatment.

<u>Score</u>	<u>Behavioural definition</u>
1	Very relaxed stance, lowered head, relaxed lower lip, eyes half closed, ears turned to side.
2	Relaxed stance, absence of restless behaviour, very little movement.
3	Neutral stance, absence of whole body movement but vigilant head and ears.
4	Active with some restless behaviour, movement of head, neck and ears including elevated head, snorting.
5	High degree of activity, very restless, raising of the head, whole body movement including feet.

5.4.10 Behavioural data analysis

Mean activity score from the twenty responses for pre sham clipping, during sham clipping and post sham clipping was calculated for each horse in addition to a mean score for each segment for all compliant horses and all non compliant horses. This was done for both the sham clipping treatment and the no clipping treatment. Following a Kolmogorov-Smirnov test a Mann Whitney-U test was conducted to investigate any difference in activity level for pre, during and post clip scores in the compliant and non compliant horses. Within the compliant group a Wilcoxon Signed Rank Test was conducted to investigate any difference in activity level between the pre and during stage, the during and post stage and the pre and post stage behaviour score. Within the non compliant group a Wilcoxon Signed Rank Test was

conducted to investigate any difference in activity level between the pre and during stage, the during and post stage and the pre and post stage behaviour score. The tests were repeated for the no clipping study data.

5.4.11 Correlation Analysis

Change in eye temperature from baseline to each subsequent sample for each horse and change in ear temperature from baseline to each subsequent sample for each horse was calculated and the relationship between the two parameters investigated (Spearman's Rank Order Correlation). In addition change in eye temperature from baseline to maximum temperature and change in salivary cortisol from baseline to maximum was calculated for each horse and a Spearman's Rank Order Correlation was used to investigate any relationship between the two physiological variables.

5.5 Data analysis for comparison of sham clipping and no clipping treatments

Data from the horses were analysed together rather than as compliant and non compliant groups as there was found to be no significant difference in physiological measures between the two groups during the sham clipping and no clipping treatments.

5.5.1 Infrared thermography

As the same horses were involved in both studies and therefore measured using the same scale on two separate occasions a Wilcoxon Signed Rank Test was used to investigate any difference in eye temperature and ear temperature between the no clipping treatment and the sham clipping treatment.

5.5.2 Salivary cortisol

A Wilcoxon Signed Rank Test was used to investigate any difference in salivary cortisol between the no clipping treatment and the sham clipping treatment.

5.5.3 Heart rate

A Wilcoxon signed rank test was conducted to investigate any difference in heart rate of the non compliant horses between the sham clipping and no clipping treatment and the heart rates of the compliant horses between the sham clipping and no clipping treatment.

5.5.4 Summary of comparisons of data

1. Sham clipping treatment – comparison of physiological and behavioural measures between the compliant and non compliant horses

2. No clipping treatment - comparison of physiological and behavioural measures between the compliant and non compliant horses

3. Comparison of sham clipping and no clipping treatment – All horses were treated as a whole group for comparison of IRT and cortisol between sham clipping and no clipping treatments as there were no significant differences in these parameters between compliant and non compliant animals in either of the studies (1 and 2).

5.6 Results

5.6.1 Infrared thermography sham clipping

The results of the Friedman test reveal a statistically significant effect of time ($\chi^2 (2, n= 10) = 31.02, p<0.001$) on eye temperature in all horses with eye temperature increasing during the sham clipping stage. There was no significant difference in eye temperature between the compliant and non compliant groups (Mann Whitney-U) however mean eye temperature of the non compliant horses was elevated above eye temperature of the compliant horses from the beginning of the study until sham clipping ceased (Figure 5.1). Eye temperature increased over the duration of the sham clipping treatment in all horses with the greatest effect of time at five minutes post onset of sham clip (Wilcoxon Signed Rank Test $p=0.008$). Mean eye temperature peaked at ten minutes post onset of sham clip and started to decline when sham clipping ceased however it had not returned to basal level by the end of the study duration. The means and standard deviations are presented in Table 5.2

A Friedman Test was conducted to investigate the effect of sham clipping on ear temperature over the duration of the sham clipping treatment. There was a statistically significant effect of time on ear temperature in all horses ($p<0.001$) during the sham clipping stage, with no difference in ear temperature between the compliant and non compliant groups (Mann Whitney-U), however ear temperature of the non compliant horses was lower than that of the compliant horses for the duration of the study (Figure 5.2). Ear temperature decreased over the duration of the sham clipping treatment in all horses with the greatest effect of time at five minutes post onset of clip (Wilcoxon Signed Rank Test $p=0.005$). Mean ear temperature began to return to basal level when clipping ceased however it had not returned to basal level by the end of the study duration. Figure 5.3 shows the change in mean eye temperature and mean ear temperature over the duration of the clipping

treatment. Details of the change in eye temperature for each individual horse can be found in Table 5.3 and details of the change in ear temperature for each individual horse can be found in Table 5.4. There was a mean (\pm SD) increase in eye temperature of 3.6°C ($\pm 5.2^{\circ}\text{C}$) and a mean decrease in ear temperature of 7.4°C ($\pm 9.9^{\circ}\text{C}$).

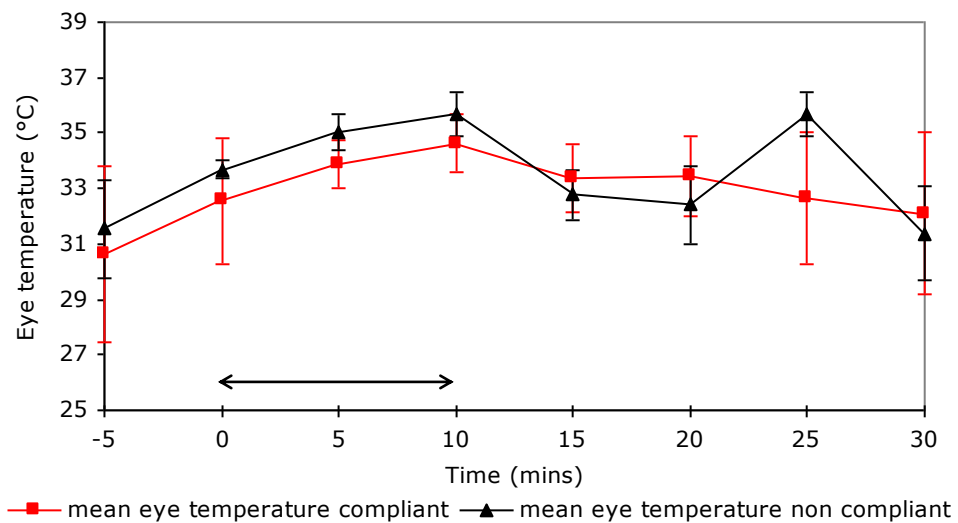


Figure 5.1 Mean (\pm SD) eye temperature ($^{\circ}\text{C}$) of the compliant and non compliant horses over the duration of the sham clipping treatment. The arrow marker indicates when sham clipping occurred starting immediately after the second pre clip sample and lasting ten minutes.

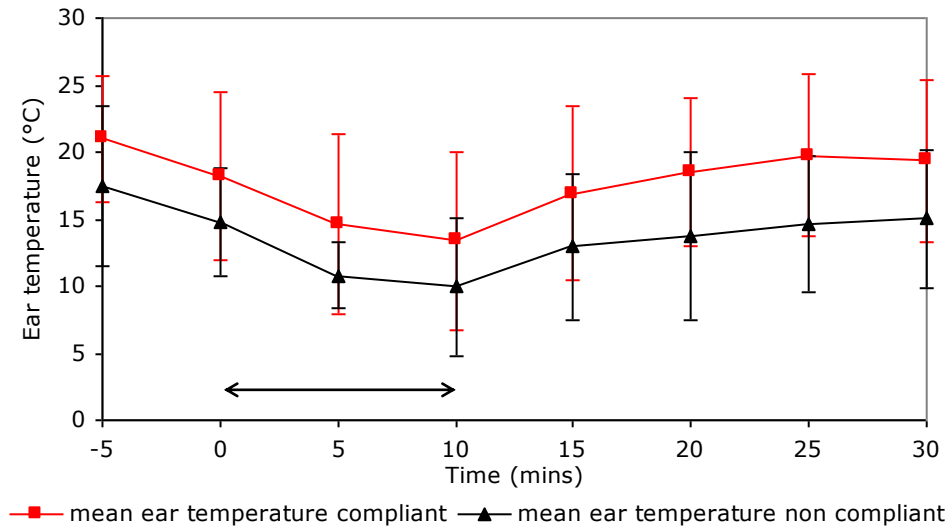


Figure 5.2 Mean (\pm SD) ear temperature ($^{\circ}$ C) of the compliant and non compliant horses over the duration of the sham clipping treatment. The arrow marker indicates when sham clipping occurred starting immediately after the second pre clip sample and lasting ten minutes.

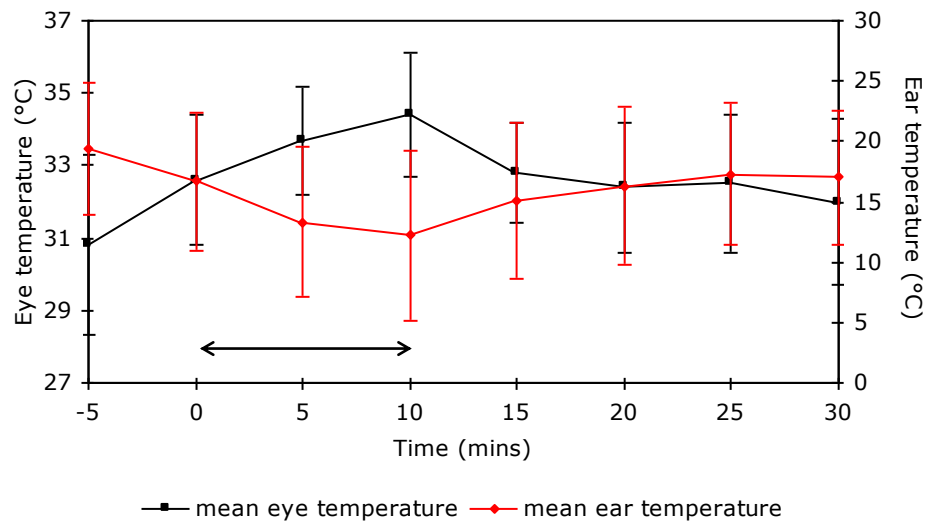


Figure 5.3. Mean (\pm SD) eye temperature ($^{\circ}$ C) and mean ear temperature ($^{\circ}$ C) for all horses ($n=10$) over the duration of the sham clipping treatment. The arrow marker indicates where sham clipping occurred starting immediately after the second pre clip sample and lasting ten minutes.

Table 5.2 Descriptive statistics for eye and ear temperature across the eight sampling points during the clipping treatment.

Time	Mean eye temperature (°C)	Standard deviation	Mean ear temperature (°C)	Standard deviation
1	30.8	2.5	19.4	5.5
2	32.6	1.8	16.7	5.7
3	33.7	1.5	13.3	6.2
4	34.4	1.7	12.2	7.1
5	32.8	1.4	15.1	6.5
6	32.4	1.8	16.3	6.5
7	32.5	1.9	17.3	5.9
8	32	2.3	17	5.6

Table 5.3 Eye temperature data for individual horses for sham clipping treatment. Data includes horse with behavioural group compliant (C) or non compliant (NC), pre stressor eye temperature, maximum eye temperature (with change in temperature), time to maximum eye temperature post onset of sham clipping and time to return to basal temperature. A * indicates that eye temperature had not returned to basal levels by the end of the study. Pre stress eye temperature was calculated as a mean of the two pre stress measures

Horse	Pre stressor eye temperature (°C)	Maximum eye temperature (°C)	Time to maximum eye temperature (mins)	Time to return to basal levels (mins)
Annie (NC)	33	36.8 (3.8)	5	10
Angus (NC)	30.9	34.7 (3.8)	5	*
Conan (NC)	29.3	31.7 (2.4)	20	*
Desmond (NC)	31.2	32.7 (1.5)	5	10
Harriet (NC)	33.3	35.9 (2.6)	5	5
Beau (C)	29	34.5 (5.5)	5	*
Kitkat (C)	28.7	35.4 (6.7)	5	*
Pie (C)	27.3	33.2 (5.9)	5	*
Tosca (C)	34.1	35.8 (1.7)	5	*
Visi (C)	32.9	34.6 (1.7)	25	*

Table 5.4 Ear temperature data for individual horses for sham clipping treatment. Data includes horse with behavioural group compliant (C) or non compliant (NC), pre stressor ear temperature, minimum ear temperature (with change in temperature), time to minimum ear temperature post onset of sham clipping and time to return to basal temperatures from minimum. A * indicates that ear temperature had not returned to basal levels by the end of the study. Pre stress ear temperature was calculated as a mean of the two pre stress measures

Horse	Pre stressor ear temperature (°C)	Minimum ear temperature (°C)	Time to minimum ear temperature (mins)	Time to return to basal levels (mins)
Annie (NC)	13.6	1.8 (11.7)	5	5
Angus (NC)	18.1	8.6 (9.5)	10	15
Conan (NC)	15.6	10 (5.6)	5	*
Desmond (NC)	12.4	8.3 (4.1)	15	10
Harriet (NC)	21.4	15 (6.4)	5	*
Beau (C)	21	9.1 (11.9)	5	*
Kitkat (C)	19.1	9.9 (9.2)	5	*
Pie (C)	15.3	5.6 (9.7)	5	*
Tosca (C)	24.1	22.1 (2)	20	*
Visi (C)	27	23.1 (3.9)	5	15

5.6.2 Infrared thermography no clipping treatment

Distribution of data was normal (Kolmogorov-Smirnov test), however non parametric tests were applied in order to compare results of the no clipping treatment to the sham clipping treatment which had data that varied significantly from normal. The results of the Friedman test indicated there was no significant effect of time on eye $X^2 (7, n = 10) = 4.837, p=0.68$ or ear temperature $X^2 (7, n = 10) = 10.901, p=0.14$ during the no clipping

treatment There was also no significant difference in eye and ear temperature between the compliant and non compliant horses (Mann Whitney-U test).

Figure 5.4 displays mean eye temperature and ear temperature for all horses across the duration of the no clipping treatment. The means and standard deviations of eye and ear temperature for the duration of the no clipping treatment are presented in Table 5.5.

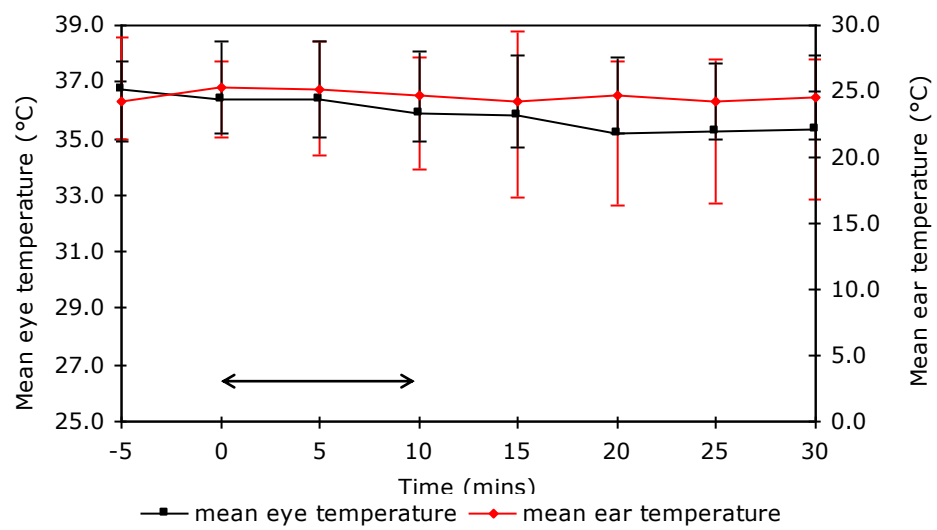


Figure 5.4 Mean (\pm SD) eye temperature ($^{\circ}$ C) and mean ear temperature ($^{\circ}$ C) of all horses over the duration of the no clipping treatment. The arrow marker indicates when sham clipping occurred during the sham clipping treatment starting immediately after the second pre clip sample and lasting ten minutes. The temperature scales on the Y axis mirror the scales used to display eye and ear temperature change in the clipping treatment (**Figure 5.7**) as close as possible to allow clearer comparison between sham clipping and no clipping studies.

Table 5.5 Descriptive statistics for eye and ear temperature across the eight sampling points during the no clipping treatment.

Time	Mean eye temperature (°C)	Standard deviation	Mean ear temperature (°C)	Standard deviation
1	36.8	1.8	24.3	3.0
2	36.4	1.3	25.3	3.4
3	36.4	2.0	25.2	3.6
4	35.9	2.0	25.6	3.4
5	34.9	2.9	25.2	3.5
6	35.2	2.6	25.7	2.8
7	35.3	2.6	25.2	2.9
8	35	2.5	25.5	3.2

5.6.3 Salivary cortisol sham clipping treatment

The results of the Friedman Test indicated there was a statistically significant effect of time on cortisol $\chi^2 (5, n= 10)= 18.214, p=0.003$ with cortisol increasing during the sham clipping stage. Mean and standard deviation for each sampling time point over the duration of the sham clipping treatment are presented in Table 5.6. There was no significant difference in salivary cortisol between the compliant and non compliant groups (Mann Whitney-U) (Figure 5.5). Mean salivary cortisol increased over the duration of the sham clipping treatment in all horses with the greatest effect of time at twenty minutes post onset of sham clip (Wilcoxon Signed Rank Test $p=0.05$). Mean salivary cortisol peaked at twenty minutes post onset of sham clipping and had begun to decrease at the final sampling point however it had not returned to basal levels by the end of the study.

The peak in mean eye temperature ten minutes post onset of sham clip and the peak in mean salivary cortisol twenty minutes post onset of sham clip resulted in a lag time of ten minutes between the maximum values of both physiological parameters (Figure 5.6).

Details of the change in salivary cortisol for each individual horse can be found in Table 5.7. There was a mean increase in salivary cortisol of 3.43ng/ml (\pm 1.2ng/ml).

Table 5.6 Descriptive statistics for salivary cortisol across the six sampling points during the sham clipping treatment.

Time	Mean	Standard Deviation
1	2.9	0.9
2	2.7	0.8
3	2.7	0.9
4	3.1	1.4
5	5.3	4.1
6	4.1	4

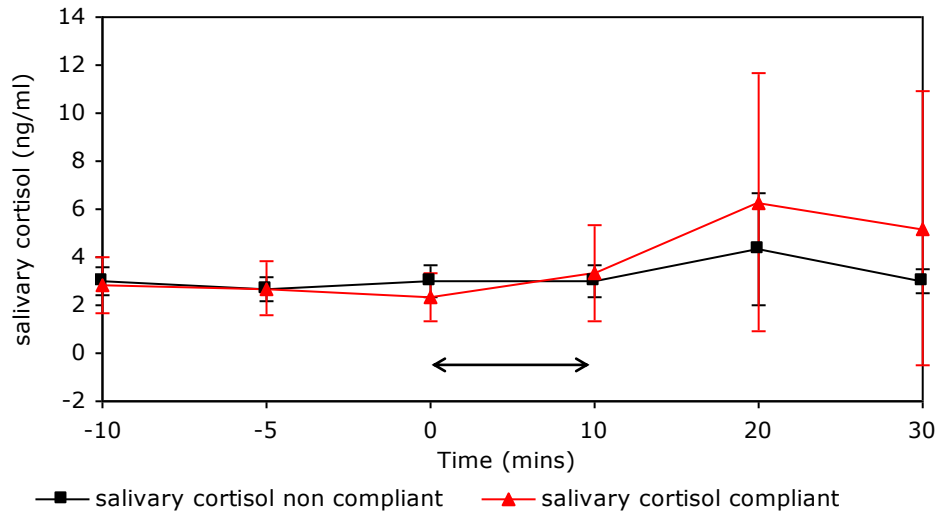


Figure 5.5 Mean (\pm SD) salivary cortisol of the compliant and non compliant horses over the duration of the sham clipping treatment. The arrow marker indicates when sham clipping occurred lasting ten minutes. Samples were taken at 10 minutes and five minutes before sham clipping and then every ten minutes thereafter

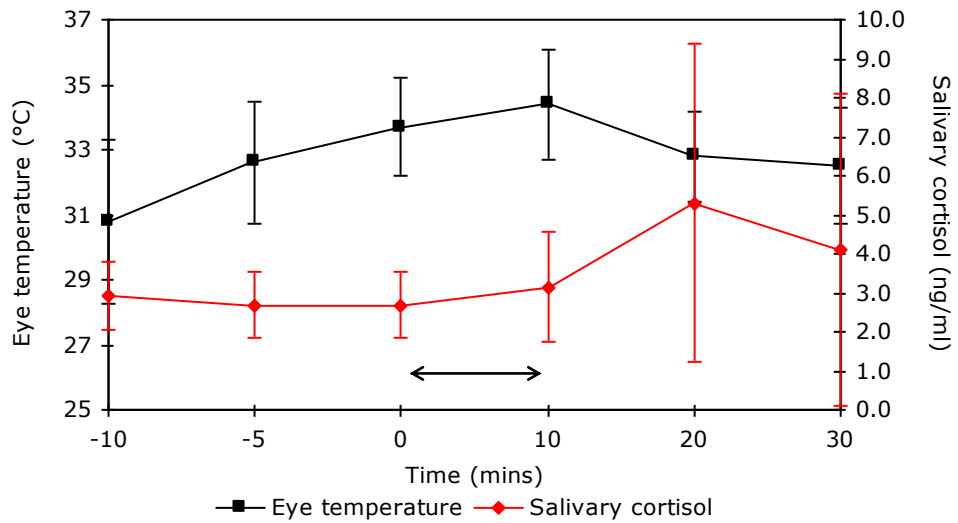


Figure 5.6 Mean (\pm SD) eye temperature ($^{\circ}$ C) and mean salivary cortisol (ng/ml) of all horses over duration of the sham clipping treatment. Arrow marker indicates when sham clipping occurred lasting ten minutes. A lag time of ten minutes between maximum value of both physiological measures can be seen at ten minutes post onset of clip for eye temperature and twenty minutes post onset of clip for salivary cortisol

Table 5.7 cortisol data for individual horses for sham clipping treatment. Data includes horse with behavioural group complaint (C) or non complaint (NC), pre stressor salivary cortisol, maximum salivary cortisol (with change), time to maximum salivary cortisol post onset of sham clipping and lag time between peak in eye temperature(ET) and peak in salivary cortisol (SC).

Horse	Pre stressor SC (ng/ml)	Maximum SC (ng/ml)	Time to maximum SC (mins)	Lag time between ET and SC
Annie (NC)	2.9	7.5 (4.6)	20	10
Angus (NC)	3.4	6.1 (2.7)	20	10
Conan (NC)	2.9	3.6 (0.7)	30	10
Desmond (NC)	2.8	3.7 (0.9)	10	5
Harriet (NC)	2.1	3.7 (1.6)	10	5
Beau (C)	1.3	5.3 (4)	20	10
Kitkat (C)	2.7	6.8 (4.1)	20	10
Pie (C)	3.9	15.6 (11.7)	20	10
Tosca (C)	2.1	2.6 (0.5)	20	5
Visi (C)	2.1	3.5 (1.4)	30	20

5.6.4 Salivary cortisol no clipping treatment

The results of the Friedman Test indicated there was no statistically significant change in cortisol over the duration of the no clipping treatment $\chi^2 (5, n=10) = 3.204$ $p=0.669$. Mean and standard deviation for each sampling time point over the duration of the no clipping treatment are presented in Table 5.8. In addition there was no significant difference in salivary cortisol between the compliant and non compliant groups (Mann Whitney-U).

Table 5.8 Descriptive statistics for salivary cortisol across the six sampling points during the no clipping treatment. The shaded row indicates when sham clipping occurred during the sham clipping treatment.

Time	Mean	Standard Deviation
1	4.2	1.8
2	4.0	2.5
3	4.1	1.8
4	3.5	1.2
5	3.8	1.8
6	3.4	1.3

5.6.5 Heart Rate sham clipping treatment

An independent samples t-test was conducted to compare the mean heart rates for the compliant and non-compliant horses over the duration of the sham clipping treatment. There was a significant difference in mean heart rate between the compliant (M=35.8, SD=2.3) and non compliant (M=53.6, SD=6.1) horses ($t(14) = 7.72, p < 0.001$), with the compliant horses displaying consistently lower heart rates throughout the duration of the study (Figure 5.7). The magnitude of the differences in the means (mean difference= 17.8, 95% CI: 12.8 to 22.7) was large (eta squared = 0.8). Mean heart rate in the non compliant horses increased by 20bpm and peaked ten minutes post onset of sham clip. Once sham clipping ceased heart rate then declined for the remainder of the sampling period however it had not returned to basal levels by the end of the study. Mean heart rate of the compliant horses remained stable throughout the duration of the study. Table 5.9 details the descriptive data for the compliant and non-compliant horses.

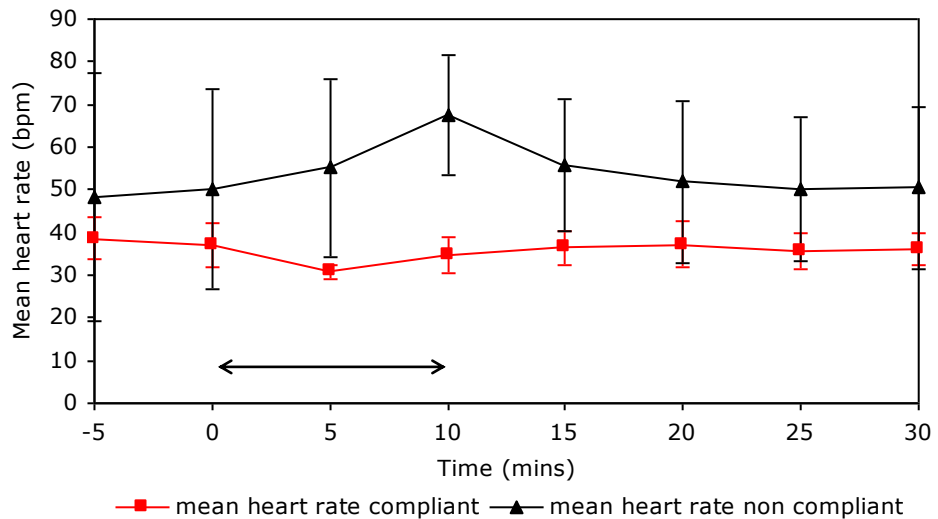


Figure 5.7. Mean heart rate (\pm SD) (bpm) of the compliant and non compliant horses for the duration of the sham clipping treatment. Arrow marker indicates when sham clipping occurred.

Table 5.9 Descriptive statistics for mean heart rate of compliant and non-compliant horses during the sham clipping treatment.

Group	Mean	Standard Deviation
Compliant horses	35.8	2.3
Non compliant horses	53.6	6.1

5.6.6 Heart rate no clipping treatment

A Mann-Whitney U test was conducted to compare the mean heart rates for the compliant and non-compliant horses. There was no significant difference in mean heart rate between the two groups at any sampling time point during the duration of the no clipping treatment. Mean heart rate remained within the range of 39.8 – 42.8bpm for both the compliant and non compliant horses for the duration of the study. Table 5.10 details the descriptive data for all horses for the duration of the no clipping treatment.

Table 5.10 Descriptive statistics for mean heart rate across the eight sampling time points of the no clipping treatment.

Time	Mean	Standard Deviation
1	40.4	3.4
2	40.9	3.1
3	41.1	3.3
4	41	3.1
5	42.1	3.4
6	41.7	2.5
7	41.4	1.6
8	40.8	1.0

5.6.7 Behavioural assessment sham clipping treatment

The results of the Mann Whitney-U Test indicate a significant difference in activity level between the compliant and non compliant horses during the pre sham clipping video segment ($p=0.03$) during sham clipping video segment ($p=0.008$) and post sham clipping video segment ($p=0.01$) with the non compliant horses displaying higher activity. The results of the Wilcoxon Signed Rank Test show no significant difference in activity between pre ($p=0.22$), during ($p=0.42$) and post ($p=0.28$) sham clipping in the compliant horses. There was a significant difference in activity level in the non compliant horses between pre ($p=0.04$), during ($p=0.04$) and post sham clipping ($p=0.04$) with the activity of the non compliant horses increasing at the onset of sham clipping (Figure 5.8).

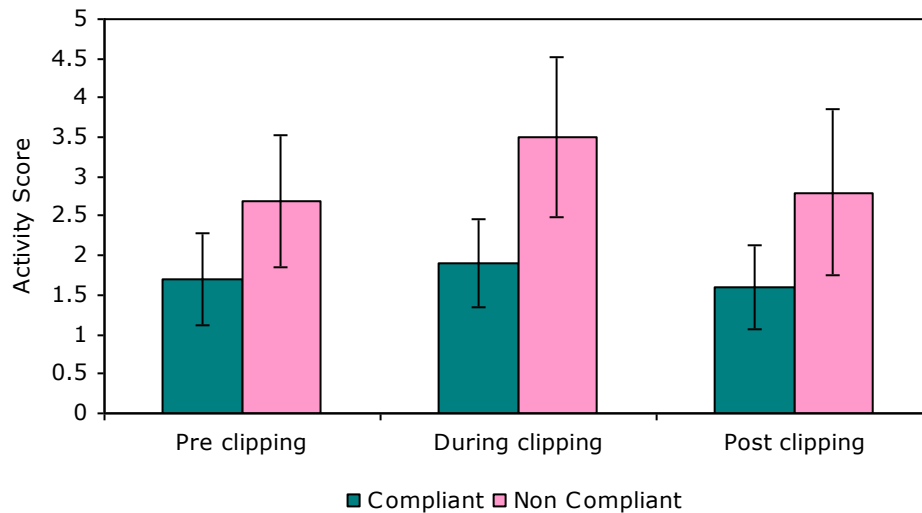


Figure 5.8. Mean (\pm SD) activity score for the compliant and non compliant horses for a five minute segment of pre sham clipping, during sham clipping and post sham clipping footage during the sham clipping treatment. Non compliant horses were significantly more active than compliant during pre ($p=.03$), during ($p=.008$) and post ($p=.01$) sham clipping.

5.6.8 Behavioural assessment no clipping treatment

The results of the Mann Whitney-U Test indicate no significant difference in activity level between the compliant and non compliant horses during the pre sham clipping video segment ($p=0.052$) during sham clipping video segment ($p=0.056$) and post sham clipping video segment ($p=0.056$) with the compliant and non compliant horses displaying similar activity. The results of the Wilcoxon Signed Rank Test show no significant difference in activity between pre ($p=0.68$), during ($p=0.63$) and post ($p=0.66$) sham clipping in the compliant horses. There was no difference in activity level in the non compliant horses pre ($p=0.09$), during ($p=0.2$) and post sham clipping ($p=0.07$) (Figure 5.9). All horses were less active during the no clipping treatment than the sham clipping treatment but during the sham clipping treatment the non compliant horses were more active than the compliant horses.

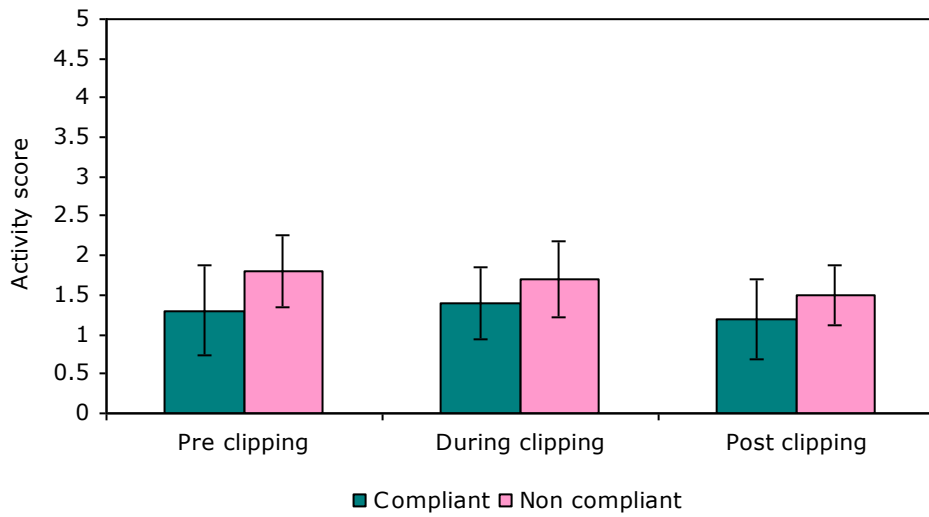


Figure 5.9 Mean (\pm SD) activity score for the compliant and non compliant horses for a five minute segment of pre sham clipping, during sham clipping and post sham clipping footage during the no clipping treatment.

5.6.9 Correlations between measures for sham clipping treatment

The relationship between change in eye temperature from baseline to each subsequent measure for each horse and change in ear temperature from baseline to each subsequent measure for each horse was investigated using Spearman's Rank Order Correlation. There was a strong negative relationship between the two variables ($r=-.568$, $P=0.000$); as eye temperature increased, ear temperature decreased (Figure 5.10).

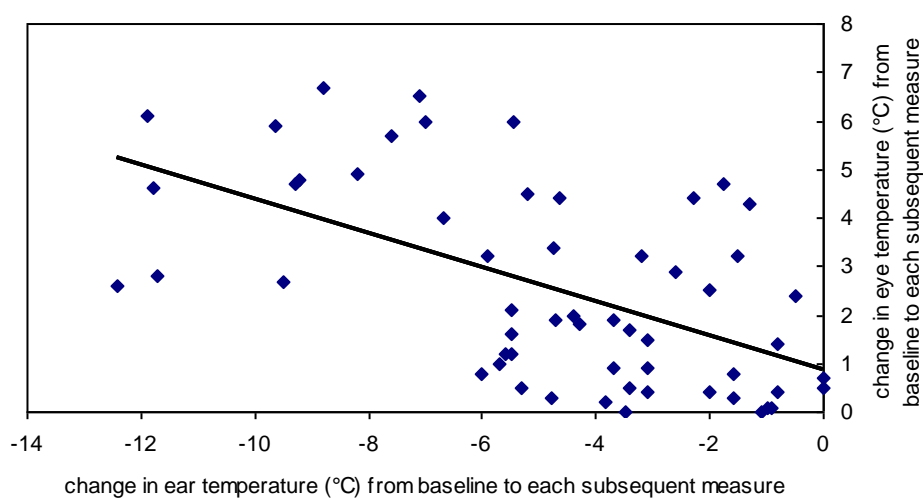


Figure 5.10 Relationship between change in eye temperature (°C) from baseline to each subsequent measure for each horse and change in ear temperature (°C) from baseline to each subsequent measure for each horse during the sham clipping treatment.

The relationship between change in eye temperature from baseline to maximum temperature and change in salivary cortisol from baseline to maximum value was calculated for each horse. Data was managed in this way rather than as in the correlation analysis of eye and ear temperature due to the lag time between the two variables. A Spearman's Rank Order Correlation was used to investigate any relationship between eye temperature and salivary cortisol. There was a positive correlation between the two variables ($r=.809$, $p=0.005$) with high levels of salivary cortisol associated with high eye temperature (Figure 5.11).

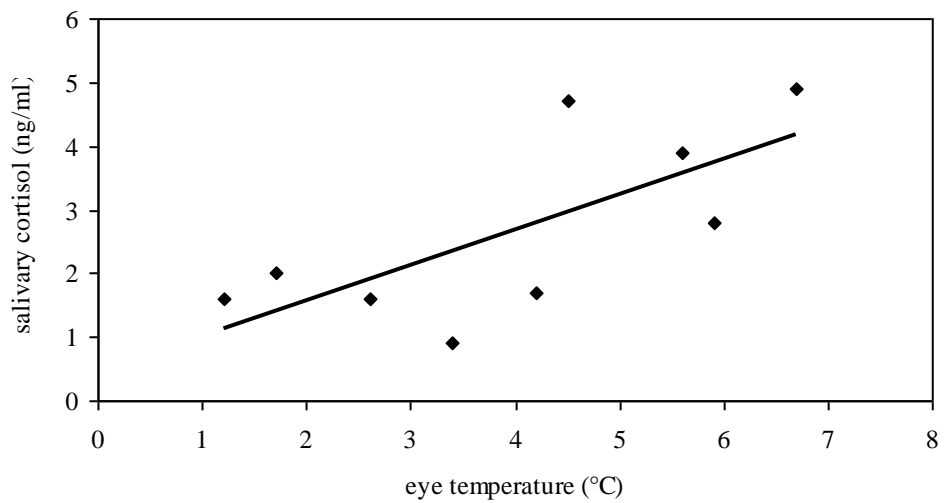


Figure 5.11 Relationship between change in eye temperature from baseline to maximum value and change in salivary cortisol from baseline to maximum value for each horse during the sham clipping treatment.

5.6.10 Core and ambient temperature

Core temperature remained within the normal range of 38-42°C for all horses throughout the duration of the treatment. As the study was carried out over a full day, air temperature fluctuated from 0°C to 5°C between the early morning and midday procedures, however it remained constant throughout each sham clipping treatment for each horse.

5.7 Comparison of sham clipping treatment data and no clipping treatment data

5.7.1 Infrared thermography

As there were no significant differences in eye temperature between the compliant and non compliant horses for both the clipping treatment and no clipping treatment, the comparison of eye temperature between the two treatments used all horses together. The results of the Wilcoxon Signed Rank Test indicate that there was a significant difference in eye temperature between the sham clipping treatment and the no clipping treatment, $z = -4.523$, $p < 0.001$. The horses displayed a higher eye temperature during the no clipping treatment than during the sham clipping treatment (Figure 5.12) however eye temperature during the no clipping treatment remained constant and there was no increase in eye temperature throughout the no clipping treatment segment as was seen in the sham clipping treatment.

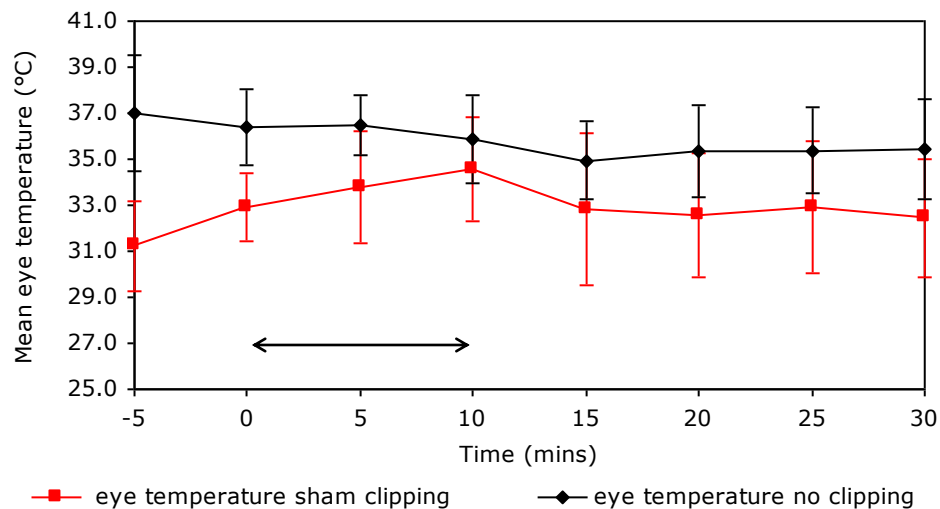


Figure 5.12 Mean (\pm SD) eye temperature ($^{\circ}$ C) of all horses during sham clipping treatment and no clipping treatment. The arrow marker indicates where sham clipping occurred during the sham clipping treatment.

5.7.2 Salivary cortisol

All horses were used to compare the sham clipping and no clipping salivary cortisol levels. The results of the Wilcoxon Signed Rank Test indicate a significant difference in salivary cortisol between the sham clipping and no clipping treatment, $z=-0.2796$, $p=0.005$. The horses displayed higher salivary cortisol levels during the no clipping treatment than the clipping treatment however, salivary cortisol did not display an increase during the no clipping treatment as it did during the clipping treatment (Figure 5.13).

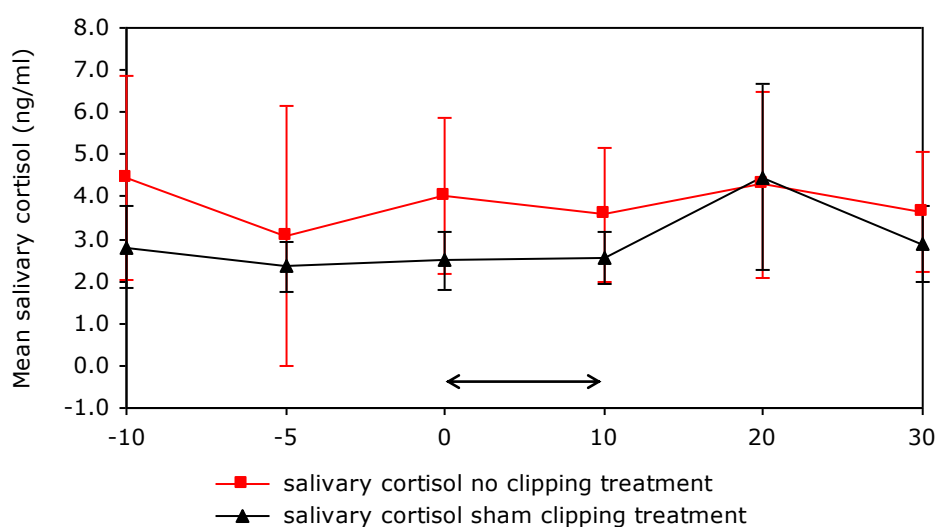


Figure 5.13 Mean (\pm SD) salivary cortisol (ng/ml) of all horses during sham clipping treatment and no clipping treatment. The arrow marker indicates where sham clipping occurred during the clipping treatment.

5.7.3 Heart rate

The results of the Wilcoxon signed rank test indicate a significant difference in heart rate between the sham clipping and no clipping treatment in the non compliant horses ($p<0.001$) and no significant difference in the heart rates of the compliant horses between the sham clipping and no clipping treatment ($p=0.076$). Figure 5.14(a) details the change in mean heart rate over time for the non compliant horses during the sham clipping and no clipping treatment and figure 5.14(b) details the change in mean heart rate over time for the compliant horses during the sham clipping and no clipping treatment.

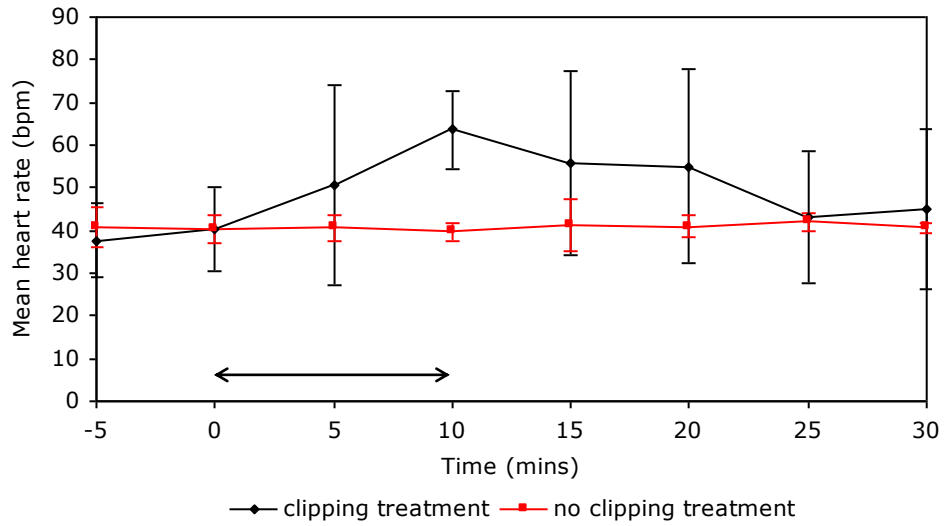


Figure 5.14(a) Mean (\pm SD) heart rate (bpm) for the non compliant horses during the sham clipping and no clipping treatment The arrow marker indicates where sham clipping occurred during the sham clipping treatment.

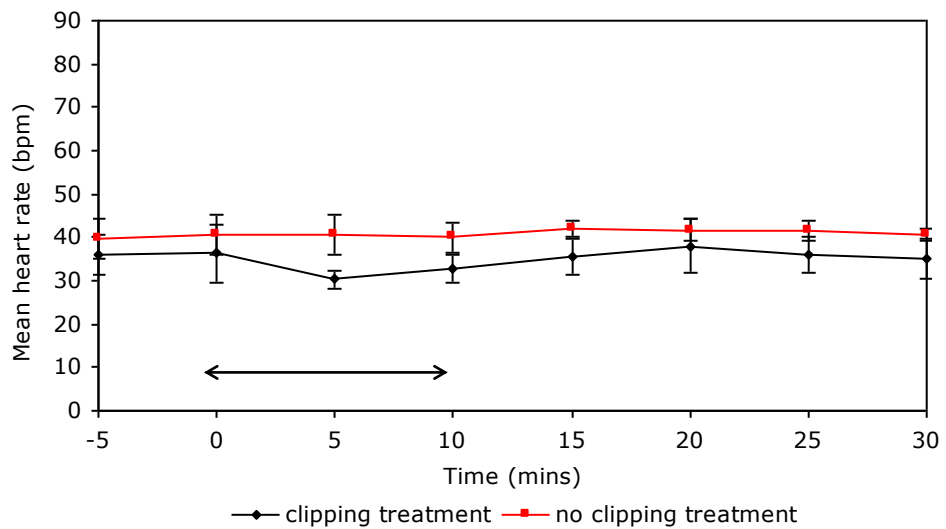


Figure 5.14(b) Mean (\pm SD) heart rate (bpm) for the compliant horses during the sham clipping and no clipping treatment The arrow marker indicates where sham clipping occurred during the sham clipping treatment.

5.8 Discussion

There was a significant ($p=0.001$) increase in eye temperature in response to sham clipping in both compliant and non compliant horses during the sham clipping treatment whereas no increase in eye temperature was recorded for either group during the no clipping study. The greatest incremental increase in eye temperature was found to be at five minutes post onset of sham clipping with a peak in eye temperature at ten minutes post onset of sham clipping as in the initial pilot study (Appendix 7). The mean increase in eye temperature of 3.6°C is larger than that reported in cows (Stewart *et al.*, 2007) and monkeys (Nakayama *et al.*, 2004) when confronted with a potentially aversive situation however the stressor in this study (sham clipping) was maintained for a longer duration. Eye temperature decreased after the ten minutes post onset of sham clipping sample which coincides with removal of the stressor therefore it may be that eye temperature would have remained elevated if the stressor had persisted, however this would need to be investigated further.

Despite the decrease in eye temperature upon removal of the stressor it had only returned to basal levels in three horses by the end of the treatment. This may be due to increased metabolic activity and therefore increased thermal output due to the effects of cortisol.

The increase in eye temperature was mirrored by the decrease in ear temperature and a strong negative relationship was found between the two parameters. As eye temperature increased, ear temperature decreased and the greatest incremental increase in temperature for both anatomical areas was five minutes post onset of sham clipping. This may suggest that both are driven by the SNS due to the rapid response at onset of the stressor. The decrease in ear temperature may be indicative of diversion of blood in response to stress-induced activation of the SNS. It may even have served to divert blood to the eye area as reported in humans when subjected to an

acoustic startle test (Pavlidis *et al.*, 2001). This initial acute response has been suggested to have the purpose of redirecting blood flow to areas with more urgent metabolic requirements and may also be a protective mechanism to reduce blood loss in the case of injury (Blessing, 2003, Vianna and Carrive, 2005). This response was also observed in the decreased ear temperature of sheep in response to transportation stress (Ingram *et al.*, 2002).

The study into temperature change in rhesus monkeys during confrontation by a handler reports that the decrease in temperature originated from an area in the uppermost portion of the nasal region and then spread to the lower regions (Nakayama *et al.*, 2004). The origin and spread of blood flow alteration and therefore temperature change was not able to be investigated during the current project. This is a limitation as it could have aided in further explaining the reasons or mechanisms behind temperature change.

There was a significant ($p=0.003$) increase in salivary cortisol in response to sham clipping in both the compliant and non compliant horses during the sham clipping study. Two horses displayed an increase in cortisol of 40% and 43% from basal levels which is suggested to be in the 'stress range' (Barnett and Hemsworth, 1990) and could potentially predispose the horse to stress related disease. This contradicts the results of Shanahan (2003) who found horses that were behaviourally compliant to loading had no increase in cortisol when compared to the behaviourally non compliant animals that displayed an increase in cortisol from seven minutes post loading. The results of the study by Shanahan (2003) may indicate that even the compliant horses in this study found the sham clipping procedure aversive. Cortisol levels were higher in the no clipping treatment when compared to the sham clipping treatment however, during the no clipping treatment cortisol levels remained constant with no increase or peak as was found in the sham clipping treatment. Salivary cortisol had begun to increase within ten minutes post

onset of stressor as found by Shanahan (2003) in horses stressed by transport and suggested by Ralston *et al* (1988) for stress induced increases in plasma cortisol. The increase in salivary cortisol within ten minutes post onset of stressor during this study supports the suggestion that salivary cortisol reflects plasma cortisol levels (Van der Kolk *et al.*, 2001) and the time taken for salivary cortisol to increase post stressor is similar to plasma cortisol (Creighton and Hughes, 2007). salivary cortisol level peaked at twenty minutes post onset of sham clipping, which is consistent with findings of past work (Marlin and Nankervis, 2002, Stewart *et al.*, 2007).

There was a positive correlation between increase in eye temperature and increase in salivary cortisol during the sham clipping treatment. This is important as the increase in salivary cortisol indicates that the horses found the procedure aversive and therefore the increase in eye temperature could suggest the same. This is reinforced by the fact that neither parameter increased during the no clipping study when the presence of the potentially aversive stimulus was removed. The large variation in mean salivary cortisol and eye temperature is due to inter animal variation in basal levels. All animals displayed a similar pattern of increase (mean increase of $15.9\% \pm 5.8\%$) and time to maximum values however they began at differing basal levels (Appendix 8) therefore it is important that pre stress measures are taken to allow each horse to act as its own control. Despite this large variation in basal levels, all horses did display a significant increase in both eye temperature and salivary cortisol in response to sham clipping.

The compliant horses' activity scores were lower than the non compliant during the sham clipping treatment, however physiological measures indicate that they found the procedure aversive. This could be due to past training and habituation towards the procedure resulting in increased behavioural compliance or it could be the horses masking stress as a survival mechanism (Berger *et al.*, 2003). It could also be that the compliant horses were

generally less active individuals. This reinforces the importance of physiological measures in order to have a robust interpretation of how the horse perceives the situation it is placed in. In this case behavioural analysis would not have been sufficient. A potential flaw in the methodology may have been that the compliant and non compliant horses were selected based on the yard managers opinion of the horses past behaviour. However the scoring of the behavioural data was carried out by individuals who were unaware of which group the horse belonged to and activity level assigned to individual horses was consistent between scorers.

Overall, eye temperature of the compliant horses was not as high as the non compliant horses and ear temperature of the compliant horses was not as low as the non-compliant horses during the sham clipping treatment however, the magnitude of increase and decrease in temperature was larger in the compliant horses than the non compliant horses. Salivary cortisol in the compliant horses also increased above cortisol levels of the non compliant horses although this was not significant. It could be that this increased physiological response in the compliant horses is a compensatory mechanism for the lack of behavioural response and that the act of masking stress is stressful in itself. As the inhibition of natural behaviour has been shown to contribute to the development of abnormal behaviour (McGreevy *et al.*, 1995, Nicol, 1999), which may be associated with stress (Mason, 1991; McBride and Cuddelford, 2001) then the training of the horse to comply with training procedures and management techniques may serve the same purpose as restriction of natural behaviour through inadequate housing and feeding regimes. This means that training of the horse may only reduce the behavioural reactivity to a stressor and not the physiological response and could still compromise health and well being.

Heart rate peaked at ten minutes post onset of sham clipping in the non compliant horses during the sham clipping treatment and was significantly higher ($p=0.001$) than the heart rate of the compliant horses which remained stable and within normal range. This may have been due to increased activity of the non compliant group compared to the compliant.

During the no clipping treatment no increase in heart rate for either group was observed.

5.9 Conclusion

The most important finding of the study was that eye temperature and salivary cortisol increased in all horses in response to clipping, despite some horses showing no behavioural signs of stress. The lag time between the two measures reflects the response time for both the SNS, which could be driving eye temperature change, and the HPA axis, which drives the cortisol response to manifest post stressor. It appears that elevated eye temperature can indicate stress in the horse during a short term potentially aversive procedure and concurs with evidence of the cortisol response which is a currently accepted measure of stress. Two horses displayed increases in salivary cortisol that reached levels that could potentially cause stress related disease if prolonged. This reinforces the need for a non-invasive and reliable measure to assess everyday management practices and improve welfare.

Domestic horses are often placed in situations that they may potentially find aversive that are of longer duration (i.e housing, transport). The positive results of this study into short term aversive stimuli prompted further investigation into the ability of IRT to assess temperature change as part of the response to more long term stress.

Chapter 6 Study D; Investigation into the use of IRT as a tool to assess thermal change associated with the physiological response to long term or repetitive stress

6.1 Introduction

Isolation is probably one of the main causes of stress in social species (Forkman *et al.*, 2007) therefore certain restrictive housing designs may have detrimental effects upon the welfare of the horse. Research suggests that a primary cause of stereotypical behaviour in horses is limited social contact and prolonged isolation through individual housing (McGreevy *et al.*, 1995). Results of both epidemiological and empirical studies show that enhancing a horse's social environment can reduce the incidence of such behaviour (McGreevy *et al.*, 1995; Cooper *et al.*, 2000; McAfee *et al.*, 2002; Mills and Davenport 2002). Irvine and Alexander (1998) report that moving horses from an open social environment into an enclosed housing design resulted in disruption of daily rhythm in plasma cortisol, which could be indicative of chronic stress. Horses involved in the study that investigated the potential limitations of IRT for this project had constant turnout in grass paddocks with their familiar companions prior to the study commencing. However, in order to standardise food intake and exercise they were placed in isolated stables with no turnout and no contact with con-specifics for a four day period. This situation could have been potentially stressful to the horses and contributed to disruption of daily rhythm of salivary cortisol.

Work so far has demonstrated that IRT is an inappropriate measure to monitor acute stress in a flight species. Data collection was difficult due to the evasive behavioural response of the horse and the application of monitoring acute stress is limited, however IRT was able to identify the thermal response to a short term (ten minute duration) potentially stressful husbandry procedure and has been shown to correlate with an increase in the stress hormone cortisol. It is now important to ascertain whether the increase in eye

temperature observed during a short term husbandry procedure is observable in periods of potentially chronic stress.

Faecal glucocorticoid analysis was used as a validatory physiological measure during this study as it is a more appropriate measure of chronic stress when compared to salivary cortisol (Queyras and Carosi 2004; Hughes and Creighton, 2007). Flight behaviour was an unreliable measure of stress during a short term husbandry procedure however, stereotypic behaviour and changes in daily activity have been shown to be indicators of stress in horses (Broom, 1991; Mason, 1991; McBride and Cuddelford, 2001; Benhajali *et al.*, 2008) therefore behavioural assessment was included in this study in an attempt to evaluate how the horses perceived the housing treatments and compare behavioural observations to any physiological (thermal, hormonal) response.

Many of the training and management techniques that domestic horses are subjected to and may potentially find stressful are of longer duration than tested so far during this project (housing, transport, foot care, training methods) and therefore validation of IRT as a measure of more long term stress would increase its usefulness as a research tool. Housing design has been found to have a substantial impact on the trainability of horses. Rivera *et al.* (2002) found group housing exerts a positive effect on behaviour. Group housed horses took less time to complete a training procedure than horses singly housed in stalls. In addition, singly housed horses showed more objectionable behaviour toward the trainer (biting and kicking) than group housed horses. Housing designs that offer increased contact with con-specifics have been shown to result in a reduction in stereotypical behaviour (McGreevy *et al.*, 1995; Cooper *et al.*, 2000) therefore housing design is an important factor in the everyday welfare of horses and may also have an impact on training performance.

Housing designs allowing differing levels of physical and social contact were used to investigate the thermal response of the eye to a potentially stressful situation of prolonged duration.

6.2 Aims and objectives

Aim

- Determine whether the change in eye temperature observed during a short term potentially stressful husbandry procedure is present in horses during periods of potentially long term or repetitive stress

Objectives

- Investigate the thermal response of the equine eye to four housing designs that allow differing levels of physical and social contact.
- Investigate faecal glucocorticoid levels between four housing designs that allow differing levels of physical and social contact and compare this to eye temperature.
- Assess activity patterns of horses housed in four housing designs that allow differing levels of physical and social contact and compare this to eye temperature

6.3 Methodology

6.3.1 Animals and Husbandry

Horses (n=16) were aged 6 to 21 years (mean age 15 years \pm 3) and breed representative of horses found in a riding school. Details of the individual horses can be found in section 2.1. The horses were divided into four groups (four horses per group) according to sex, with two groups of four geldings and two groups of four mares. The study was conducted during the summer (August) and one month prior to it commencing the horses were turned out in grass paddocks in their experimental groups of four to remove the effect of a new social group. Horses were brought in for one hour per day to be given approximately 3kg of meadow hay. This was to prevent any digestive upset due to a rapid change in forage type and availability when the study began. Horses were managed as previously discussed in section 2.1 and had all been stabled in each of the housing designs used in this study at some point prior to the study commencing.

6.3.2 Housing treatments

The four housing treatments used were single housed no physical contact (SHNC), single housed semi contact (SHSC), paired housing full contact (PHFC) and group housing full contact (GHFC). Figure 6.1 (a-d) displays the four housing treatments as viewed from the cameras used to record behaviour in each treatment. Horses had access to water at all times however hay was withheld throughout the day whilst in their experimental housing. Horses were bedded on shavings and rubber mats in the indoor housing treatments. Table 6.1 details each of the four housing designs.

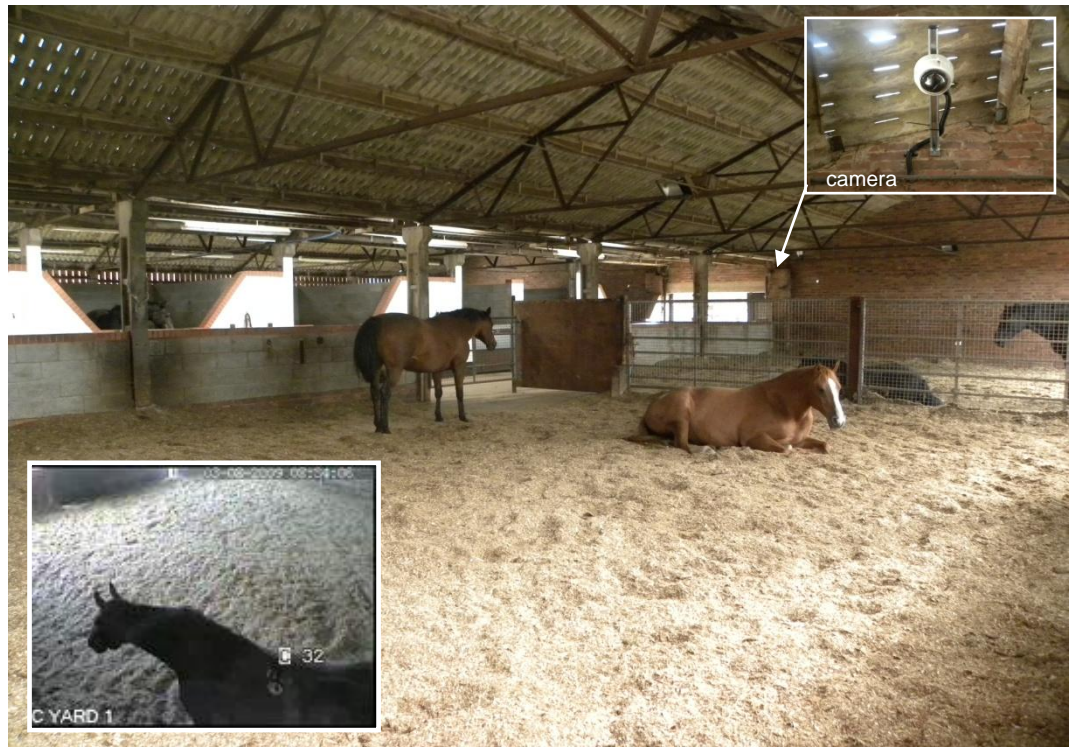


Figure 6.1 (a) Paired housed full contact housing design. Image shows the barn housing each pair of horses with position of camera. The smaller image in the bottom left hand corner shows the view produced from the camera.



Figure 6.1 (b) single housed semi contact design. Image shows the stables housing each horse with position of camera. The smaller image in the bottom right hand corner shows the view produced from the camera.



Figure 6.1 (c) single housed no contact design. Image shows the stables housing each horse with position of camera. The smaller image in the bottom right hand corner shows the view produced from the camera.



Figure 6.1 (d) group housed full contact. Image shows the paddock housing each experimental group of four horses with position of camera. The smaller image in the bottom right hand corner shows the view produced from the camera.

Table 6.1. Four housing designs used in the study with differing levels of social and physical contact.

Housing Design	Description
Group housing full contact (GHFC)	Horses were turned out in their experimental group of four into a paddock which had been grazed down prior to the study commencing. The horses had full physical contact with all other members of the group and had visual and auditory contact with horses in nearby paddocks.
Paired housing full contact (PHFC)	Horses were housed in pairs in the barn previously discussed in section 4.2.2. The barn lies adjacent to indoor single box stables which allowed the study horses visual and auditory contact with the horses stabled in them. In addition there were two horses housed in the neighbouring barn (from the same experimental group of four) which allowed visual and auditory contact through a wire partition separating the two enclosures. Each pair of horses had full physical contact with one another.
Single housing, semi contact (SHSC)	Horses were individually housed in box stables measuring 3 x 3.6 metres with a solid wall to ceiling height at the rear. The front, sides and integrated sliding door of the stable measured a total height of 2.5 metres with solid walls to 1.2 metres high and vertical metal bars spaced at 5cm apart for the remainder of the height. Visual, auditory and tactile communication with the neighbouring horse at either side was possible through the bars and the horses were also able to see their companions stabled opposite in the same housing treatment.
Single housed no contact (SHNC)	Horses were housed in box stables measuring 3 by 3.6 with 2.5 metre high solid brick walls to the rear and side. The horses only had visual contact if they and a neighbouring horse looked over the traditional half door. No physical contact with other horses was possible.

6.3.3 Experimental design

Horses were exposed to each housing treatment for a period of five days. They were brought from their paddocks where they were turned out in their experimental groups of four by the same handler at 0800h every day and walked the short distance to the relevant housing treatment. Horses remained in the housing treatment until 1600h at which point they were walked back to their paddock where they spent the night. At the end of the five day period they were turned out in their paddocks in their experimental group for two days and then the groups rotated to the next housing treatment. Table 6.4 details the four groups and the order they were rotated through the treatments. Human disturbance was limited to three times daily for approximately ten minutes when physiological measures were taken.

Table 6.2. Order of rotation of the four experimental groups through the four housing treatments

	Week 1	Week 2	Week 3	Week 4
Group 1	GHFC	PHFC	SHNC	SHSC
Group 2	PHFC	SHSC	GHFC	SHNC
Group 3	SHNC	GHFC	SHSC	PHFC
Group 4	SHSC	SHNC	PHFC	GHFC

Key

GHFC Group housed full contact (paddock)
PHFC Paired housed full contact (barn)
SHSC Single housed semi contact
SHNC Single housed no contact.

6.3.4 Data collection

The horse order in which the physiological measures were sampled remained the same each day regardless of the housing treatment the horse was in at the time. This ensured all images were captured as close as possible to the same time every day for each horse. Each horse was caught with a head collar and tied to a tie ring for physiological samples to be taken safely and also to standardise the procedure regardless of the housing treatment. The horses could move around freely but were not able to walk away whilst tied up. Two handlers were responsible for taking physiological measures and each handler was responsible for two of the four groups of horses and remained so for the duration of the study.

6.3.4.1 Infrared thermography data collection

Thermal images of the left and right lateral aspect of the head were captured three times per day for each horse at approximately 0830h, 1200h and 1530h. As the whole profile of the head was captured this allowed eye temperature, which had shown to be the most reliable measure of temperature change, to be extracted and measured. It would also allow ear temperature to be extracted if required at a later date.

6.3.4.2 Faecal collection.

Faeces were collected for glucocorticoid analysis from two randomly chosen horses in each group. One sample per day was collected from each horse on an opportunistic basis during each rotation, however the sampling protocol used for the study guaranteed the sample would be less than two hours old. Faeces were collected into a 100ml sterile plastic container and refrigerated at 4°C for no longer than two hours before they were transferred to a freezer and frozen at -20°C until analysis.

Samples from day 1, 2 and 3 in each housing treatment were assayed for the eight horses. This would allow sufficient time for any hormonal response to each housing treatment to appear in the faeces due to gut transit time (Van Weyenberg *et al.*, 2006).

A validation assay test was carried out by Chester zoo as described in chapter 2.2.1.2. The results of the test revealed the assay for corticosterone to be the most appropriate (Appendix 2). Extraction and analysis was then carried out by Chester Zoo (see Appendix 9 for extraction and analysis protocol).

6.3.4.3 Core temperature data collection

Core temperature was taken using the method previously described in chapter 2.2.2.1 Temperature was measured three times daily using the same timing parameters as the thermal image collection. Temperatures were recorded by hand onto the horses data sheet and transferred to an Excel spreadsheet at a later date.

6.3.4.4 Ambient temperature data collection

Ambient temperature was monitored for the duration of the study (Lascar EL-USB-2). The temperature loggers were set to record at thirty minute intervals Data was uploaded and processed using the methods previously discussed in chapter 2.2.2.2 This would allow any thermal changes in the eye to be fully investigated and ambient temperature ruled out or confirmed as a contributing factor.

6.3.4.5 Behavioural activity data collection

The camera system installed throughout the equestrian centre was utilised to record the behaviour of the horses in each housing treatment (Figure 6.2). The system works by having cameras fed through cables to a digital video recorder (DVR) in a central location which accepts high quality footage and records it in a compressed format (DAV). This footage can then be downloaded from the unit via a USB device or a DVD. For the purpose of this study the system was set to record in high definition quality. The DVR can store data for over a week in this format, allowing the footage to be downloaded when required. Figure 6.1 (a-d) details the positioning of the cameras in each of the housing treatments and also shows the corresponding footage produced which was used at a later date to record behavioural observations. Recording the studies allowed objective behavioural analysis to be carried out post data collection and reduced observer effects. It also ensured that all horses were visible and could clearly be observed for the whole of the study.

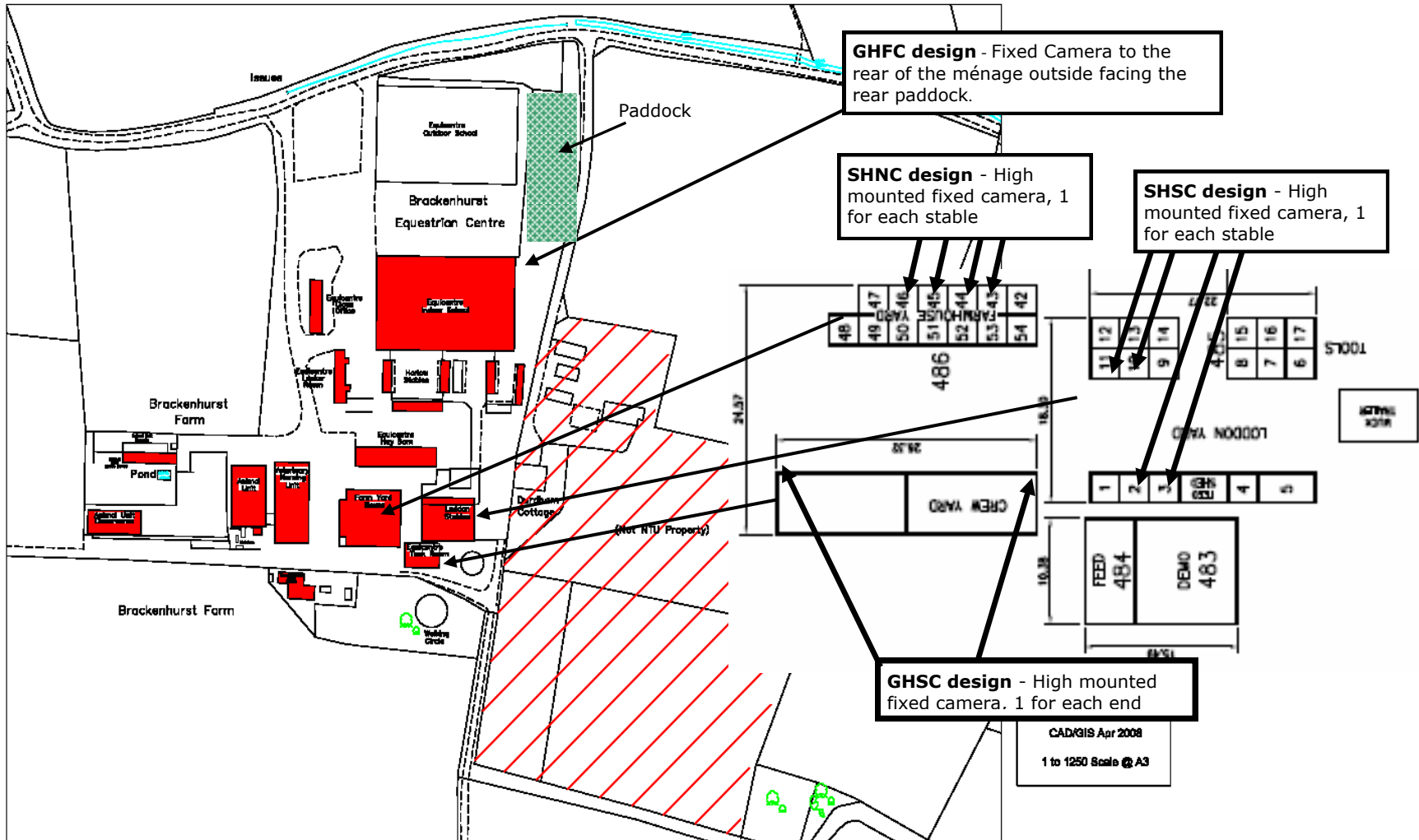


Figure 6.2 The camera system at Brackenhurst Equestrian centre

The video footage was used to form a time budget of the horses' activity in each housing treatment. An ethogram (Table 6.2) adapted from Cooper *et al.* (2000) and Heleski *et al.* (2002) was used. The listed categories were recorded and the sub-categories used to define what is included in each category. Only behaviours lasting longer than five seconds were recorded. Behavioural observations were obtained for seven hours for ten of the horses (three from group 1, three from group 2, two from group 3 and two from group 4) on the fourth day the horses spent in each of the housing treatments. The fourth treatment day was the last complete day the horses spent in each housing treatment having already been housed there for three complete days. Data were not collected whilst the horse was having physiological measures taken.

The behaviour of each horse and the time of initiation were recorded so the minutes spent in each behavioural state could be calculated and then expressed as a percentage of the total time observed. Two of the four housing treatments prevented the expression of social behaviour however it was included in the ethogram in order to investigate whether horses would participate in social behaviour in the other two housing treatments where they were able to interact with con-specifics and whether the time spent was similar to horses in their natural environment.

Table 6.3 Ethogram of behavioural states measured for analysis of activity patterns in ten study horses with definitions and sub categories to define the behaviors Adapted from Heleski et al, (2002) and Cooper et al. (2000).

Behavioural state	Sub category	Definition
Feed	Concentrate	Ingestion of concentrate food
	Forage	Ingestion of grass or hay
	Bedding	Ingestion of bedding
	Drinking	Intake of water
Standing	Standing alert apart	Standing with eyes fully open, ears forward, and body position showing alertness, more than a metre (approx.) away from another horse
	Standing alert together	Standing with eyes fully open, ears forward, and body position showing alertness, within a metre (approx.) away from another horse
	Standing resting apart	Stood still, one or both ears back, relaxed neck (lower than when alert) and eyes fully or partially closed, more than a metre (approx.) away from another horse
	Standing resting together	Stood still, one or both ears back, relaxed neck (lower than when alert) and eyes fully or partially closed, within a metre (approx.) away from another horse
Lying	Lying sternally	Lying with the sternum in contact with the ground
	Lying prone	Lying stretched out on their side
	Rolling	Number of times the horse rolls
Active	Walk	Four beat gait of forward movement
	Trot	Two beat diagonal gait of forward movement
	Canter	Three beat gait of forward movement
Social	Positive social	Interactive behaviour; nuzzling/sniffing another horse or mutual grooming
	Negative social	Aggressive behaviour, laid back ears, lowered head and neck, dominant body position, threat to kick/bite or actual kick/bite
Stereotypic behaviour	Box walking	Repetitive pacing around the perimeter of the enclosure
	Weaving	Repeated lateral movement of the head from side to side
	Nodding	Horse swings its head up and down
	Crib biting	Grasps an object with teeth and draws air into oesophagus
	Licking	Repetitive licking of surfaces
Other	Self grooming	Scratching body using teeth, foot or object
	Defecate/urinate	times the horse defecates/urinates.

6.3.4.6 Ease of handling data collection

The video footage from the fourth day of each housing treatment was utilised for this test. This was the last complete day the horses were stabled in each treatment having already been stabled there for three complete days. During the seven hour recording period each horse had a thermal image and core temperature taken and was swabbed for saliva at 0830h, 1200h and 1530h by the same handler. Saliva was swabbed to provide an example of a handling procedure and samples were kept in case they were needed for a secondary hormonal measure at a later date. An ease of handling score was designed to assess whether housing design affected the level of behavioural compliance toward human handlers as evasive behaviour can suggest discontent (Anderson *et al.*, 1999; Minero *et al.*, 2006; Christensen *et al.*, 2008). Scores were first attributed to a behavioural definition according to the behaviour of the horse and the ease of which the saliva sample was obtained (Table 6.3). The definitions used were kept as objective as possible with a scale of evasive head and foot movement only. One BSc equine science undergraduate that was unaware of the study was shown the footage of each sample point for each horse in a random order. A mean score from the three sampling points for each horse for each housing treatment was calculated. A mean score for all horses in each housing type was then calculated and plotted using Excel.

Table 6.4 Ease of handling score with scores ranging from 1 (compliant to swabbing for saliva) to 5 (resistant to swabbing for saliva). Evasive movement of head is an elevated head carriage or lateral movement away from the handler.

<u>Score</u>	<u>Behavioural definition</u>
1	Sample obtained with no evasive movement of head or steps away from handler
2	Sample obtained after 1-3 attempts due to evasive movement of head with no steps away from handler
3	Sample obtained after 3-5 attempts due to evasive movement of head and/or steps away from handler.
4	Sample obtained after more than five attempts due to evasive movement of head and/or steps away from handler.
5	Sample obtained after more than five attempts due to evasive movement of head and steps away from handler

6.3.5 Data analysis

6.3.5.1 Infrared thermography data analysis

Temperature was extracted from the thermal images as previously discussed. Mean temperature of left and right eye was calculated for each horse for each sampling time point. Mean eye temperature for all horses for each sampling point was calculated for each housing design. Distribution of data was normal (Kolmogorov- Smirnov) therefore a one way repeated measures ANOVA was conducted to examine any difference in eye temperature. A pairwise comparison (Bonferroni) was used to test for differences between each housing treatment.

6.3.5.2 Faecal corticosterone analysis

Raw data for each horse was provided in the form of a spreadsheet by Chester zoo (Appendix 10).

Initially faecal corticosterone for day 1, 2 and 3 were plotted for each horse in each of the four housing treatments using excel (Appendix 11). Faecal samples were unable to be collected on certain days for one horse. This horse was removed from the analysis. Mean faecal corticosterone for all horses on day 1, 2 and 3 were then plotted for each housing treatment.

Distribution of data was normal (Kolmogorov- Smirnov) therefore a one way repeated measures ANOVA was conducted to examine any difference in faecal corticosterone levels. A pairwise comparison (Bonferroni) was used to test for differences between each housing treatment.

6.3.5.3 Core temperature data analysis

Distribution of data was normal (Kolmogorov- Smirnov) therefore a one way repeated measures ANOVA was conducted to examine any difference in core temperature. A pairwise comparison (Bonferroni) was used to test for differences between each housing treatment.

6.3.5.4 Ambient temperature data analysis

Distribution of data was normal (Kolmogorow- Smirnov) therefore a one way repeated measures ANOVA was conducted to examine any difference in ambient temperature. A pairwise comparison (Bonferroni) was used to test for differences between each housing treatment.

6.3.5.5 Activity pattern data analysis

Following a Kolmogorov-Smirnov test for normality a Friedmann test was conducted to test for differences in feeding, standing, lying, social positive, social negative, stereotypic and other behaviours between housing treatments. A one way repeated measures ANOVA was conducted to investigate any difference in active behaviour between housing conditions. A pairwise comparison (Bonferroni) was used to test for differences between each housing treatment.

6.3.5.6 Ease of handling data analysis

Mean ease of handling score for all horses during each treatment was plotted using Excel. A one way repeated measures ANOVA was conducted to examine any difference in ease of handling between housing treatments.

6.3.5.7 Correlation analysis

The relationship between eye, core and ambient temperature was investigated using Pearson product-moment correlation coefficient. This was carried out for each group of four horses using temperature data from each horse from all four housing treatments. Mean ease of handling and mean corticosterone for all horses for each housing treatment were also plotted to investigate any relationship between the two measures.

6.4 Results

6.4.1 Infrared thermography

A one way repeated measures ANOVA was conducted to compare eye temperature measured using IRT between housing treatments. The means and standard deviations are presented in Table 6.5. There was a significant effect of housing treatment on eye temperature, Wilks Lambda = 0.68, $F(3, 221) = 34.1$, $p < 0.001$, multivariate partial eta squared = 0.32.

A pairwise comparison (Bonferroni) was used to investigate differences between treatments (Table 6.6). There was no significant difference in eye temperature between the PHFC and SHNC treatments and no significant difference between the SHSC and SHNC housing treatments. Significant differences in eye temperature were found between all other treatments

Table 6.5 Descriptive statistics for eye temperature during the four housing treatments

Housing treatment	N	Mean (\pmSD) eye temperature °C
GHFC	224	36.8 (\pm 1.5)
PHFC	224	38.2 (\pm 1.2)
SHSC	224	38 (\pm 1.1)
SHNC	224	38 (\pm 1.3)

Table 6.6 Pairwise comparison (Bonferroni) to investigate difference in eye temperature between housing treatments. Significance levels highlighted in bold indicate a significant difference in eye temperature between housing treatments.

Housing treatment	Housing treatment	Mean Difference	Std. Error	Significance
GHFC	PHFC	-1.408	.142	.000
	SHNC	-1.114	.137	.000
	SHSC	-1.115	.139	.000
PHFC	GHFC	1.408	.142	.000
	SHNC	.293	.117	.077
	SHSC	.292	.110	.049
SHSC	GHFC	1.115	.139	.000
	PHFC	-.292	.110	.049
	SHNC	.001	.109	1.000
SHNC	GHFC	1.114	.137	.000
	PHFC	-.293	.117	.077
	SHSC	-.001	.109	1.000

Key

GHFC - Group housed full contact (paddock)

PHFC – Paired housed full contact (barn)

SHSC – Single housed semi contact

SHNC – Single housed no contact

6.4.2 Faecal corticosterone

Mean faecal corticosterone levels for all horses for each housing treatment were plotted. Corticosterone levels were higher in the SHNC treatment for all three sample days in comparison to the other three treatments (Figure 6.3).

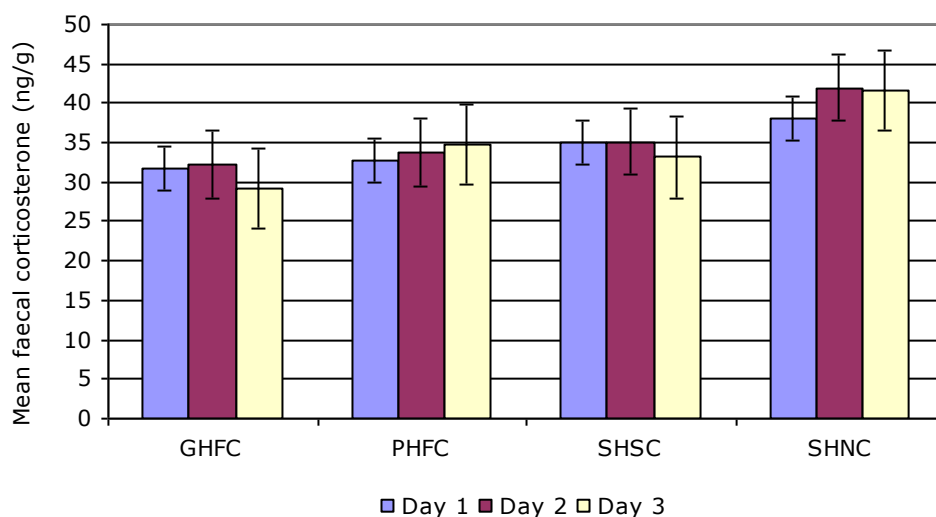


Figure 6.3 Mean (\pm SD) faecal corticosterone concentrations for seven horses for three sample days in each housing treatment.

A one way repeated measures ANOVA was conducted to compare faecal corticosterone between housing treatments. The means and standard deviations are presented in Table 6.7 There was a significant effect of housing treatment on faecal corticosterone, Wilks Lambda = 0.58, $F(3, 18) = 4.29$, $p=0.01$, multivariate partial eta squared = 0.42. with higher levels of faecal corticosterone during the single housed no contact housing treatment which was the most restricted.

Table 6.7 Descriptive statistics for faecal corticosterone during the four housing treatments

Housing treatment	Number of samples	Mean (\pm SD) faecal corticosterone (ng/g)
GHFC	21	31.0 (\pm 7.8)
PHFC	21	33.6 (\pm 10.3)
SHSC	21	34.4 (\pm 13.6)
SHNC	21	41.1 (\pm 16.5)

6.4.3 Core temperature

A one way repeated measures ANOVA was conducted to compare core temperature between housing treatments. The means and standard deviations are presented in Table 6.8 There was a significant difference in core temperature between housing treatment, Wilks Lambda = 0.38, $F(3, 220) = 118$, $p < 0.001$, multivariate partial eta squared = 0.62.

A pairwise comparison (Bonferroni) was used to investigate differences between treatments (Table 6.9). There was no significant difference in core temperature between the PHFC and SHSC treatments and no significant difference between the SHSC and SHNC housing treatments.

Table 6.8 Descriptive statistics for core temperature during the four housing treatments

Housing treatment	N	Mean core temperature °C	Standard Deviation
GHFC	224	36.9	0.5
PHFC	224	38.3	1.2
SHSC	224	38	1.1
SHNC	224	38	1.3

Table 6.9 Pairwise comparison (Bonferroni) to investigate difference in core temperature between housing treatments. Significance levels highlighted in bold indicate a significant difference in eye temperature between housing treatments.

Housing treatment	Housing treatment	Mean Difference	Std. Error	Significance
GHFC	PHFC	-1.445	.095	.000
	SHNC	-1.151	.100	.000
	SHSC	-1.146	.085	.000
PHFC	GHFC	1.445	.095	.000
	SHNC	.294	.117	.079
	SHSC	.300	.110	.041
SHSC	GHFC	1.151	.085	.000
	PHFC	-.294	.110	.041
	SHNC	.006	.109	1.000
SHNC	GHFC	1.151	.100	.000
	PHFC	-.294	.117	.079
	SHNC	.006	.109	1.000

6.4.4 Ambient temperature

A one way repeated measures ANOVA was conducted to compare ambient temperature between housing treatments. The means and standard deviations are presented in Table 6.10. There was a significant difference in ambient temperature between housing treatment, Wilks Lambda = 0.88, $F(3, 220) = 9.8$, $p < 0.001$, multivariate partial eta squared = 0.12. with ambient temperature higher in the GHFC treatment

A pairwise comparison (Bonferroni) was used to investigate differences between treatments (Table 6.11). There was a significant difference in ambient temperature between the GHFC housing treatment and all other housing treatments ($p < 0.001$). There were no other significant differences in ambient temperature between any of the other housing treatments

Table 6.10 Descriptive statistics for ambient temperature during the four housing treatments

Housing treatment	Mean (\pmSD) temperature °C	N
GHFC	21.2 (\pm 4.7)	224
PHFC	19.48 (\pm 4)	224
SHSC	19.48 (\pm 4)	224
SHNC	19.44 (\pm 4)	224

Table 6.11 Pairwise comparison (Bonferroni) to investigate difference in ambient temperature between housing treatments.

Housing treatment	Housing treatment	Mean Difference	Std. Error	Significance
GHFC	PHFC	1.767	.423	.000
	SHNC	1.812	.406	.000
	SHSC	1.771	.379	.000
PHFC	GHFC	-1.767	.423	.000
	SHNC	.045	.372	1.000
	SHSC	.004	.412	1.000
SHSC	GHFC	-1.771	.379	.000
	PHFC	-.004	.412	1.000
	SHSC	.040	.414	1.000
SHNC	GHFC	-1.812	.406	.000
	PHFC	-.045	.372	1.000
	SHNC	-.040	.414	1.000

6.4.5 Activity pattern assessment

There was no significant difference in time budget for lying and social positive behaviours between housing conditions however there was a significant difference in time budget for feeding, standing, active, social negative, stereotypy and other behaviours between housing conditions (Table 6.12). Standing behaviour was significantly reduced in the group housed full contact condition in addition to a significant increase in feeding behaviour (Figure 6.4 a-d). There was also an increase in active and social negative behaviour in the group housed full contact and paired housed full contact housing conditions.

Table 6.12 Data for the effect of housing design on time budget (%) of ten representative horses in the four housing treatments

	Feed	Stand	Lying	Active	Social positive	Social negative	STB	Other
Single housed no contact	0.91 ±1.0 A	88.52 ±7.7 A	2.97 ±9.0	1.76 ±0.5 A	0.00 ±0.0	0.00 ±0.0 A	3.94 ±4.4 A	1.90 ±1.3 A
Single housed semi contact	3.65 ±4.5 A	89.84 ±10.9 A	2.84 ±7.4	2.06 ±0.7 A	0.00 ±0.01	0.04 ±0.1 A	0.23 ±0.5 B	1.35 ±1.3 A
Paired housed full contact	5.86 ±7.8 A	81.18 ±6.6 A	3.70 ±5.9	5.42 ±4.2 B	1.39 ±1.9	1.56 ±2.3 B	0.07 ±0.2 B	0.83 ±0.5 AB
Group housed full contact	34.89 ±14.3 B	56.27 ±14.4 B	0.08 ±0.1	7.36 ±2.7 B	1.34 ±1.9	0.02 ±0.03 A	0.00 ±0.0 B	0.02 ±0.2 B

Values are means ± SD

Within a column means with different letters are significantly different (p<0.05).

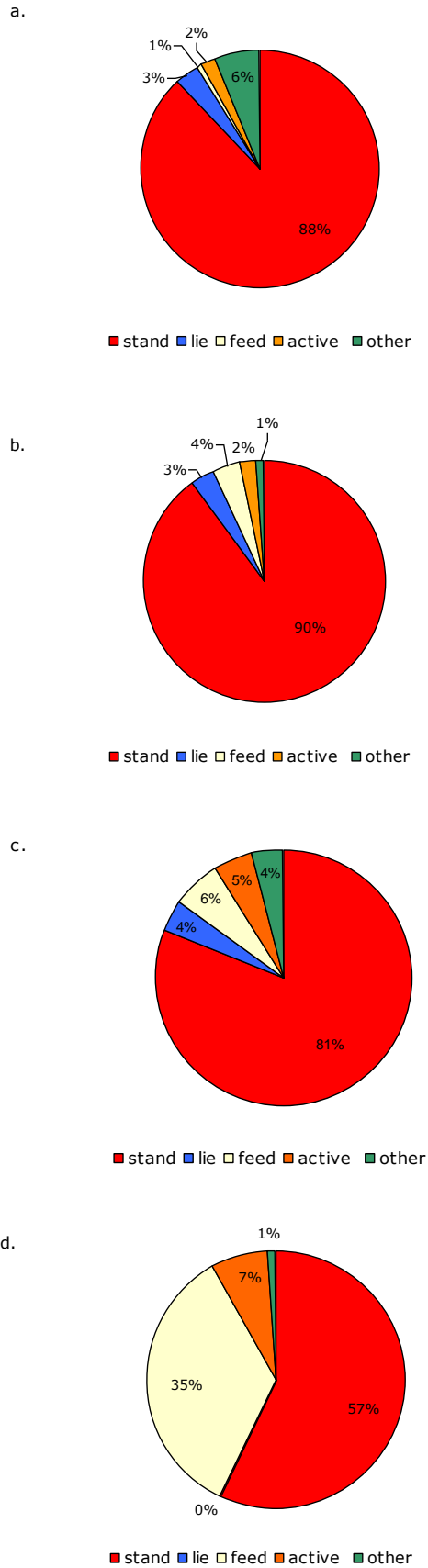


Figure 6.4 Average time budget of ten study horses in each housing treatment. a) single housed no contact, b) single housed semi contact, c) paired housing full contact d) group housed full contact. Other category is the average time spent performing social positive, social negative and stereotypic behaviours.

Significantly more stereotypical behaviour was observed in the single housed no contact housing treatment. The stereotypies performed were box-walking, head nodding, weaving and crib-biting. Stereotypical behaviour was observed in seven of the ten study horses used for time budget assessment. Only two of the ten horses displayed stereotypical behaviour in both the single housed semi contact and paired housed full contact treatments. No stereotypical behaviour was observed in the group housed full contact housing treatment.

6.4.6 Behavioural ease of handling

Figure 6.5 details the mean ease of handling score for all horses for each housing treatment. The group housed full contact and paired housed full contact treatments scored 1.1 with the single housed semi contact and single housed no contact scoring 1.2 and 1.6 respectively. As the level of isolation increased the difficulty of handling the horses due to evasive behaviour also increased. No horse was attributed a score higher than 3 from the footage used to assess handling on the fourth day in each treatment however, informal observation suggests horses did display higher levels of aggression and non-compliance with handlers throughout the trial in certain housing designs. It was noted by handlers that three horses in group A and one horse in group C displayed high levels of aggression and non-compliance in the single housed semi contact and single housed no contact treatments in addition to displaying stereotypic behaviour however in contrast they were compliant when being handled in the paired and full contact housing treatments.

A one way repeated measures ANOVA was conducted to compare behavioural ease of handling score between the four housing treatments. The means and standard deviations are presented in table 6.13. There was a significant difference in ease of handling between housing treatment, Wilks Lambda = 0.36, $F(3, 13) = 7.63$, $p = 0.003$, multivariate partial eta squared = 0.63.

A pairwise comparison (Bonferroni) showed the significant difference in ease of handling across the four treatments was between the single housed no contact design and each of the other three designs, Group housed full contact ($p = 0.003$), Paired housed semi contact ($p = 0.001$) and single housed semi contact ($p=0.02$). Horses were more difficult to handle in the SHNC design when compared to the other designs.

Table 6.13 Descriptive statistics for behavioural ease of handling score during the four housing treatments in sixteen horses

Housing treatment	Mean (\pmSD)
GHFC	1.08 (\pm 0.13)
PHFC	1.13 (\pm 0.18)
SHSC	1.24 (\pm 0.25)
SHNC	1.64 (\pm 0.5)

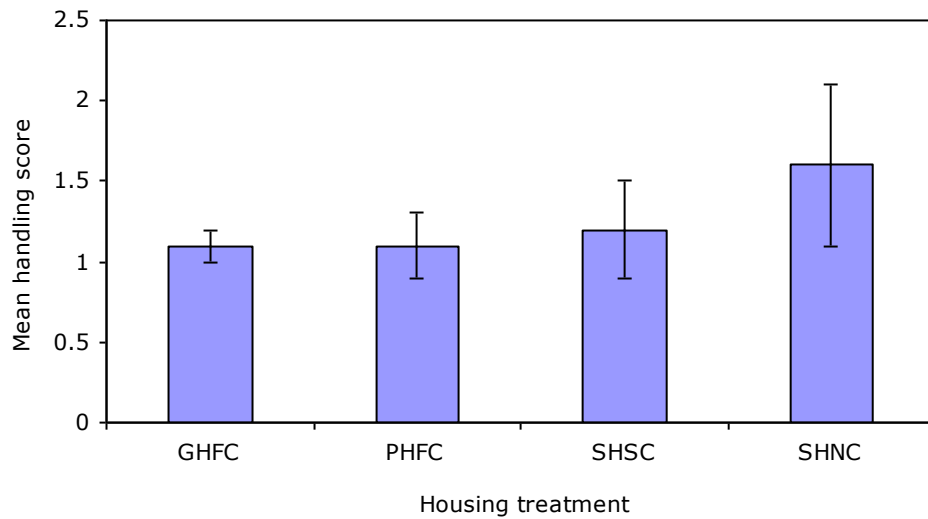


Figure 6.5 Mean (\pm SD) ease of handling score for all horses during the fourth day in each housing treatment

6.4.7 Correlation analysis

6.4.7.1 Temperature data

The relationship between core, eye and ambient temperature during all housing treatments was investigated for each group of four horses using Pearson product-moment correlation coefficient. Horses were assessed in their individual experimental groups as each group were housed in each treatment at a different time. This means ambient temperature was not standard for each treatment and it was not possible to compare temperatures of all horses together.

Group 1. There was a negative correlation between core and ambient temperature, $r=-.325$, $n = 224$, $p<.001$. As ambient temperature decreased, core temperature increased.

There was a positive correlation between eye and core temperature, $r=0.133$, $n = 224$, $p=0.046$.

There was no correlation between eye and ambient temperature ($p=0.934$).

Group 2. There was a positive correlation between eye and core temperature, $r=0.177$, $n = 224$, $p=0.008$ with high core temperatures associated with high eye temperatures. There was no correlation between core and ambient temperature ($p=0.315$) or eye and ambient temperature ($p=0.231$)

Group 3. There was a positive correlation between eye and core temperature, $r=0.223$, $n = 224$, $p=0.001$ with high core temperatures associated with high eye temperatures. There was no correlation between core and ambient temperature ($p=0.919$) or eye and ambient temperature ($p=0.09$).

Group 4. There was a negative correlation between core and ambient temperature, $r=-0.164$, $n= 224$, $p=0.014$. As ambient temperature decreased, core temperature increased. There was no correlation between eye and core temperature ($p=0.16$) or eye and ambient temperature ($p=0.47$).

6.4.7.2 Faecal corticosterone and behaviour

Mean ease of handling score for all horses in each housing treatment was plotted alongside mean faecal corticosterone level of the eight selected horses for each housing treatment (Figure 6.6). It is clear that both measured parameters increased as the housing type became more restrictive.

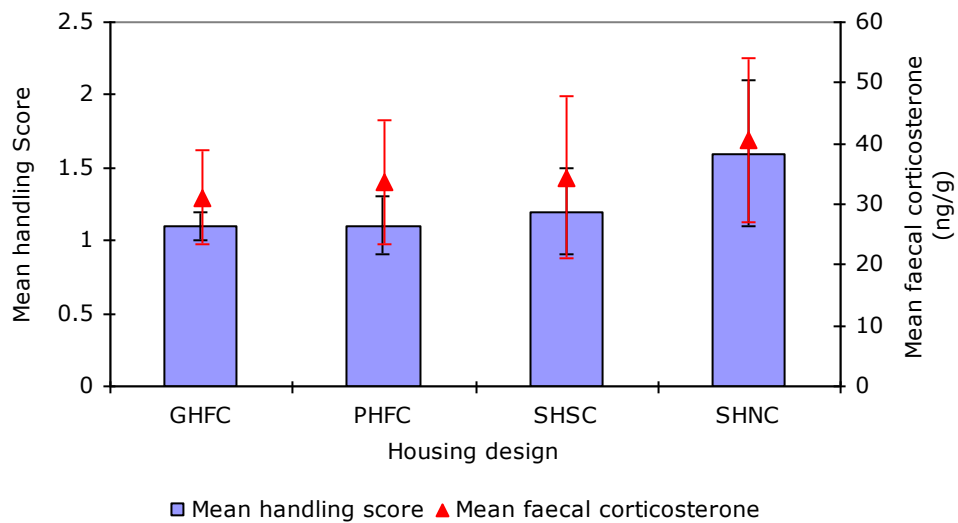


Figure 6.6 mean (\pm SD) ease of handling score for all horses in each housing treatment and mean (\pm SD) faecal corticosterone of seven horses for each housing treatment

6.5 Discussion

Mean eye temperature was significantly ($p < 0.001$) lower in the group housed full contact (paddock) treatment compared to all other housing treatments. In addition to recent work in other species (Levine *et al.*, 2001; Pavlidis *et al.*, 2001; Nakayama *et al.*, 2004; Stewart *et al.*, 2008) the study into short term potentially aversive stimuli found that increased eye temperature is indicative of a horse perceiving a situation to be stressful. Based on eye temperature alone this suggests the horses found the paddock housing treatment less stressful in comparison to the more restrictive housing types, however, this is in no way conclusive. Eye temperatures recorded during the group housed treatment were similar to many of the pre stressor eye temperatures recorded in the study into a potentially aversive husbandry procedure. However, due to large variation between horses during the study into a potentially aversive husbandry procedure there were also post stressor eye temperatures recorded that are similar to temperatures recorded during the group housed treatment. The initial study design was to compare eye temperature between housing treatments therefore it is impossible to say whether eye temperature increased upon entering any of the treatments as pre housing measures are not available. In addition, potential factors affecting temperature measurement must be taken into account. Mean paddock ambient temperature was significantly ($p = < 0.001$) higher than in all other housing treatments. There was no correlation between eye and ambient temperature in any of the groups of study horses which initially suggests any difference in eye temperature was not a direct result of ambient temperature change. However, there was a negative correlation between core and ambient temperature in two of the experimental groups of horses. As ambient temperature decreased core temperature increased.

A positive correlation in eye and core temperature was found in three of the experimental groups of horses. An increase in eye temperature may be a

reflection of increased core temperature due to the effects of thermoregulation and could explain why eye and core temperature were significantly lower in the paddock where the ambient temperature was at its highest and significantly higher in the indoor housing where ambient temperature was at its lowest.

From a physiological point of view it seems that eye temperature may be driven by the SNS due to its rapid response upon presentation of a stressor as eye temperature mirrored the SNS driven cardiovascular response in chapter 4 and 5 of this project. In evolutionary terms this makes sense as enhanced sensory awareness and a mechanism to facilitate rapid eye movements during preparedness for flight and escape are reasons suggested for increased eye temperature (Levine *et al.*, 2001; Pavlidis *et al.*, 2001; Sapolsky *et al.*, 2001). Such mechanisms are required immediately in a potentially dangerous or stressful situation. During this study the horses were subjected to potential stress of a more chronic nature. Horses may not have perceived isolation due to initial stabling in the various treatments to be stressful and by the time it became apparent that they were to remain in an inadequate environment an immediate reaction would be of no use.

Faecal corticosterone was significantly ($p=0.01$) higher in the SHNC housing treatment in comparison to all other treatments for the three days that samples were taken and supports the results of the behavioural assessment. Horses displayed significantly more stereotypic behaviour in this treatment (Table 6.13) in addition to the highest handling score. There was no significant difference in corticosterone between any of the other housing treatments. It may be that the change in level of restriction between these treatments was not severe enough to evoke a hormonal response. The difference in restriction of social contact is clearly large between the group housed full contact treatment and the treatment that imposed total isolation

upon the horses. Another reason for no difference in corticosterone between the other housing treatments is that all horses had been housed in them at some point prior to the study commencing. Perhaps if horses were exposed to the single housed treatments having never been housed there previously it may have potentially been more stressful. The horses involved in this study are riding school horses that are often stabled and isolated. This may reduce the perceived aversiveness of semi restrictive housing. It cannot be ruled out that a stressful incident in the holding paddock during the two days between treatments may have stimulated a hormonal response before the horses were placed in their housing. Elevated levels may be indicative of this prior event rather than the aversiveness of the housing design. Despite a potentially stressful situation in the paddock potentially contributing to elevated levels of corticosterone in the first sample, it was decided that samples from day 1, 2 and 3 would be the most appropriate samples to use with faecal corticosterone levels reflective of the past two days due to rate of passage of digesta (Van Weyenberg *et al.*, 2006).

In addition to gut passage rate, corticosterone levels may not have remained elevated into the fourth and fifth day as the horses may have become habituated to their housing treatment. Horses were still displaying stereotypic behaviour and altered time budgets during the fourth day however this may have been a coping mechanism for a sub optimal environment (Rietmann *et al.*, 2004) which would possibly not be reflected physiologically.

As the housing treatment became increasingly restricted and isolated for the horses they became increasingly difficult to handle. The ease of handling assessment revealed that horses were significantly more difficult to handle ($p=0.003$) in the single housed no contact housing treatment compared to all other treatments. This was the most restrictive and isolated treatment and supports the findings of Rivera *et al.* (2002), who found singly housed horses

showed significantly more objectionable behaviour (biting and kicking) toward their trainer than group housed horses.

Assessment of time budget revealed horses displayed significantly less stereotypical behaviour in the group and paired housed treatments. No stereotypical behaviour was observed in any of the ten horses in the group housed full contact treatment however seven out of the same ten horses observed in the single housed no contact treatment displayed stereotypic behaviour. This number was reduced to two horses during the single housed semi contact and paired housed treatments. Informal observations include one horse displaying chronic head nodding in the single housed treatments that was not displayed in the group or paired housed treatments. Stereotypical behaviour observed in the single housed treatments included box walking, weaving and crib biting. This supports the suggestion that increasing access to con-specifics and providing opportunity to display natural behaviour reduces stereotypical behaviour and subsequently improves welfare (McGreevy *et al.*, 1995; Cooper, *et al.*, 2000; McAfee, *et al.*, 2002). Horses also displayed time budgets similar to feral horses in the group housed condition. Grazing (mean 34.89 ± 14.3) was below that reported in feral herds (Duncan, 1980; Boyd, 1988) however Przewalski horses have been reported to peak in grazing behaviour between 2000h and 0000h in summer (Boyd, 1991). As the time budget recordings stopped at 1600h this peak in grazing would not have been seen, indicating that for the recording time, 0830-1600h during August, the level of feeding may be representative of that seen in feral horses. The time spent active in the group housed and paired housed treatments conditions was also similar to that observed in feral horses (Duncan, 1980; Boyd, 1988) and highlights the restriction of movement in the single housed treatments. There was also an increase in active and social negative behaviour in the group and paired housing conditions, play and agonistic behaviour are a predominant part of the horse's behavioural

repertoire (McDonnell and Haviland, 1995) and the demonstration of play behaviour is thought to be an indicator of good welfare (Christensen *et al.*, 2002). Horses in this study were moved through a series of housing designs which included exposure to a group of horses, a single horse and isolation. This may have disrupted dominance hierarchies and contributed to elevated aggression.

The primary aim of this study was to investigate and potentially validate IRT as a measure of chronic stress. Although eye temperature was significantly lower in the group housed treatment which was consistent with low faecal corticosterone and the behavioural assessment, the confounding effects of ambient temperature cannot be ruled out. The correlation between core and ambient temperature and core and eye temperature suggest that thermoregulatory mechanisms responding to an increased ambient temperature result in a decrease in core temperature which is reflected in eye temperature. The increased duration of the study could possibly have allowed a greater chance for changes in ambient temperature and subsequently core temperature to confound results. During the study into the potentially aversive husbandry procedure ambient temperature remained constant over the sampling period which was much shorter duration. In addition it makes physiological and evolutionary sense that eye temperature change be short term. Increased cognitive vigilance and rapid eye movements are associated more with acute and short term stress (e.g predation) and it would be impractical and of great energetic cost to maintain increased sensory awareness over a longer duration. In this case faecal corticosterone was a more appropriate measure to assess potential chronic stress when compared to eye temperature.

6.6 Conclusion

During this study horses showed a wider range of behaviour similar to that of free ranging horses, in addition to decreased incidence or absence of stereotypic behaviour when in the social housing treatments. These treatments provided an environment where horses were able to display natural behaviour and allowed contact with con-specifics. The behavioural findings imply that the social housing treatments were less aversive than the single housing and provided an improved standard of equine welfare. In addition horses were easier to handle in these treatments. Faecal corticosterone results support the behavioural findings with significantly higher levels reported in the isolated treatment, however it is unclear whether differences in eye temperature were a result of housing design or due to the confounding factors of ambient temperature and subsequent thermoregulatory mechanisms. It appears that IRT may not be suitable to monitor chronic stress due to the physiological mechanisms that drive such change. This study has highlighted the importance of housing design and its contribution to the physiological and behavioural welfare of domestic horses.

Chapter 7. General discussion

7.1 Main findings of the project

Horse owners and handlers are often unaware of the elements of domestication that animals may find stressful. Stress is often caused by factors associated with an animal's evolutionary history, including natural habitat, behaviour and social structure being disrupted or altered through captivity. Acute stress has an adaptive role however if this stress becomes repetitive or chronic then stress related disease and behavioural problems may emerge and compromise welfare. It is therefore necessary from both a legal and ethical point of view to objectively assess the domestic environment in order to identify these potentially stressful situations and alter them if required in order to improve welfare.

The results of existing work that measured temperature change using infrared thermography as a measure of the stress response prompted this project. The aim of the project was to determine whether a thermal response to stressful stimuli was present in the horse and whether it could be measured using IRT.

Existing work reports an instant change in the nasal and eye temperature of monkeys (Nakayama *et al.*, 2004) and the eye temperature of cows (Stewart *et al.*, 2008) in response to acute stress. This project began by investigating whether the same response was present in the horse using a startle response test. Data from one horse revealed the same rapid change in eye temperature when the horse was exposed to an acute stressor, however capturing this response was difficult due to the species specific behavioural response of flight. A modified experimental design may make capturing this response possible however the use of IRT to monitor such acute stress is limited as many of the potentially stressful management practices and training procedure that domestic horses are exposed to and that need to be objectively assessed are of longer duration. The evasive flight behaviour

displayed by the horses was sufficient to confirm that they found the situation they were in to be stressful.

Eye temperature measured using IRT increased during a short term potentially stressful situation (clipping) and correlated with an increase in the currently accepted stress measure of salivary cortisol. However, eye temperature measured using IRT was shown to be unsuitable as a measure of chronic stress.

7.2 Temperature change in response to acute stress

Initially the thermal response of the eye to acute fright was investigated. This was a logical place to begin as existing work had measured temperature change in response to acute or very short duration aversive stimuli. In the bovine species an initial decrease in eye temperature was reported followed by a significant rise above basal levels when calves were exposed to an acute husbandry stressor (Stewart *et al.*, 2008). When the response to acute stress was investigated in the horse during this study it became apparent that the use of IRT to measure eye temperature change inappropriate. Thermal recordings of the eye were difficult to capture due to sudden movement of the horse caused by species specific flight behaviour. Eye temperature data from a single horse did indicate a similar response of eye temperature to that reported cows (Stewart *et al.*, 2008) and humans (Levine *et al.*, 2001; Pavlidis *et al.*, 2001) when subjected to acute stress.

The one thermal profile available displayed a decrease in eye temperature of 1.8°C which is consistent with the drop in eye temperature found following acute fright (Schaefer *et al.*, 2006) and pain during disbudding (Stewart *et al.*, 2008) in cattle. The magnitude of the decrease in eye temperature was also consistent with the drop in nasal temperature in monkeys that were threatened by a handler (Nakayama *et al.*, 2004). Eye temperature of the one study horse then increased by 1.1°C back to basal level however it did not

mirror the increase above basal levels reported in the disbudded cows (Stewart *et al.*, 2008).

Two explanations for the absence of an eye temperature increase above basal levels in the continuous recording may be the acute nature and immediate removal of the stressor. This may have been interpreted by the horse as the passing of danger therefore the stress response was no longer stimulated. In addition, the stressor used in cattle was a disbudding procedure (Stewart *et al.*, 2008), which is longer in duration and eye temperature possibly indicated stress caused by pain and not fright. The single horse from which thermal data was available was also assigned the lowest behavioural reactivity score and displayed the smallest increase in salivary cortisol. The reduced physiological response and behavioural reaction could indicate that the horse did not perceive the procedure to be as stressful as the other study horses and this could also account for the absence of any subsequent rise in eye temperature. Despite a small increase in salivary cortisol and a reduced behavioural response eye temperature did display a change consistent with that found in other species (Nakayama *et al.*, 2004; Schaefer *et al.*, 2006; Stewart *et al.*, 2008). This may suggest that eye temperature is a more sensitive measure of the stress response compared to cortisol measurement and behavioural assessment.

Thermal data from only one horse meant that findings were inconclusive and it is unlikely that any further investigation would capture enough data to allow validation of the response. Past studies into the thermal response to acute stress involved animals that displayed forms of defence behaviour other than flight (Nakayama *et al.*, 2004) or employed restraint of the test subject (Nakayama *et al.*, 2004; Stewart *et al.*, 2008). It would be possible to restrict horses' movement however the level of restraint could prove dangerous to handlers and detrimental to the horse, in addition to being stressful in itself which could confound results. IRT would have limited use in the horse in

potentially stressful situations that last a few seconds. None of the equine management practices and training procedures currently under scrutiny by are of longer duration and include transportation, husbandry procedures, housing design and ridden training methods.

7.3 Temperature change in response to short term stress

Chapter 5 (study C) revealed that IRT can identify an increase in eye temperature associated with the stress response when horses are exposed to aversive stimuli of short duration. Eye temperature increased in ten horses when exposed to ten minutes of sham clipping and correlated with an increase in the stress hormone salivary cortisol. This is an important finding of the project as many of the husbandry procedures that could potentially cause stress are of short duration (clipping, transport, farriery, dentistry, abrupt weaning).

Eye temperature response in other species is immediate when presented with acute aversive stimuli (Levine *et al.*, 2001; Pavlidis *et al.*, 2001; Nakayama *et al.*, 2004; Stewart *et al.*, 2008) and during this project it has been shown to occur within five minutes of exposure to aversive stimuli. It is therefore unlikely that the hypothalamic-pituitary-adrenal system is responsible for initial temperature change as this system takes longer to be fully functional and for physiological signs to show post stressor (Nelson, 2005). Rapid thermal change was mirrored by the cardiovascular response during this project (chapter 5, section 5.6.5). As the cardiovascular response is driven by the sympathetic nervous system then it seems logical that the same system is driving the mechanisms to facilitate eye temperature change. An explanation for an increase in eye temperature was put forward by Levine *et al.* (2001) and Pavlidis *et al.* (2001). Both authors suggest that the immediate increase in eye temperature in response to stressful stimuli make physiological and evolutionary sense as it could represent a mechanism to facilitate rapid eye

movements during preparedness for flight and escape. This is particularly relevant in the horse which is a flight animal and relies on enhanced sensory function to survive predation and danger (Sapolsky *et al.*, 2000).

Eye temperature increase during sham clipping was greater (up to 6.7°C, Table 5.3 chapter 5) and remained elevated for longer (>20 minutes post removal of stressor) than in other species including monkeys (Nakayama *et al.*, 2004) and cows (Stewart *et al.*, 2008). This may be due to species specific differences in survival mechanisms. The horses primary defence mechanism is flight and they have evolved bio-mechanically to accommodate this response. In comparison monkeys have alternate primary forms of defence including aggressive behaviour, facial expressions and vocalisations. As flight is so important to survival in horses and their innate reaction is to flee from danger, the supporting physiological mechanisms of increased sensory function and rapid eye movement will be equally as important as the behavioural response. If reliance on these defence mechanisms is paramount to the horse, this could explain the higher or longer reported temperatures when compared to other species that rely on other types of defence. Eye temperature remained elevated for the duration of the stressor and declined once the stressor was removed. It is not possible to say how long eye temperature would have remained elevated if the stressor were maintained, however this is an important factor to consider during future work as it will highlight the working limits of IRT and the types of procedure it can assess.

An alternate theory to species specific survival mechanisms resulting in a larger increase in eye temperature in comparison to other species may simply be that the differences in eye temperature are a reflection of the longer duration for which the stressor was maintained. Monkeys subjected to aversive stimuli were exposed for three minutes (Nakayama *et al.*, 2004) and eye temperature had returned to baseline four minutes post stressor. Cows

and humans were subjected to acute stress of a few seconds (Levine *et al.*, 2001; Pavlidis *et al.*, 2001; Stewart *et al.*, 2008). During this study the stressor was maintained for ten minutes which could have allowed eye temperature to continue rising until removal of the aversive stimulus. The longer duration of exposure could account for eye temperature being elevated above levels previously reported. Finally the type of stressor may have contributed to the larger reaction in horses. The placement of the clippers represented the target areas for predator attack in this species (Farmer-Dougan and Dougan, 1999) which could have evoked an innate and stronger fear. Disbudding in cows (Stewart *et al.*, 2008) and human handling in monkeys (Nakayama *et al.*, 2004) may not have been stressful enough to produce a response of the same magnitude

Ear surface temperature was recorded using IRT during study C. There was a negative correlation with eye temperature in response to sham clipping. As eye temperature increased, ear temperature decreased. This is consistent with findings of a study by Ingram *et al.* (2002) who found a negative correlation between ear temperature and heart rate of sheep during the potentially stressful situation of transportation. As ear temperature decreased, heart rate increased. Ingram *et al.*, (2002) suggest the decrease in peripheral temperature may be due to vasoconstriction and diversion of blood in response to stress-induced activation of the SNS. This initial acute response acts to redirect blood flow to areas with more urgent metabolic requirements (skeletal muscle, heart and lungs) and may also be a protective mechanism to reduce blood loss in the case of injury (Blessing, 2003; Vianna and Carrive, 2005) both of which would be beneficial to the horse as a prey species. Stress related decrease in ear pinna temperature (measured with temperature sensors) that correlated with an increase in stress hormones has also been

reported in sheep subjected to isolation (Lowe *et al.*, 2005) and rabbits when subjected to a startle test (Yu and Blessing, 1997).

The decrease in ear temperature of horses during study C ranged from 2°C to 11.9°C which is a greater range than eye temperature (1.5°C to 6.7°C) and may suggest that ear temperature is affected more by environmental temperature change due to its role in thermoregulation. Despite providing another potential anatomical area to assess the thermal response to stress it seems that during this study, eye temperature provided a more reliable measure of the stress response.

7.4 Temperature change in response to chronic stress

The explanation put forward by Levine *et al.* (2001) and Pavlidis *et al.* (2001) that the immediate increase in eye temperature in response to stressful stimuli could represent a mechanism to facilitate rapid eye movements during preparedness for flight and escape, implies that the difference in eye temperature between housing treatments during the study into chronic stress (chapter 6) are likely to be as a result of thermoregulatory mechanisms rather than as a stress response. The immediate need for enhanced sensory function and eye movement in a prey species is likely to be required during acute or short term stress (predation, aggressive encounters). Activation of this mechanism for longer duration would be impractical and energetically costly. Furthermore, horses may not have perceived isolation due to initial stabling in the various treatments to be stressful and by the time it became apparent that they were to remain in an inadequate environment an immediate reaction would be of no use.

It seems that horses may use behavioural coping mechanisms including stereotypic behaviour and alterations in time budget rather than flight and related defence mechanisms to combat stress of chronic duration. Study D that investigated the thermal response to chronic stress for this project

indicated that faecal cortisol is an appropriate measure to assess how animals perceive management practices of longer duration and supports the findings of extensive work that has used this established technique as a measure of long term welfare in horses (Berghold *et al.*, 2007; Merl *et al.*, 2000) and other species including African wild dogs (Monfort *et al.*, 1998), spotted hyaena (Goymann *et al.*, 1999) and leopards (Wielebnowski *et al.*, 2002).

7.5 Hormonal response to stress

The time to maximum salivary cortisol level during the study B (chapter 4) is consistent with previous work that reports a peak in cortisol between ten and thirty minutes post stressor (Colborn *et al.*, 1991, Shanahan, 2003, Stewart *et al.*, 2007). The highest cortisol level displayed was 81.5ng/ml which was an increase of 69.6ng/ml in horse number 4 and is similar to the increase in salivary cortisol reported by Moon *et al.* (2004) in abruptly weaned foals. The remaining cortisol levels and magnitudes of increase were similar to the change in cortisol reported in cows during disbudding (Stewart *et al.*, 2007). Two horses displayed an increase in cortisol prior to the presentation of the aversive stimulus (Horse 2 and 5, Appendix 6) which may be due to anticipatory stress. In both horses cortisol increased post stressor. This increase in salivary cortisol supports the idea that the horses found the situation to be stressful and supports the findings of the one thermal profile available. As previously discussed this single horse did display an increase in salivary cortisol however this was the smallest increase from all of the study horses and suggests that eye temperature is a more sensitive measure of the stress response when compared to salivary cortisol.

Salivary cortisol also increased in response to sham clipping during study C and demonstrated peak concentrations consistent with those reported in existing work (Marlin and Nankervis, 2002, Stewart *et al.*, 2007). In two study horses levels increased by 40% and 43% from basal level which is

suggested to be within the 'stress range' (Barnett and Hemsworth, 1990) and could potentially predispose the horse to stress related disease. This highlights that a stressful procedure lasting only ten minutes can still be of detriment to the health of domestic horses. Salivary cortisol increased within ten minutes post onset of stressor which is similar to plasma cortisol levels (Van der Kolk *et al.*, 2001) and the time taken for salivary cortisol to increase post stressor is similar to that reported in plasma cortisol by Creighton and Hughes, (2007). Salivary cortisol can therefore offer a less invasive and stressful means of collecting reliable hormonal data from the horse.

The results of the faecal corticosterone assessment in chapter 6 (study D) support the suggestion that isolated housing which limits interaction with conspecifics can be stressful for horses (McGreevy *et al.*, 1995; Cooper *et al.*, 2000). Higher levels of corticosterone were displayed when the horses were housed in the restricted stable designs when compared to housing that mirrored the natural habitat and allowed increased contact with conspecifics. During study D faecal corticosterone was suitable to assess how the horses perceived each housing design. Hormonal results were supported by the behavioural findings of increased stereotypic and objectionable behaviour in the restricted housing designs which have been associated with reduced welfare or stress caused by an inadequate environment (Broom, 1991; Mason, 1991; McBride and Cuddelford, 2001) or indicative of a situation in which a horse lacks a certain degree of control (McAfee *et al.*, 2002). Despite collection of faeces being on an opportunistic basis the experimental design and sample times of the other physiological measures meant that each sample was less than two hours old. In this case faecal hormone analysis was shown to be a good method of assessing well being in terms of long term welfare and could also be suitable for assessing well being in free ranging species including horses.

7.6 The relationship between temperature change and hormonal response to stress

During this project in all cases of an increased eye temperature in response to a potentially stressful situation an increase and positive correlation with the stress hormone cortisol was also reported.

Both eye temperature and salivary cortisol increased in response to the potentially stressful procedure of sham clipping with a positive correlation between the two physiological variables. This is important as the increase in salivary cortisol indicates that the horses found the procedure aversive and therefore the increase in eye temperature could suggest the same. This is reinforced by the fact that neither parameter increased during the no clipping study when the presence of the potentially stressful stimulus was removed.

There was a lag time between the peak in eye temperature and the peak in salivary cortisol which reflects the timings of the physiological mechanism behind each response. Cortisol release is controlled by the hypothalamic-pituitary-adrenocortical (HPA) axis and the response takes several minutes to be fully functional (Nelson, 2005). Eye temperature mirrored the cardiovascular response during this project which is facilitated by the sympathetic nervous system. This mechanism is activated within seconds of perceiving a stressor which is reflected in the timing of eye temperature change and explains why a peak in eye temperature is observed followed by a peak in cortisol level. This means that eye temperature offers a more immediate assessment of how the horse perceives its situation without the need for prolonged sampling.

7.7 Behavioural response to stress

During this project behavioural assessment did not always reflect the physiological stress response. The single horse from which thermal data was available during study B was assigned the lowest behavioural reactivity score despite physiological measures indicating it found the situation to be stressful. Horses appeared tolerant and behaviourally compliant with sham clipping despite physiological indication that they found the procedure to be stressful. Therefore in both cases, behavioural assessment was not a reliable method of assessing how the horses perceived the procedure. The inconsistency between behavioural and physiological measures could be due to training by human handlers resulting in horses learning not to react to these particular stressors. These results support the suggestion that physiological measures, which cannot be masked in the way behaviour can, are needed to allow a robust interpretation of equine welfare (Anderson *et al.*, 1999; Strand *et al.*, 2002; Momozawa *et al.*, 2003; Pritchett *et al.*, 2003; Minero *et al.*, 2006) and overcome the behavioural limitations of masking stress, habituation or training effects in the horse.

An increase in cortisol of 40% from basal levels is suggested to be contributory to stress related disease (Barnett and Hemsworth, 1990). Two horses involved in this project displayed cortisol levels that increased by 40% and 43% from pre stress measures and both were in the group that were behaviourally compliant with the clipping procedure. This again highlights the importance of physiological measures in assessing current management and training procedures and may even suggest that masking any behavioural response is just as stressful for the horse or maybe more stressful than displaying the behavioural response. Despite this horses are trained to learn that such a behavioural reaction will not be tolerated and this may have reinforced the masking of the behavioural stress response and could ultimately compromise the horses' health and well being. As clipping is usually

limited to between one and four occasions per year it is unlikely that this particular repeated stimulation of the stress response and subsequent rise of cortisol into "stress levels" would impact on the horses' health. However, there are other husbandry procedures of a more repetitive nature that may evoke the same response and that may not be identified by behavioural observation alone therefore the effects of such procedures may be going unnoticed.

Despite these findings behavioural assessment still has a place in assessing welfare. It can be used to differentiate between 'non-threatening' stress and threatening stress for example a stallion covering a mare. During study D behavioural assessment during potentially chronic stress revealed an increased incidence of stereotypic behaviour in the restrictive housing designs which can be associated with reduced welfare or stress caused by an inadequate environment (Broom, 1991; Mason, 1991; McBride and Cuddelford, 2001). When used in the appropriate situation alongside physiological measures behaviour still has a place in assessing equine welfare.

7.8 Limitations of Infrared thermography

The main limitation of IRT during this study was the capability of the thermal camera (Mobir® GuidIR M4 static thermal image camera). Study B utilised a camera capable of continuous thermal recording (FLIR ThermoVision A40M) however this camera was only available for the one study. The camera utilised for the remainder of the research (Mobir® GuidIR M4 static thermal image camera) was not capable of continuous recording and captured only static thermal images. This meant that any instant thermal response reported in existing work (Stewart *et al.*, 2008) was potentially overlooked. It was therefore impossible to report on any thermal change within the period between initial presentation of the stressor and the first static thermal image for example any sudden temperature change between the pre stressor and

first post stressor thermal images during the study into short term stress (study C). Nakayama *et al.* (2004) reports that nasal temperature started its descent between 10 and 110 seconds post onset of confrontation in all monkeys and had returned to pre stressor levels within four minutes post removal of confrontation therefore at the time of the first post stress static thermal image for study B at five minutes post stressor, any changes in eye temperature had possibly dissipated. This theory is supported by Stewart *et al.* (2007) who state that it is possible that studies which have only reported increases in temperature in response to acute stress may have failed to detect an initial decrease in eye temperature due to its instantaneous nature. It is likely that the static thermal sampling during this project was too infrequent and therefore could not capture such an immediate temperature change.

The study into temperature change in rhesus monkeys during confrontation by a handler reports that the decrease in temperature originated from an area in the uppermost portion of the nasal region and then spread to the lower regions (Nakayama *et al.*, 2004). The authors were able to investigate the origin of temperature change and how it spread through use of a thermal camera with video recording capabilities and subsequent pixel analysis of the footage. The origin and spread of blood flow alteration and therefore temperature change was not able to be investigated during the current project. This is a limitation as it could have aided in further explaining the reasons or mechanisms behind temperature change. The stress induced change in monkeys was due to fear of a threatening handler. It may be that pain and environmental stress elicits a different pattern of temperature change to fear induced stress and without a continuous thermal recording these potential differences cannot be investigated.

Another functional limitation of the camera was the effect of sunlight during outdoor measures. The glare of the sun on the camera screen (which previewed any captured images to the user) made the positioning of the study horse difficult and increased the length of time needed to capture an image. The camera preview screen had to be shaded by hand or moved out of direct sunlight in order to confirm that the image was adequately positioned and of sufficient quality to assess at a later date. This would have been more problematic if temperatures were required to be read straight from the screen. The distance capability of the camera was also a limitation. Once the camera was held approximately five metres from the horse the image produced by the camera was greatly reduced in quality making it impossible to select the specific eye area accurately. At greater distances the horse appeared as a blurred shape making it impossible to select even larger anatomical areas. This limitation did not impact on this project as images were captured at a set distance of 1-1.5 metres from the study horse, however it does have implications for this particular model of camera being used in a free ranging setting where it may be impossible to get close to the horses.

Aside from these limitations the image quality of the thermal images, if images were taken carefully within the set distance from the study horse, was sufficient to extract the required temperatures. The Mobir[®] GuidIR thermal camera has a thermal sensitivity of $\leq 0.1^{\circ}\text{C}$ and the FLIR ThermoVision A40M thermal camera a thermal sensitivity of 0.08°C . Therefore both cameras can reliably measure the temperature changes reported during this project and would also be capable of measuring the temperature change of 0.6°C seen in cattle (Stewart *et al.*, 2008) and 0.2°C in monkeys (Nakayama *et al.*, 2004).

Individual variation in basal eye temperature questioned the value of using group means, however despite this variation, all horses in study C displayed

similar patterns of increase and time to maximum temperature (Appendix 8) and a significant ($p < 0.001$) increase in mean eye temperature was reported therefore mean data may still have value when assessing horses as a group. It is, however, important that pre-stress temperature measures are taken to allow each horse to act as its own control and enable investigation of individual animals to be carried out if necessary.

Existing work has cited ambient temperature as a possible confounding factor during the use of IRT (Kastelic *et al.*, 1996; Eddy *et al.*, 2001; van Hoogmoed and Snyder 2002). Ambient temperature remained stable with only minor fluctuations throughout study C and there was no correlation between eye temperature and ambient temperature during preliminary study 1 (Appendix 4). However, it appears that ambient temperature may have confounded results of the study into long term stress by activating thermoregulatory mechanisms. If core temperature had not been measured in addition to eye temperature this may have gone unnoticed, however a correlation between core and eye temperature suggests that changes were due to changing environmental temperature rather than as a response to stress. As no definitive answer can be given on the effect of ambient temperature upon eye temperature measured during this project, it is important that environmental temperatures are monitored in a research setting when utilising IRT in order to better interpret any physiological thermal change.

If the main limitation of IRT is the confounding effects of ambient temperature then this is something that can be monitored and taken into account during scientific research. In comparison many of the limiting factors of existing methods to assess the stress response cannot be controlled. These include the effects of horse temperament and past experience during behavioural assessment and the time to analyse physiological samples in the laboratory. Advances in technology mean that the one off costs of high

specification cameras which offer better quality images are lower in comparison to the ongoing costs of laboratory based studies.

7.9 Application of IRT as a tool to assess the stress response in the horse

After consideration of the practical limitations of thermal imaging in the domestic equine environment it is clear that as a research tool IRT is of most use in assessing the thermal response to stress during short term potentially stressful situations. Despite some of these short term management practices and training procedures being sporadic or rare on an individual basis (clipping up to twice per year lasting approximately one hour), it may be that the total biological cost of such procedures has an impact on equine welfare. If horses are being subjected to many different situations involving potential short term stress then the stress response may be being repeatedly or even chronically activated. Often horse owners plan aspects of horse care and management practices to occur on the same day from an organisational and time management point of view. A possible scenario may be that a horse will have new shoes fitted, a dental check and be clipped and then placed in an isolated stable all within a twenty four hour period. If the horse found more than one of these procedures to be stressful then the stress response could be activated for a prolonged period and this could predispose the horse to health problems. IRT could be used as a tool to assess these short term practices in order to evaluate their impact upon the horse. If it was apparent that the horse found more than one procedure to be stressful then one or more of the procedures could be carried out at a separate time. This could minimise the effects of the activated stress response by allowing the horse time to recover and cortisol levels to return to basal level.

IRT would be most appropriately used as a research tool to identify which of the many short term husbandry procedures evoke a stress response. These

procedures could then be limited or altered if necessary and avoided altogether if more than one procedure needed to be carried out on the same day.

IRT has been used to assess the stress caused by pain (Cook *et al.*, 2006; Stewart *et al.*, 2008). However, IRT also offers an objective means of investigating environmental causes of stress which are often more difficult to identify than stress caused through pain. Environmental causes of short term stress are common and cover diverse situations including abrupt weaning, transportation, grooming techniques and ground and ridden training techniques. A horse that finds any of these situations stressful may mask the stress response, either due to training or simply as a survival mechanism and so environmental stress is of potentially greater risk to equine health than pain which is often site specific and produces obvious behavioural signs. Pain can also be controlled or even alleviated in the domestic situation however; environmental stress cannot be managed or avoided without altering the procedure which is causing the stress. This is difficult if outward signs are not always apparent.

7.10 Future work

It is not possible to say how long eye temperature would have remained elevated if the stressor of sham clipping were maintained during study C, however this is an important factor to consider as it will highlight the working limits of IRT and the types of procedure it can assess with regards to duration of the stressor. It may be that after a certain period of time eye temperature will begin to decline or it may remain elevated until removal of the stressor. Longer studies are therefore needed both with short term stressors with prolonged thermal imaging post stressor and longer term stressors to assess how long eye temperature remains elevated.

As discussed earlier an increase in cortisol of 40% from basal levels is suggested to be contributory to stress related disease (Barnett and Hemsworth, 1990). It may be that there is an equivalent eye temperature increase that indicates the same. The actual temperature increase itself is unlikely to cause detrimental effects to health however it may reflect the corresponding increase in cortisol. Further work would be required to investigate if specific increases in cortisol correlate with rise in eye temperature. If this is the case then IRT would be a non-invasive tool to measure whether stress caused by specific procedures would increase cortisol levels and thus have detrimental consequences for equine health.

There are many short term husbandry and training procedures involved in the equine industry which could be perceived as stressful to the horse and only one (clipping) has been investigated during this project. IRT could now be utilised to assess other aspects of equine domestication for example weaning methods and transportation. Now that better quality cameras are becoming available, with improved capabilities of capturing temperature from a greater distance, it should be possible to apply IRT to free ranging horses to assess welfare.

7.11 Conclusion

The Animal Welfare Act 2006 is in place to ensure that the main welfare needs of animals are met. This includes the provision of suitable housing and protection from illness. The Federation Equestrian Internationale (FEI) has called for an objective method to identify 'the happy equine athlete' however horse owners and trainers are not sure what the signs of a stress free ridden horse are, or what are the most suitable methods of husbandry and management of horses. Interpreting short term stress in terms of welfare is difficult as it is essentially a normal protective response. However assessing short term stress can serve to help horse owners and trainers decide which are the most appropriate management and training methods in terms of how stressful horses perceive them to be and subsequently improve welfare. This project has revealed that although IRT is not appropriate to assess acute or chronic stress in the horse, it can be utilised as an indicator of stress during short term potentially aversive management and training practices. IRT of the eye area overcomes the confounding effects of hair and dirt, as well as the invasive and time consuming limitations of existing laboratory based methods. IRT is non-invasive, instant and measures a physiological stress response that cannot be masked in the same way that behaviour can. IRT can also be used as an objective physiological measure to help identify which behaviours are most reliable in indicating that the horse finds the situation it is in to be stressful.

It is always difficult to predict the impact of training and management practices on a population of horses as each individual horse will perceive procedures differently due to the influencing factors of past experience and temperament. However, IRT can be used in an experimental setting with horses acting as their own controls to carefully observe and monitor the thermal response to elements of the domestic environment. This will allow

horse owners and trainers to decipher which procedures may be causing stress at a given place and time and alter them accordingly.

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Appendices

Appendix 1	Assessment of salivary cortisol collection methods
Appendix 2	Validation for cortisol assays
Appendix 3	Standard curves of cortisol assays
Appendix 4	Preliminary study 1
Appendix 5	Preliminary study 2
Appendix 6	Individual physiological data for chapter 4.
Appendix 7	Preliminary study 3
Appendix 8	Individual physiological data for chapter 5
Appendix 9	Extraction and assay protocol used by Chester Zoo
Appendix 10	Raw data from faecal glucocorticoid analysis
Appendix 11	Individual faecal corticosterone data
Appendix 12	Ethical approval and risk assessments

Appendix 1

Assessment of salivary cortisol collection methods

During the preliminary stages of the study various methods were explored to allow collection of saliva from the horses for cortisol analysis. In order to assay each sample in duplicate a minimum volume of 200 μ l of saliva was required. Five horses were used to investigate the efficacy of each swabbing method. Horses were allowed to chew the swab for one minute. This length of time was chosen as it was anticipated that some studies would require quick and frequent sampling in addition to any longer sampling duration potentially resulting in destruction of the swab through the horses chewing action. Swabs were taken at the same time of day (0800h) from the same horses and frozen at -4°C. Each swab was then thawed and centrifuged (Rotina 380, DJB Labcare) at 1000g for ten minutes. Saliva was transferred into 1.5ml microtubes via pipette. 100 μ l increments had been marked onto the microtubes by pipetting set amounts of distilled water into the tubes and marking the level. This allowed volume of saliva to be calculated to the nearest 50 μ l once it was transferred. Swab types used were cotton buds, cotton gauze wrapped around a wooden stick that was held in the mouth and a salivette. Table 1.1 displays the amount of saliva collected using each method.

Table 1.1 Amount of saliva (μ l) collected using each of three sampling methods in five horses.

	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5
Cotton Bud	0	0	100	100	50
Cotton gauze	150	50	150	200	200
Salivette	750	650	700	800	800

Once centrifugation ceased the cotton bud and gauze swab both remained in the same container as the saliva and it is possible that some of the extracted saliva was re absorbed before it was able to be removed. The gauze swab was partially destroyed by the horses chewing action within the allotted one minute period which resulted in an unpleasant and unhygienic collection method. The unique design of the salivettes offered a hygienic and efficient collection method that yielded adequate amounts of saliva and collected saliva remained separate from the cotton swab post centrifugation. The salivettes withstood the chewing action however there was a concern that they could be swallowed by the horses. It was concluded that they should be modified from their design intended for human use by stitching cotton thread through the swab that could be held as the horses chewed to prevent swallowing.

Appendix 2

Validation for cortisol assays

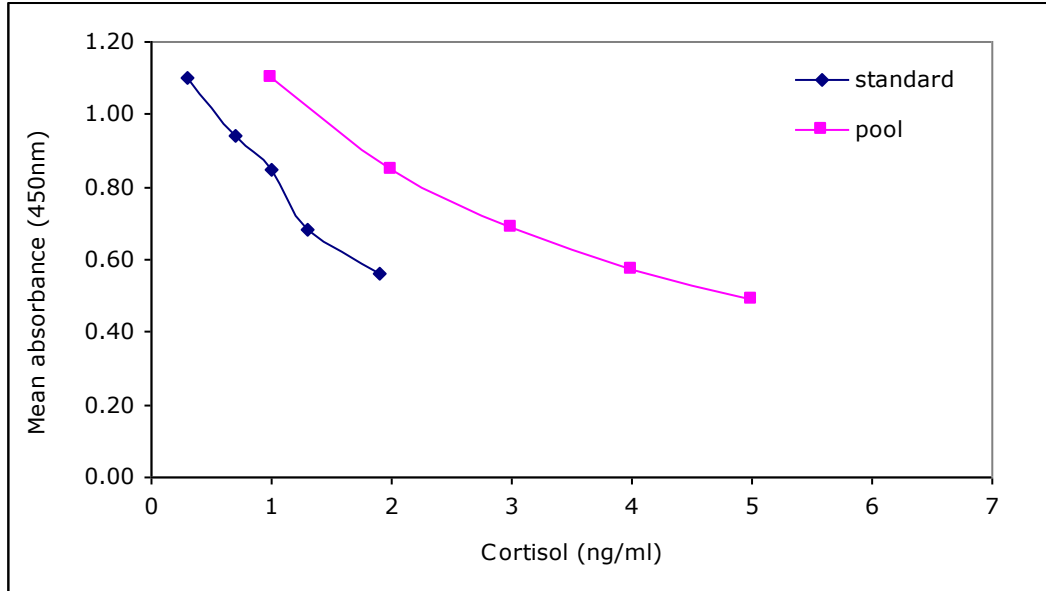


Figure 2.1 Standard curve for pooled sample of saliva assayed neat (10ng/ml) and diluted to 8ng/ml, 6ng/ml, 4ng/ml, 2ng/ml and 1ng/ml alongside the standards provided with the kit.

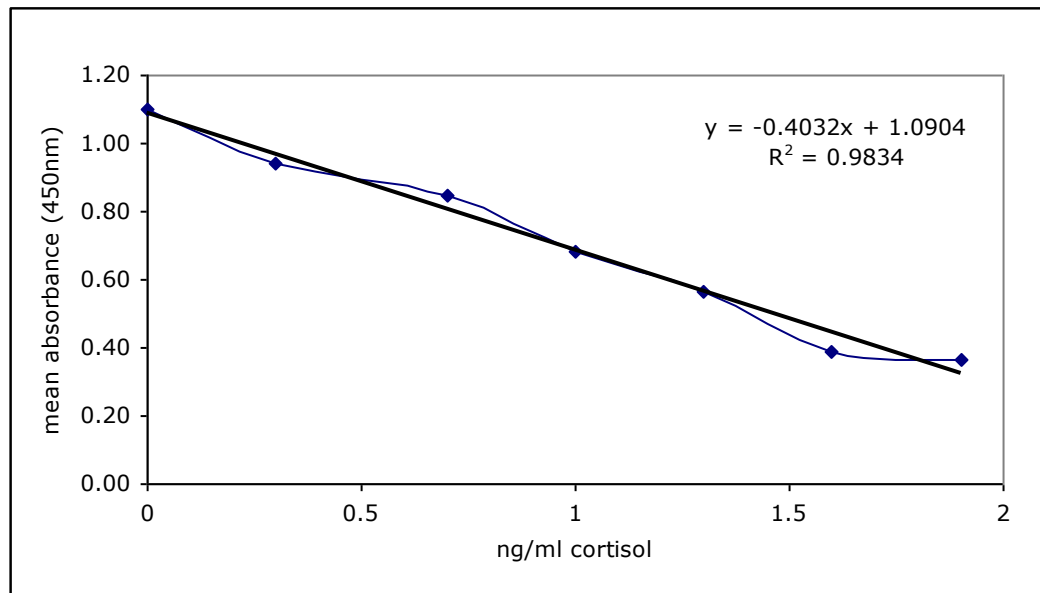


Figure 2.2 Semi log plot for pooled sample of saliva assayed neat (10ng/ml) and diluted to 8ng/ml, 6ng/ml, 4ng/ml, 2ng/ml and 1ng/ml alongside the standards provided with the kit.

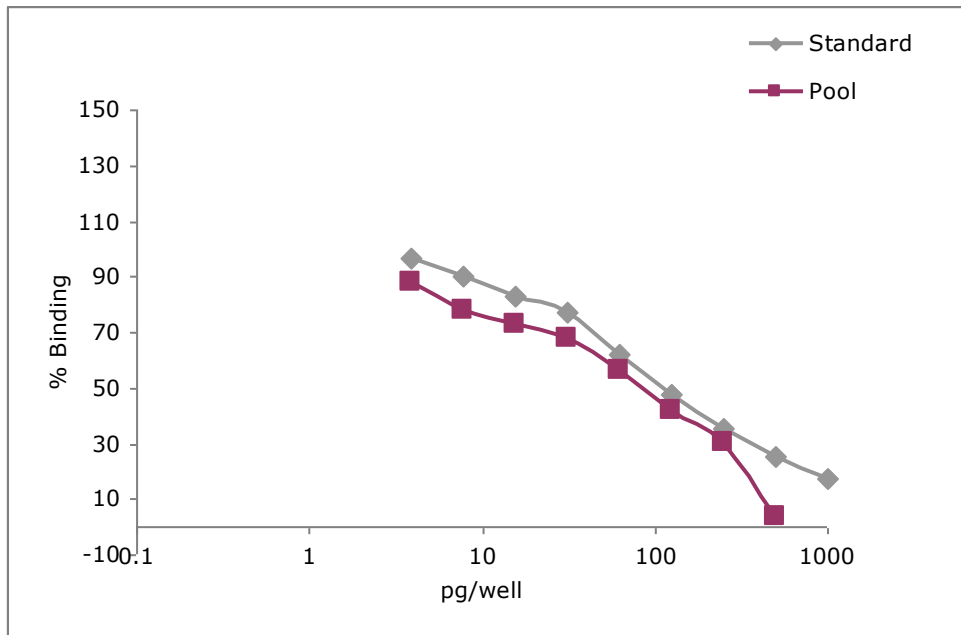


Figure 2.3 Standard curve of pooled equine faecal samples and corticosterone standards. Analysis carried out at Chester Zoo, see Appendix 8 for protocol

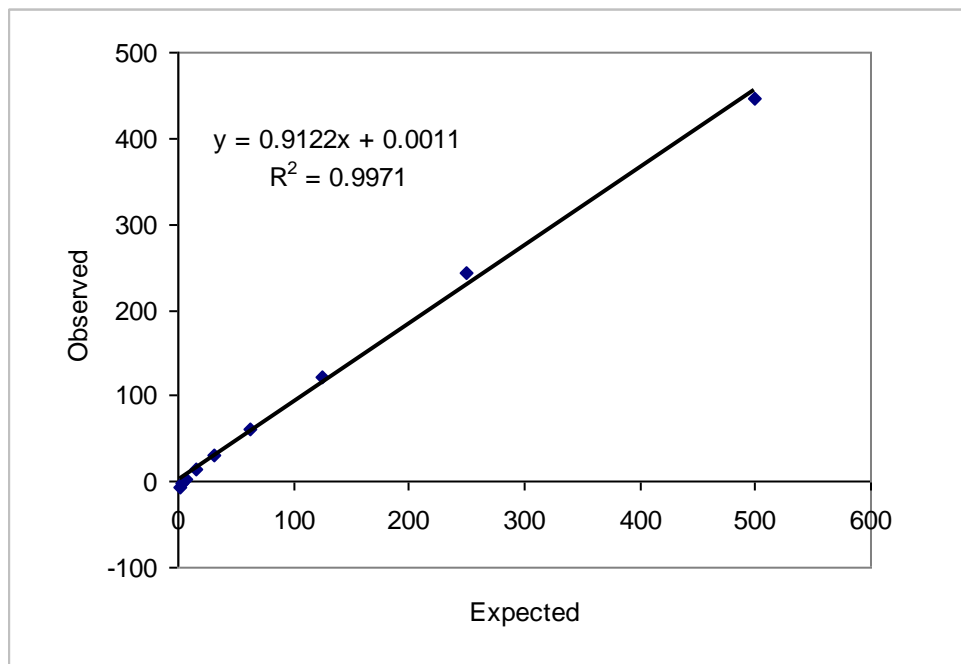


Figure 2.4 Semi log plot of pooled equine faecal samples and corticosterone standards. Analysis carried out at Chester Zoo, see Appendix 8 for protocol

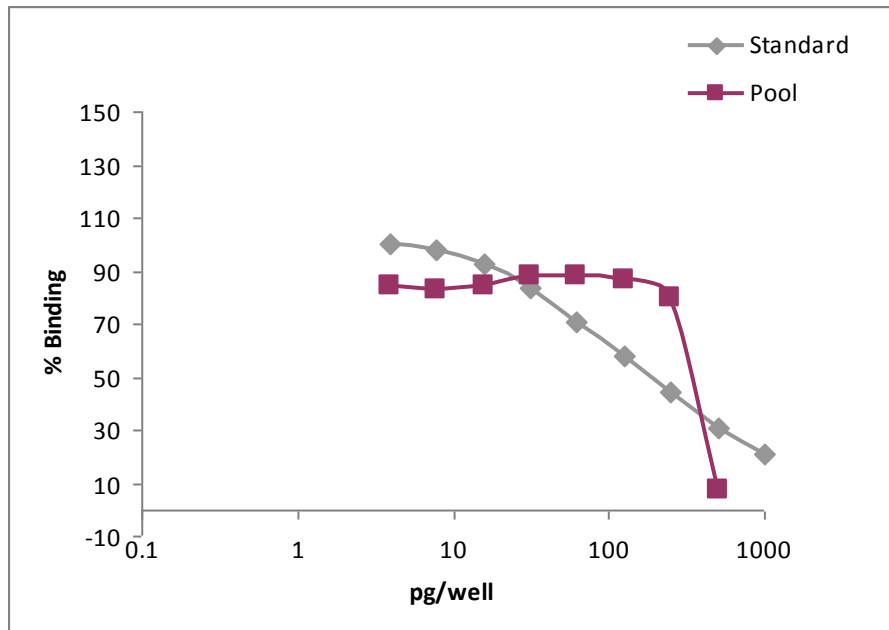


Figure 2.5 Standard curve for pooled equine faecal samples and cortisol standards. Curve shows that analysis of faeces for cortisol is an unsuitable method. Analysis was carried out at Chester Zoo, see Appendix 8 for protocol.

Appendix 3

Standard curves of cortisol assays

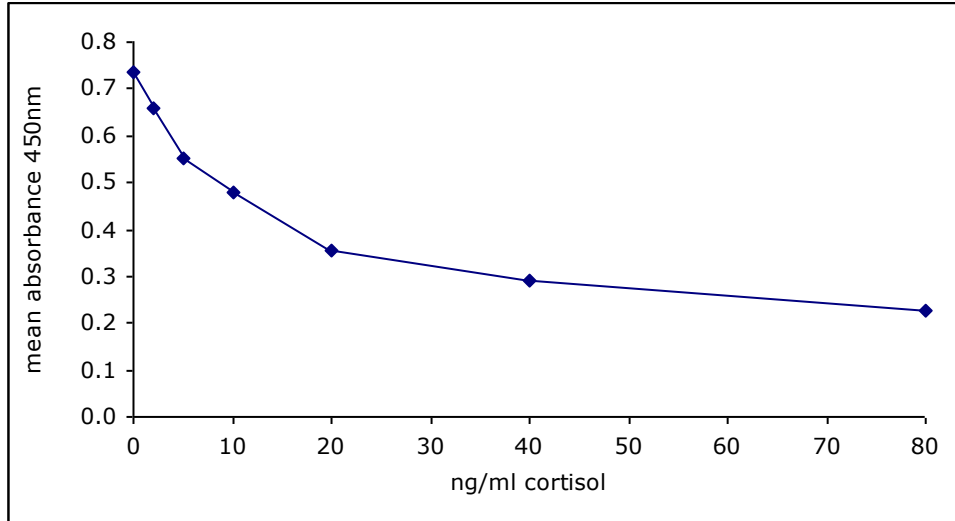


Figure 3.1(a) Standard curve of cortisol assay for circadian study A

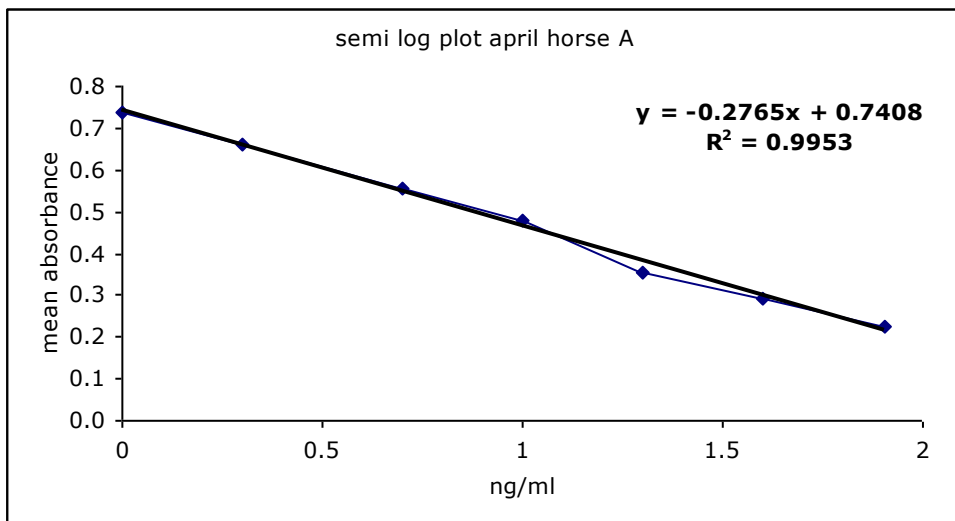
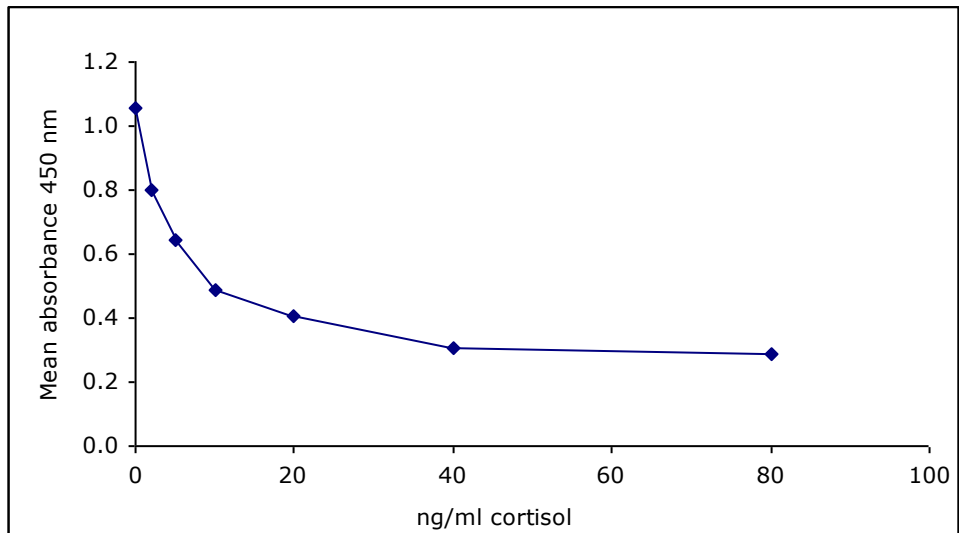
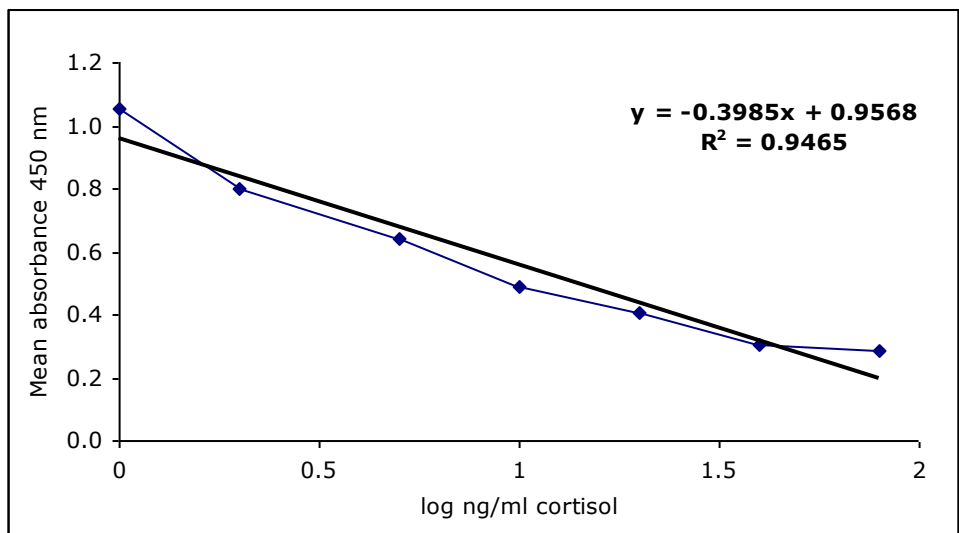


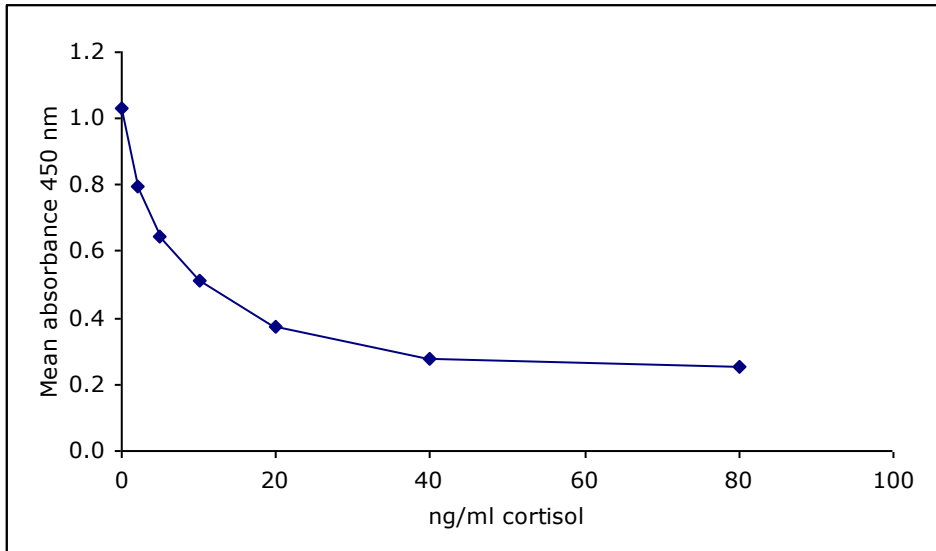
Figure 3.1(b) Semi log plot of cortisol assay for study A



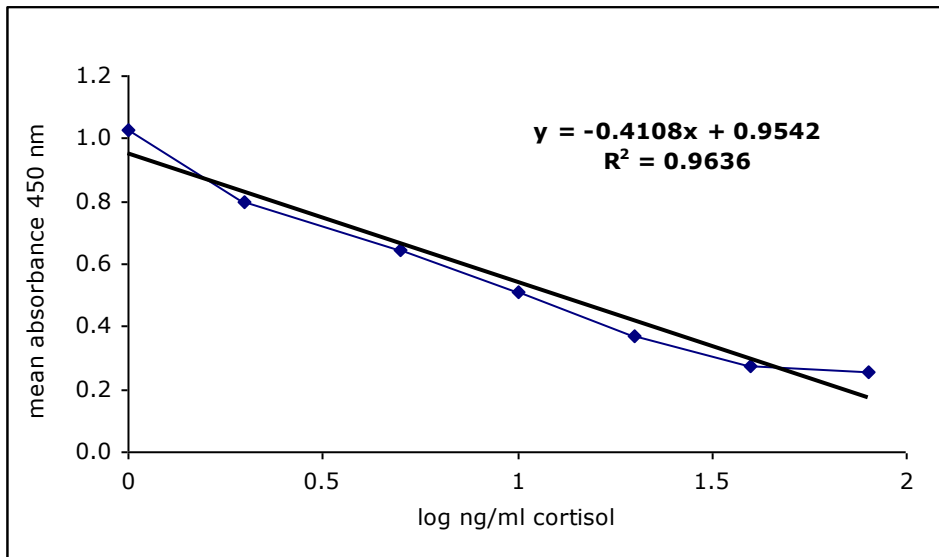
3.2(a) Standard curve for cortisol assay of saliva from study B



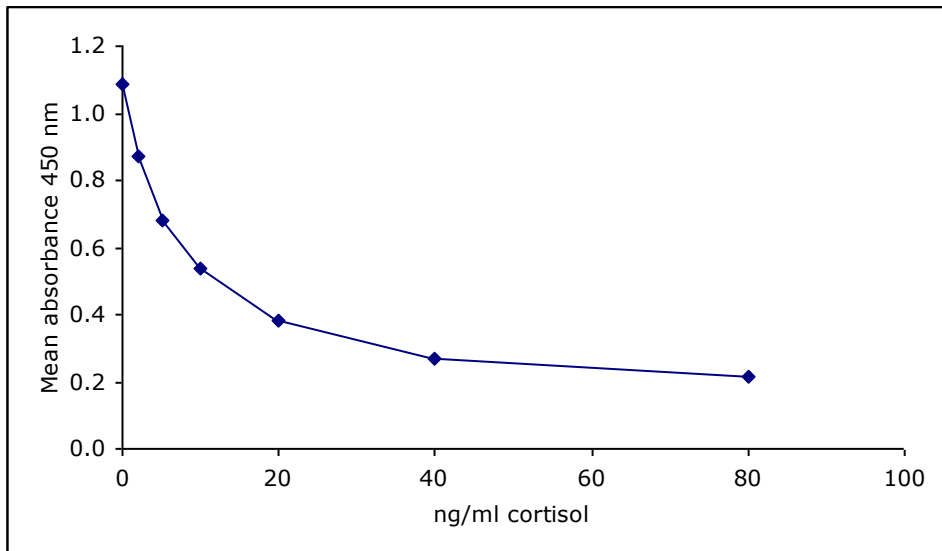
3.2(b) Semi log plot of cortisol assay for study B



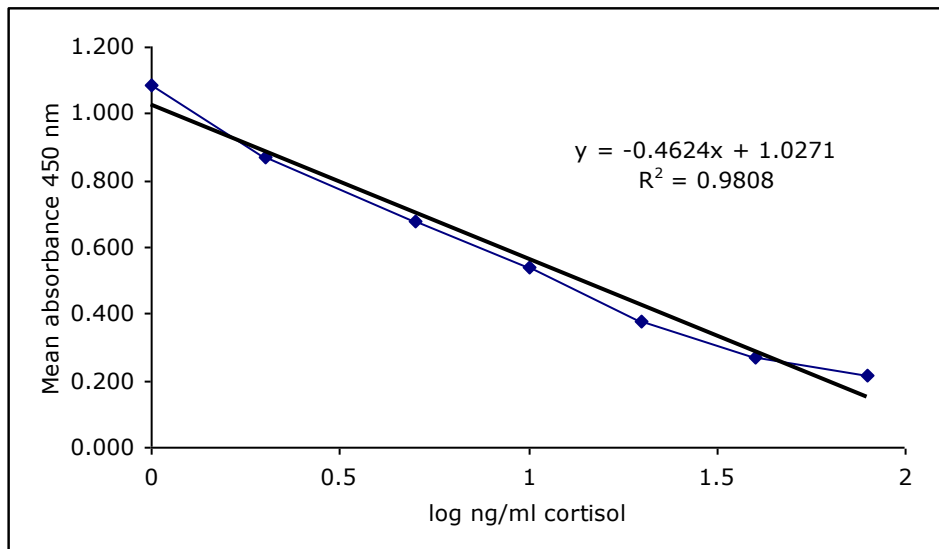
3.3(a) Standard curve of cortisol assay (plate 1) study C



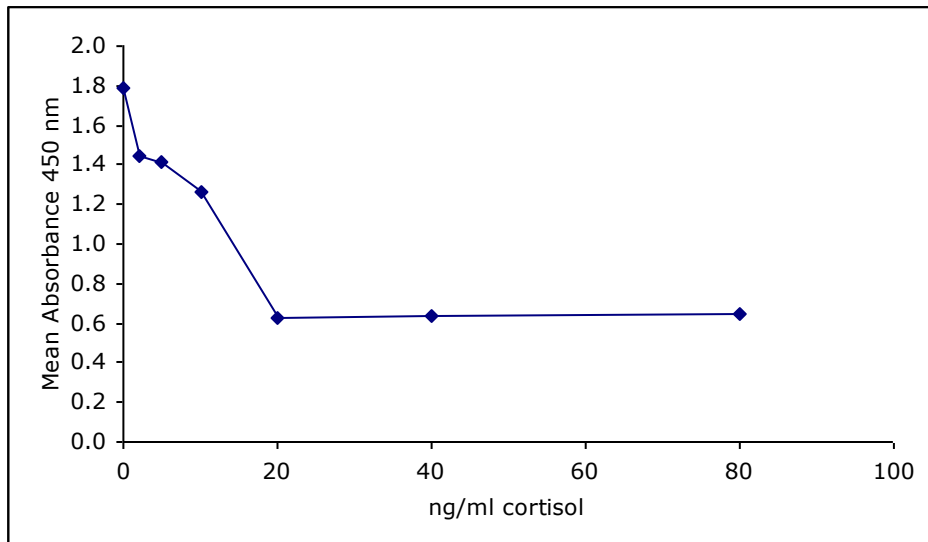
3.3(b) Semi log plot of cortisol assay (plate 1) for study C



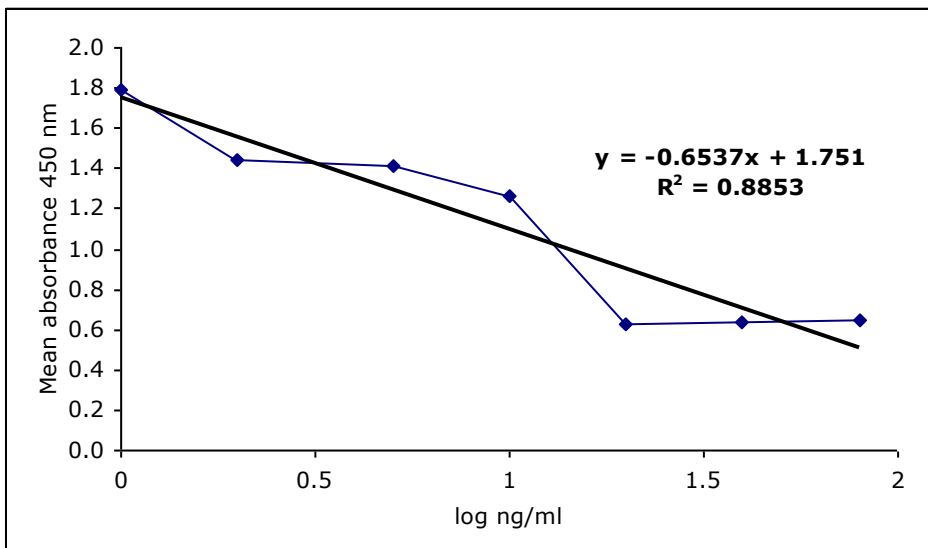
3.4(a) Standard curve of cortisol assay (plate 2) for study C



3.4(b) Semi log plot of cortisol assay (plate 2) for study C



3.5(a) Standard curve of cortisol assay (plate 3) for study C



3.5(b) Semi log plot of cortisol assay (plate 3) for study C

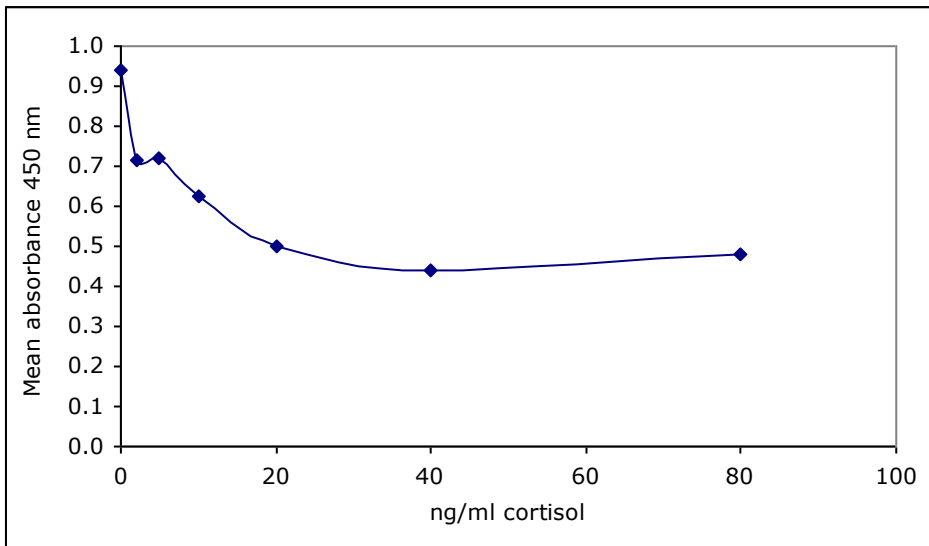


Figure 3.6(a) Standard curve of cortisol assay (plate 1) for study C (control)

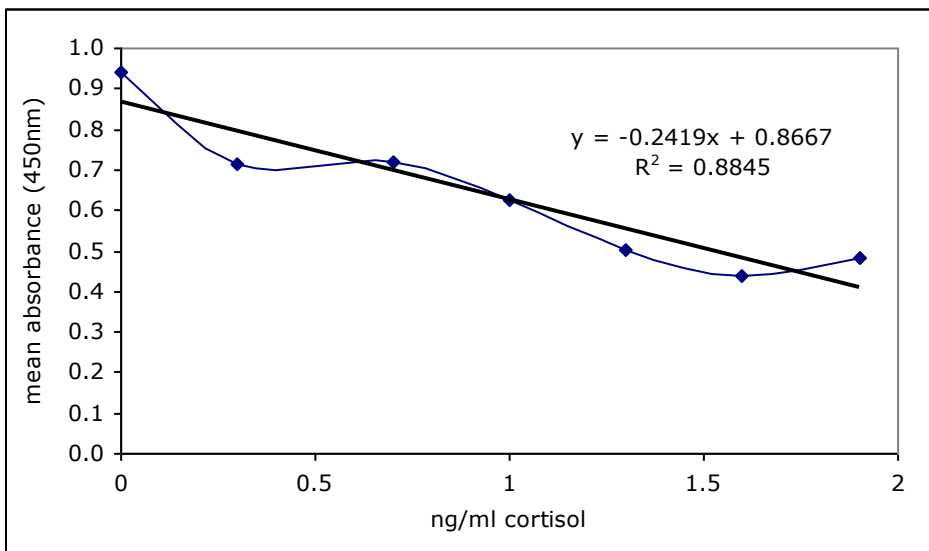


Figure 3.6(b) Semi log plot of cortisol assay (plate 1) study C (control)

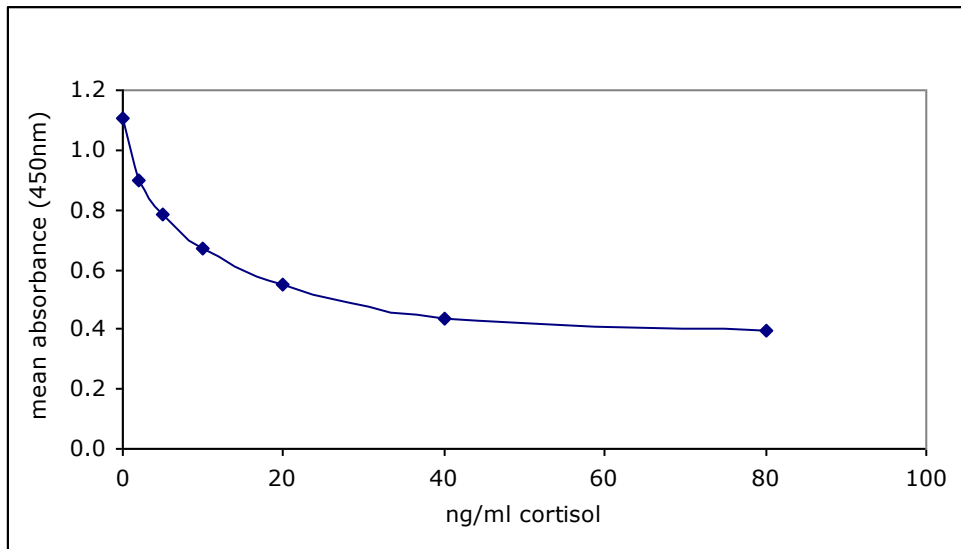


Figure 3.7(a) Standard curve of cortisol assay (plate 2) for study C (control)

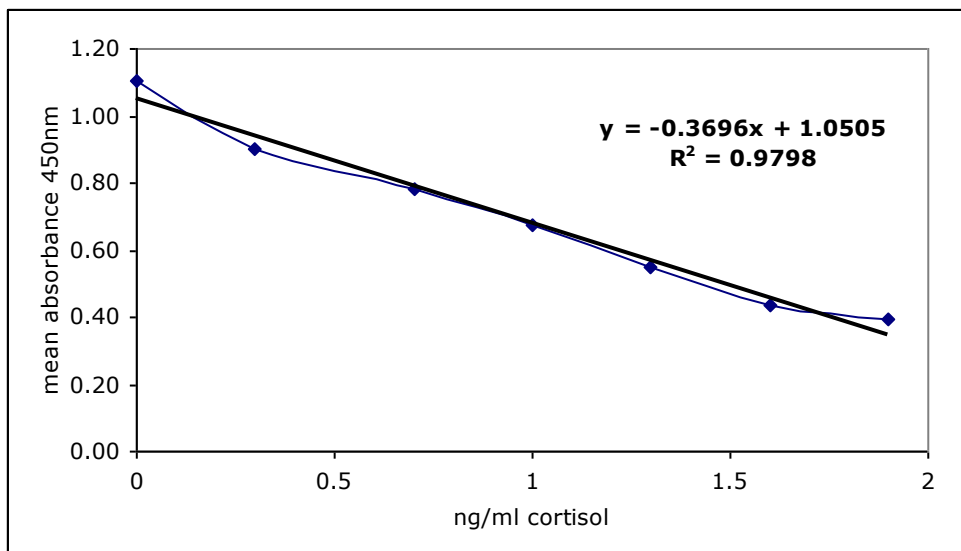


Figure 3.7(b) Semi log plot of cortisol assay (plate 2) for study C (control)

Appendix 4

Preliminary study 1

This preliminary study was carried out to investigate whether changing the distance that a thermal image is captured from the horse will affect the recorded temperature. It also investigated the effect of ambient temperature on eye temperature.

Methods and materials

Horses

Ten horses from the University equestrian centre were involved in the study. The horses were a mixture of breed representative of riding school horses, details of the horses can be found in table 2.1. Horses were managed as previously discussed in section 2.0 and were exercised for approximately two hours per day.

Test area and apparatus

The test area was an enclosed grass arena measuring 20x40 metres. A jump pole was placed on the ground to mark where the horse was to stand and then six further wooden jumping poles were placed on the ground at set distances of 1 metre, 3 metres, 6 metres, 9 metres and 12 metres from the horse (Figure 4.1).

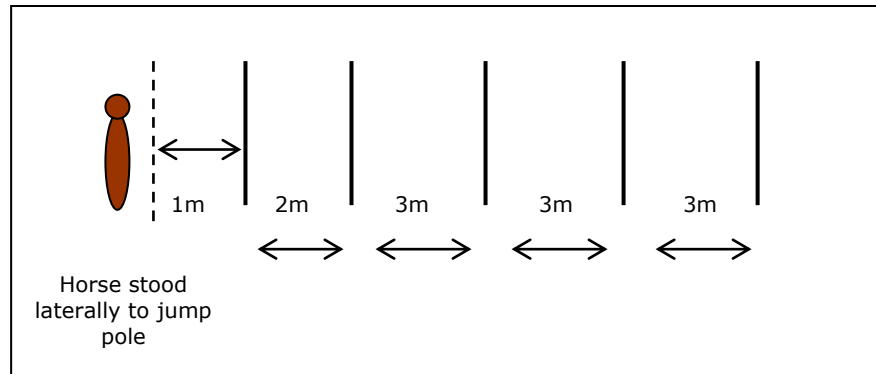


Figure 4.1 Test area with jumping poles placed at set distances of 1, 3, 6, 9 and 12 metres laterally from horse.

Data Collection

Horses were led one at a time to the test area by the same familiar handler and positioned laterally to the marker pole (Figure 4.1). Thermal images of the eye were taken at each jumping pole. This was carried out for both left and right lateral aspect. Thermal data was captured, uploaded and temperature extracted using the method previously discussed in section 2.0

Core temperature was taken three times for each horse using the method previously discussed in section 2.0. Core temperature was measured when the horse first entered the arena, during the experiment (at the 6 metre marker pole) and before the horse left the arena. Ambient temperature was also recorded each time a thermal image was captured.

Data Analysis

Eye temperature

Distribution of eye temperature data was normal (Kolmogorov-Smirnov, $p=0.2$) A one way repeated measures ANOVA) was carried out to investigate any difference in eye temperature when images were captured at different distances (1= 1 metre from horse, 2= 3 metres from horse, 3= 6 metres from horse, 4= 9 metres from horse and 5 = twelve metres from horse).

Core temperature

Distribution of core temperature data was normal (Kolmogorow-Smirnov, $p=0.16$).) A one way repeated measures analysis ANOVA was carried out to investigate any difference in the three core temperature measurements over the study period (when the horse first entered the arena, during the experiment, at the 6 metre marker pole and before the horse left the arena)

Correlation

The relationship between eye temperature measured using IRT and distance the image was captured from the horse was investigated using Pearson product-moment correlation in addition to any relationship between ambient temperature and eye temperature.

Results

Eye temperature

A one way repeated ANOVA indicated that there was a significant effect of distance on eye temperature (Wilks Lambda=.05, $F(4, 6) = 28.637$, $p=0.001$, multivariate partial eta squared =.95). Post hoc tests using the Bonferroni correction revealed that there was a decrease in mean temperature between each distance which was significant (Table 4.1)

Core temperature

A one way repeated ANOVA indicated that there was no significant effect of time on core temperature (Wilks Lambda= .56, $F(2, 8) = 3.206$, $p=0.095$). Core temperature remained. Table 4.2 details the descriptive statistics for core temperature for before, during and after the study

Table 4.1 Results of ANOVA test used to investigate the effect of distance on eye temperature when measured using IRT (1= 1 metre from horse, 2= 3 metres from horse, 3= 6 metres from horse, 4= 9 metres from horse and 5 = twelve metres from horse) Table shows significant effect of distance on eye temperature.

(I) distance	(J) distance	Mean Difference (I-J)	Std. Error	Sig. ^a	95% Confidence Interval for Difference ^a	
					Lower Bound	Upper Bound
1	2	3.570 [*]	.666	.005	1.112	6.028
	3	7.998 [*]	.902	.000	4.669	11.326
	4	10.393 [*]	.946	.000	6.902	13.883
	5	12.828 [*]	1.088	.000	8.811	16.844
2	1	-3.570 [*]	.666	.005	-6.028	-1.112
	3	4.427 [*]	.493	.000	2.610	6.245
	4	6.822 [*]	.842	.000	3.715	9.930
	5	9.258 [*]	1.157	.000	4.987	13.528
3	1	-7.998 [*]	.902	.000	-11.326	-4.669
	2	-4.427 [*]	.493	.000	-6.245	-2.610
	4	2.395 [*]	.563	.021	.317	4.473
	5	4.830 [*]	.861	.003	1.652	8.008
4	1	-10.393 [*]	.946	.000	-13.883	-6.902
	2	-6.822 [*]	.842	.000	-9.930	-3.715
	3	-2.395 [*]	.563	.021	-4.473	-.317
	5	2.435 [*]	.459	.005	.740	4.130
5	1	-12.828 [*]	1.088	.000	-16.844	-8.811
	2	-9.258 [*]	1.157	.000	-13.528	-4.987
	3	-4.830 [*]	.861	.003	-8.008	-1.652
	4	-2.435 [*]	.459	.005	-4.130	-.740

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Bonferroni.

Table 4.2 Descriptive statistics descriptive statistics for core temperature for before, during and after the study

	Mean	Std. Deviation	N
before	36.5100	1.04078	10
during	36.5600	.79610	10
after	36.3300	.81656	10

Correlation

Pearson product-moment correlation revealed that there was a strong negative correlation between eye temperature measured using IRT and distance the image was captured from the horse, ($r = -0.783$, $n=55$, $p < 0.001$). As the distance the image was captured from the horse increased, eye temperature decreased (Figure 4.2).

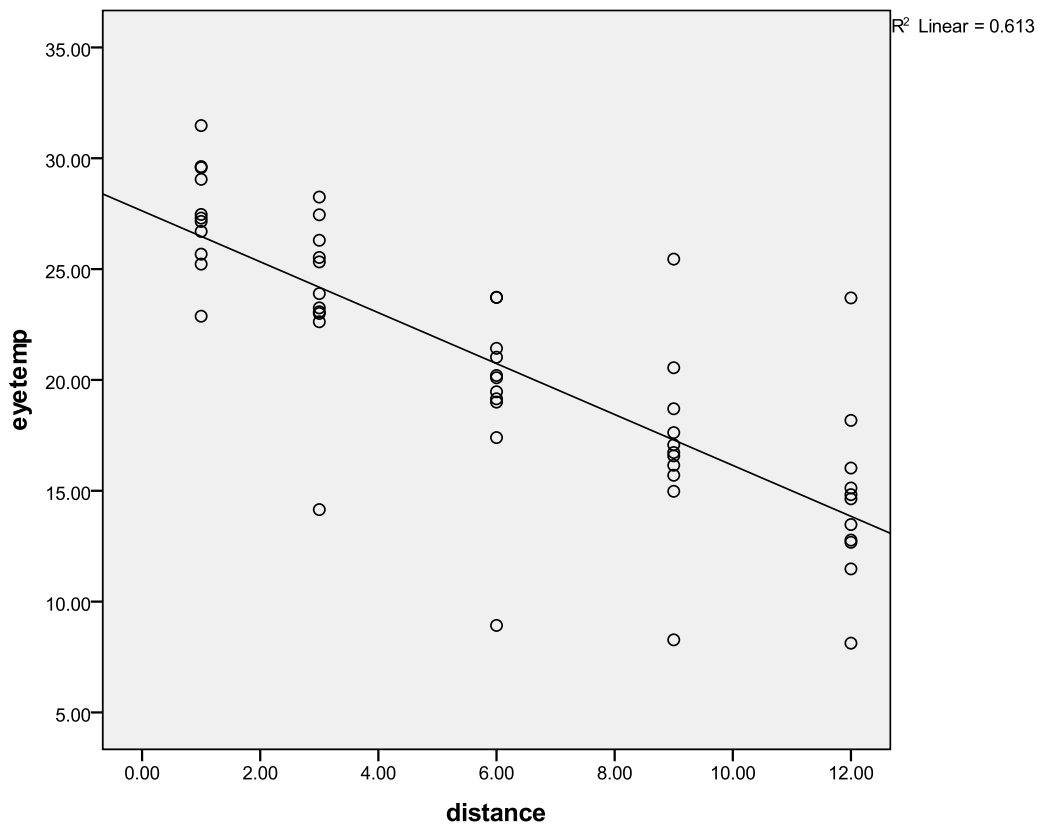


Figure 4.2 Negative correlation between eye temperature (°C) measured using IRT and distance the image was captured from the horse (metres)

Pearson product-moment correlation revealed that there was no correlation between eye temperature and ambient temperature ($r = -0.2$, $n=50$, $p < 0.25$) (Figure 4.3).

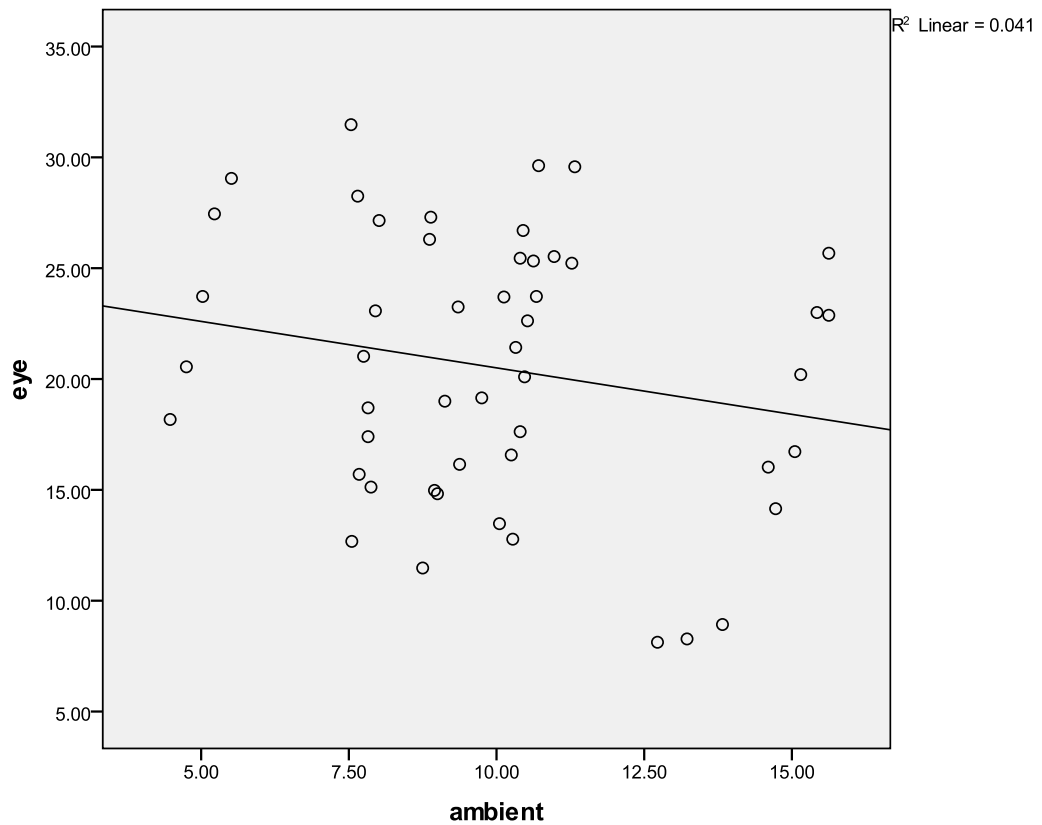


Figure 4.3 No relationship between eye temperature (°C) and ambient temperature (°C) throughout the duration of the study.

Conclusion

Recorded eye temperature decreased significantly when thermal images were taken at a greater distance from the horse. This highlights the importance of a standardised distance when capturing thermal images during this project as accurate temperatures are required. Furthermore, images captured at 1 metre from the horse were clear and placement of the polygon for temperature extraction could be carried out easily and accurately. The decrease in eye temperature appears to not be a direct result of ambient temperature as no correlation was found between the two measures.

Appendix 5

Preliminary study 2

This preliminary study was carried out to investigate eye and ear temperature output of horses in their usual environment measured using IRT.

Materials and Methods

Horses

Five horses from the University riding school were selected based on their availability throughout the week at the sampling times required. Details of the horses can be found in chapter 2.0. All horses were fed concentrate feed twice daily, offered ad libitum hay and water and were all familiar with the yard management routine.

Data collection

All horses were brought from their stable by the same familiar handler and loosely tied in the same area in order for thermal images to be taken.

Static thermal images of the left and right eye and left and right ear pinna were taken of each horse using the method previously discussed in chapter 2.0. Images were captured three times daily at 0800h, 1300h and 1800h over five subsequent days. Thermal data was uploaded and temperature extracted as previously discussed in chapter 2.0. The maximum temperature of eye and ear pinna were extracted for each horse and entered into an Excel spreadsheet. Initially this was carried out for each individual horse and then subsequently the mean eye and ear pinna temperatures for all horses were calculated for each sampling time point. Temperatures were then plotted against time in order to investigate any daily patterns or changes.

Data analysis

Eye temperature data analysis

Distribution of data did not vary significantly from normal (Kolmogorov-Smirnov test $p=0.2$) therefore a two way within subjects ANOVA was carried out to investigate any difference in eye temperature between days and also between sampling time points.

A one way repeated measures ANOVA was used to investigate any difference in eye temperature between the five 0800h eye temperatures taken during the course of the study. This was carried out as initial investigation revealed eye temperature to be higher on the third 0800h sample.

Ear pinna temperature data analysis

Distribution of data was normal ($p=0.2$) therefore a two way within subjects ANOVA was carried out to investigate any difference in ear temperature between days and also between sampling time points.

Results

Eye temperature

The results of the two way repeated measures ANOVA revealed no significant difference in eye temperature between days ($F(2, 8) = 1.111$, $p=0.375$), however there was a significant effect of time on eye temperature ($F(2, 8) = 10.149$, $p=0.006$). Post hoc tests using the Bonferroni correction revealed that there was a decrease in mean temperature between times 1 and 2 (08.00h and 13.00h) (33.8 ± 0.6 °C vs. 31.1 ± 0.4 °C) which was statistically significant ($p=0.04$).

Ear temperature

The results of the two way repeated measures ANOVA revealed no significant difference in ear temperature between days ($F(2, 8) = 0.308$, $p=0.743$) or between individual time points ($F(2, 8) = 0.237$, $p=0.795$). Mean ear temperature displayed larger variation between horses than mean eye temperature (Figure 5.1). Table 5.1 details maximum, and minimum temperatures for both eye and ear pinna temperature.

Table 5.1 Minimum, maximum and range in temperature recorded for the eye, and ear pinna (°C).

	Minimum temperature (°C)	Maximum temperature (°C)	Range (°C)
Eye temperature	32	35	3
Ear temperature	22	32	10

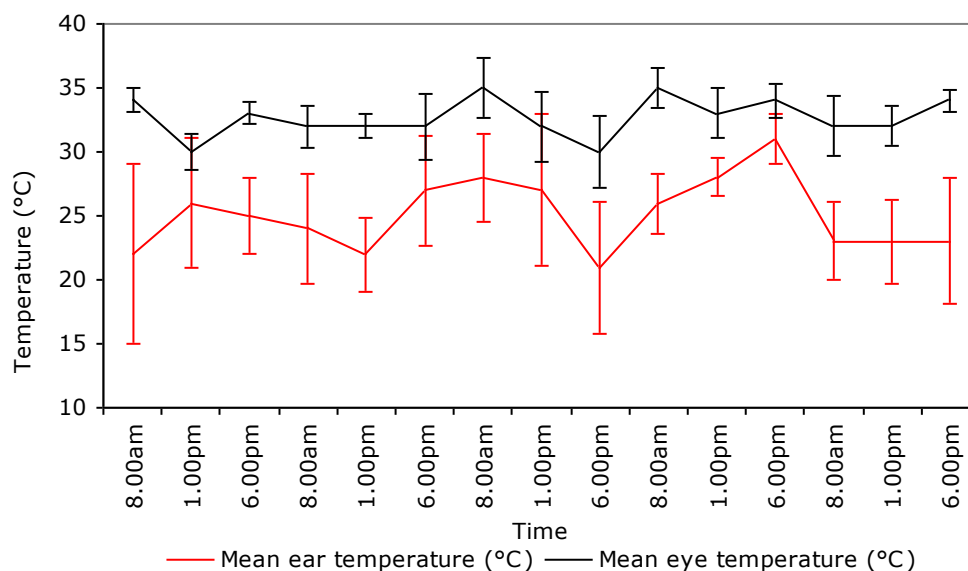


Figure 5.1 Mean (\pm SD) eye and ear temperature of five study horses.

Further observations

80% of the thermal images (75 images in total) displayed maximum eye temperature at the lacrimal gland (Figure 5.2).

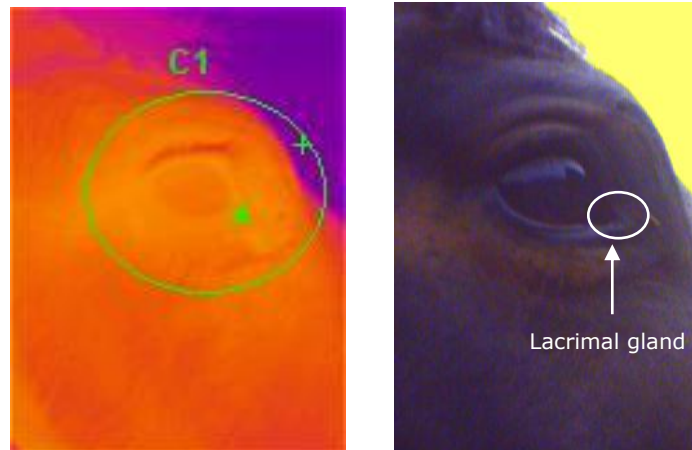


Figure 5.2 Thermal image displaying location of maximum eye temperature at the lacrimal gland with corresponding photograph.

During the third day of the study eye temperature increased above temperatures recorded during the two previous days (sample 7). The increase above basal levels was observed in four of the five study horses and eye temperature did not increase again to the levels shown at any other point during the study (Figure 5.3). A one way repeated measures ANOVA revealed that this increase was not significant (Wilks Lambda = .013, $F(4, 1) = 18.907$, $p=0.17$).

Ear pinna temperature did not display any changes greater than those observed during the two previous days or at any other point during the study.

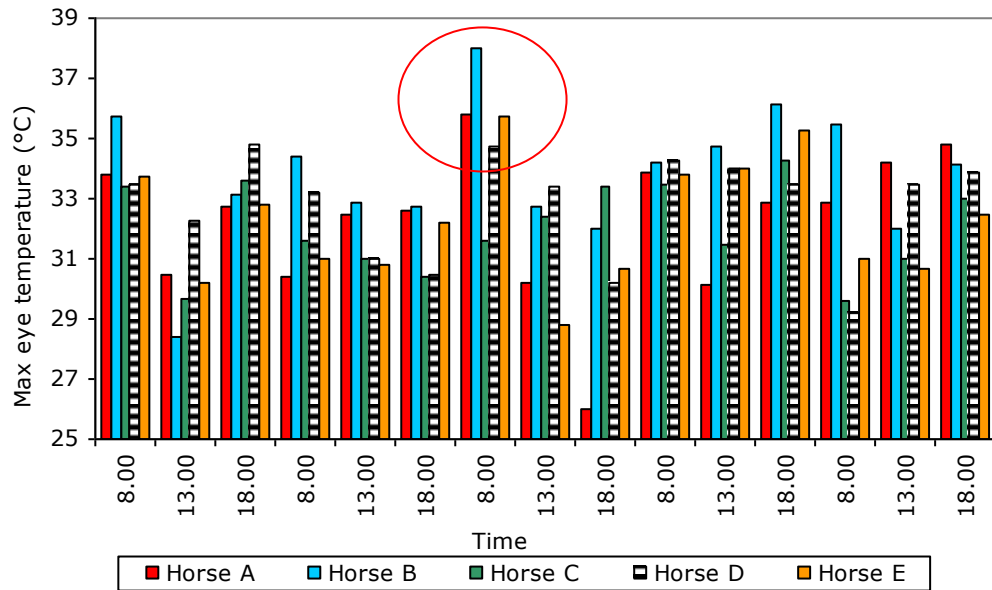


Figure 5.3. Maximum eye temperature for each horse throughout the duration of the study. The red circle highlights elevated eye temperature in four of the five study horses on the third study.

Discussion

A large proportion of the thermal images displayed maximum eye temperature at the lacrimal gland (Figure 5.2). This is in agreement with the study by Stewart *et al.* (2007) and may be because the vessels that supply the eye with blood are close to the skin surface in this area.

In this preliminary study ear temperature fluctuated across a greater range of temperature when compared to eye temperature. This may be due to the ear being an outer extremity and therefore affected more by climate in addition to playing a role in dissipation of heat for thermoregulation.

During the third day of the pilot study eye temperature increased above temperatures recorded during the two previous days although this increase was not significant ($p=0.17$). The increase above basal levels was observed in four of the five study horses. High winds resulted in noise and sudden movements amongst plastic covered hay bales in the vicinity where the horses were tied for their thermal images to be taken. All four horses exhibited flight behaviour. One of the horses was stabled adjacent to the bags

and it was therefore possible that it had become habituated to the noise and movement and subsequently did not exhibit behavioural signs of stress or an increase in maximum eye temperature.

The increase in eye temperature in response to exposure to a startling stimulus (in this case sudden movement and noise caused by plastic bags) supports findings of existing work into the thermal response of the eye to acute aversive stimuli (Pavlidis *et al.*, 2001; Cook *et al.*, 2006).

Eye temperature displayed a significant decrease between morning and midday measure, however the frequency of eye temperature measurements was limited to three times over each twenty four hour period. It is impossible to know what temperature changes occurred if any during the five hour periods between daytime measures in addition to any changes overnight. Data were limited for this study due to the small sample size of horses and temperature measures therefore a larger more detailed study was required.

Conclusion

Preliminary results show that eye temperature fluctuates less over time when compared to ear temperature. This is in agreement with existing work that has used eye temperature as a measure of temperature change associated with the stress response in other species (Cook *et al.*, 2001; Pavlidis *et al.*, 2002; Cook *et al.*, 2006; Stewart *et al.*, 2008T). Less fluctuation in temperature of the eye area will be beneficial as it will allow clearer interpretation of any stress related changes.

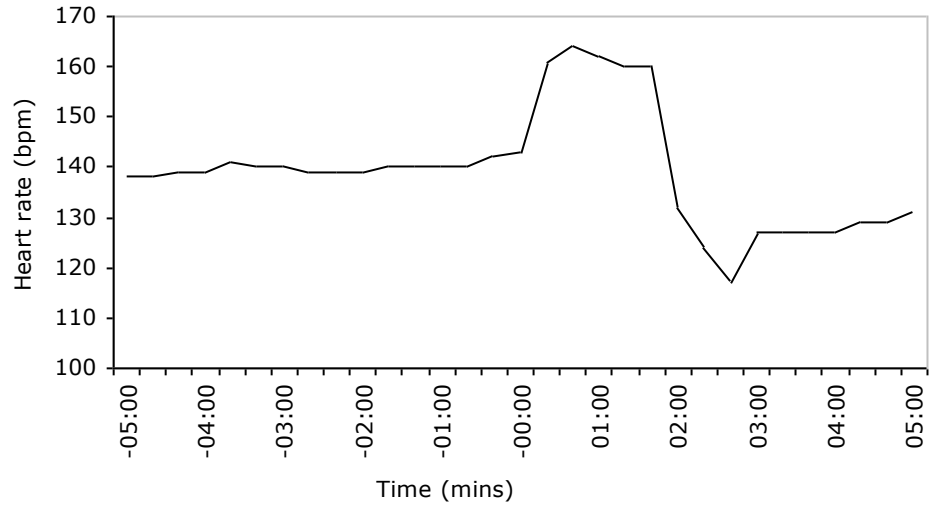
A significant effect of time was found in eye temperature but a limited number of samples (3 per day) were available. A larger and more detailed investigation into the daily pattern of eye temperature was now needed, in addition to salivary cortisol measurements, in order to investigate diurnal rhythm and allow better interpretation of the thermal and hormonal data collected as a measure of stress. It appears that sudden potentially aversive

stimuli may result in warming of the eye area which supports the objective of investigation into the thermal response of the equine eye to a startling stimulus.

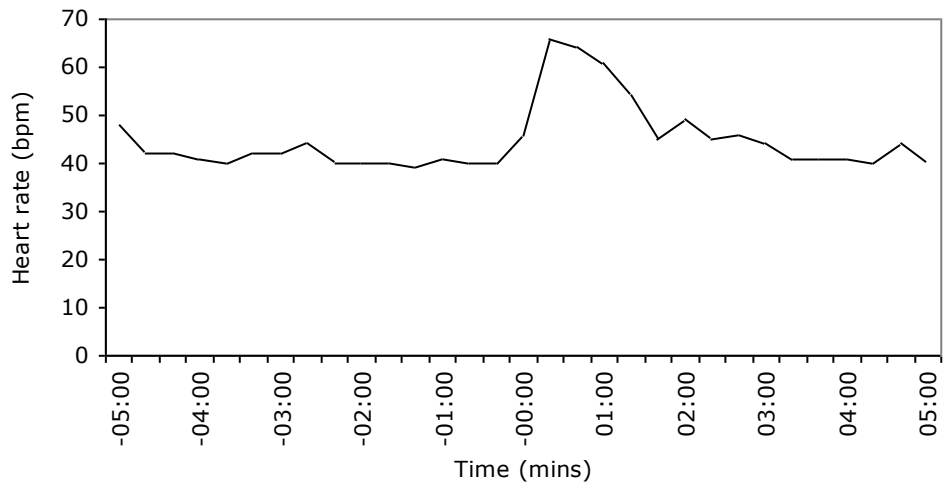
Appendix 6

Individual physiological data for chapter 4.

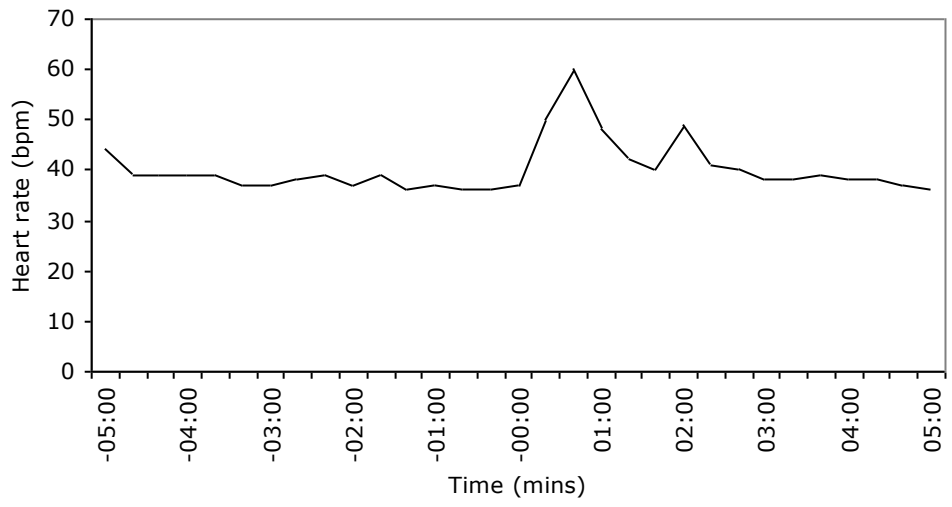
Horse 1



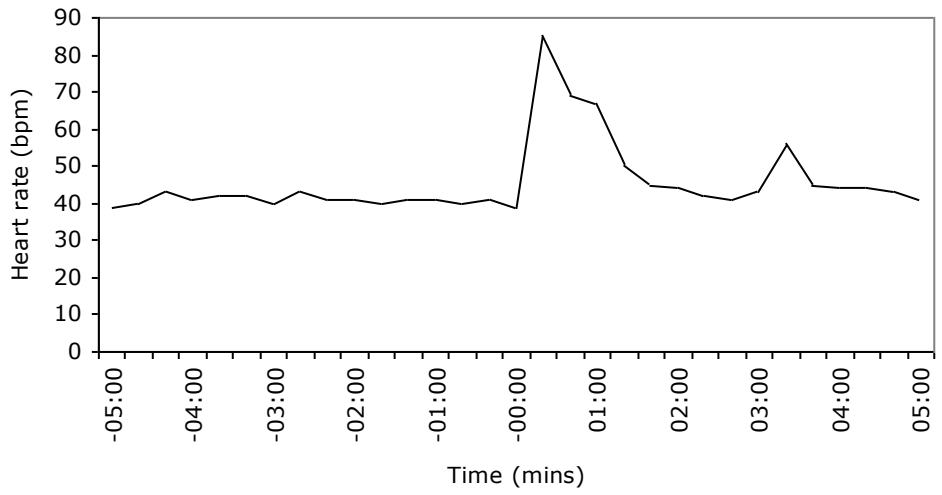
Horse 2



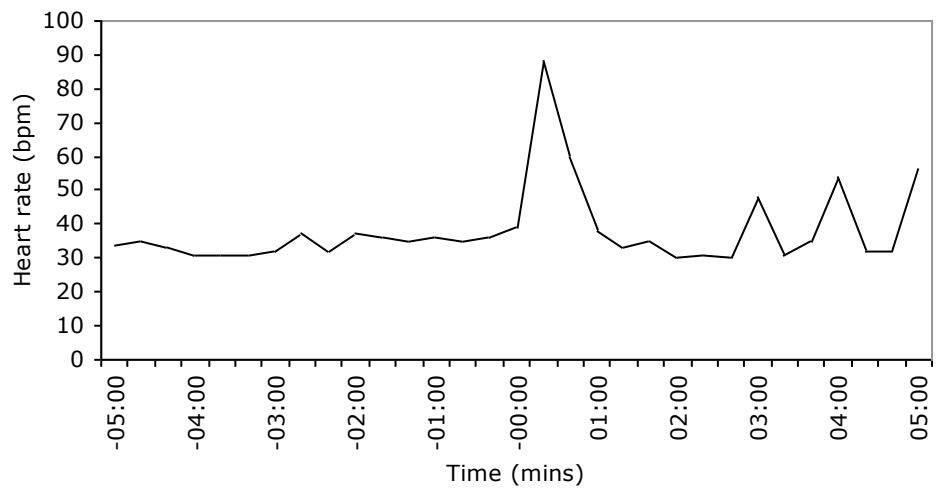
Horse 3



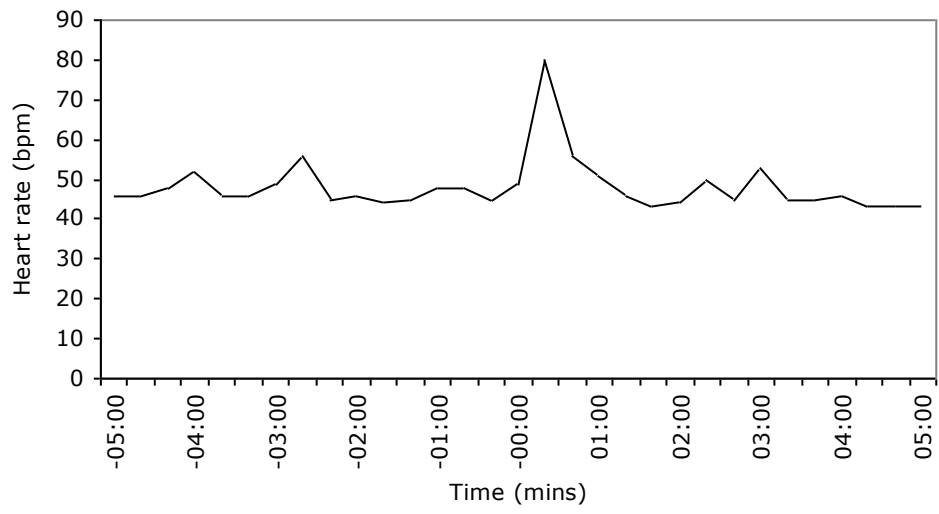
Horse 4



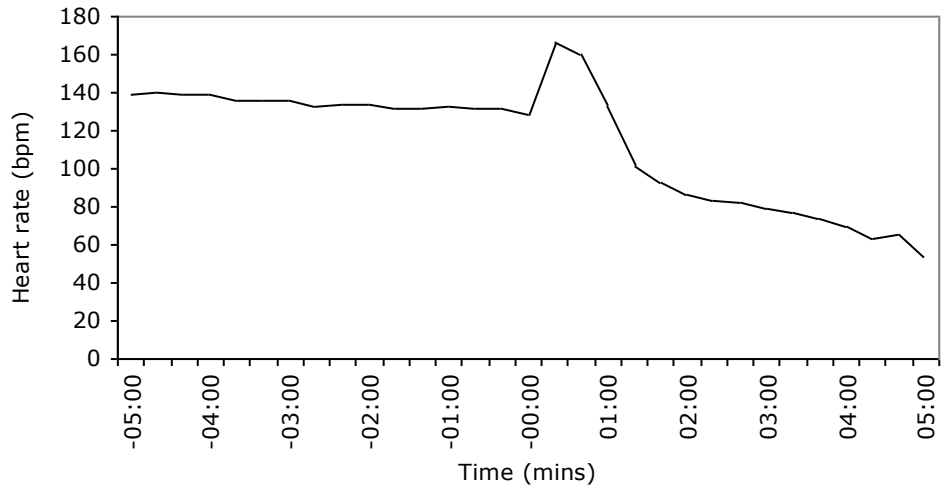
Horse 5



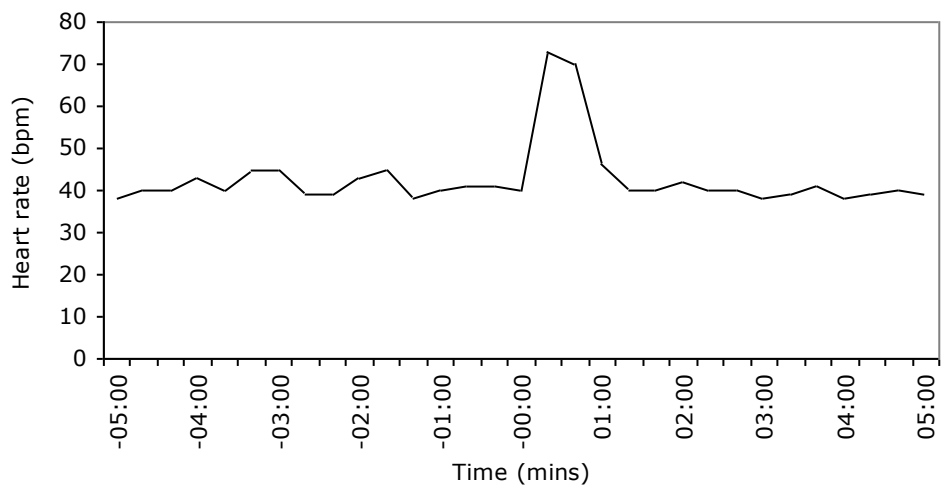
Horse 6

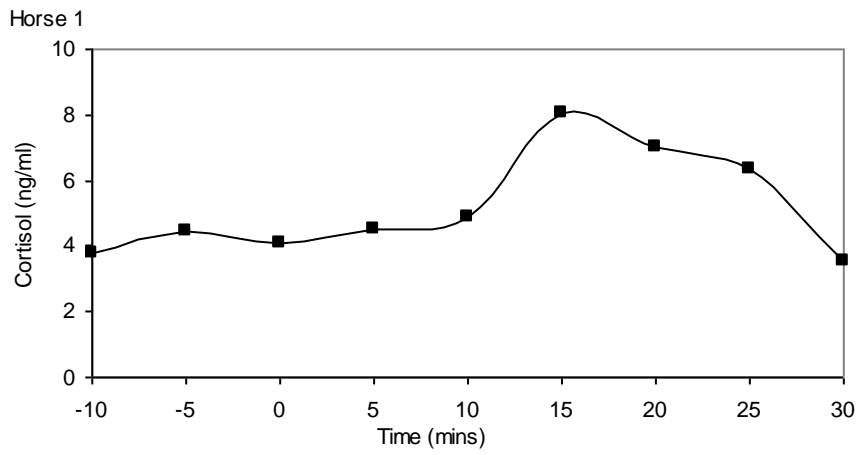
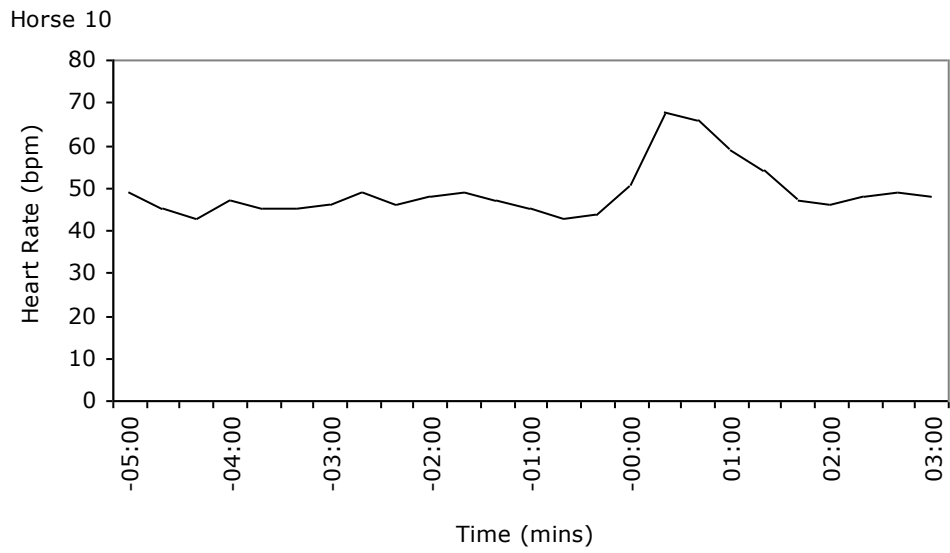
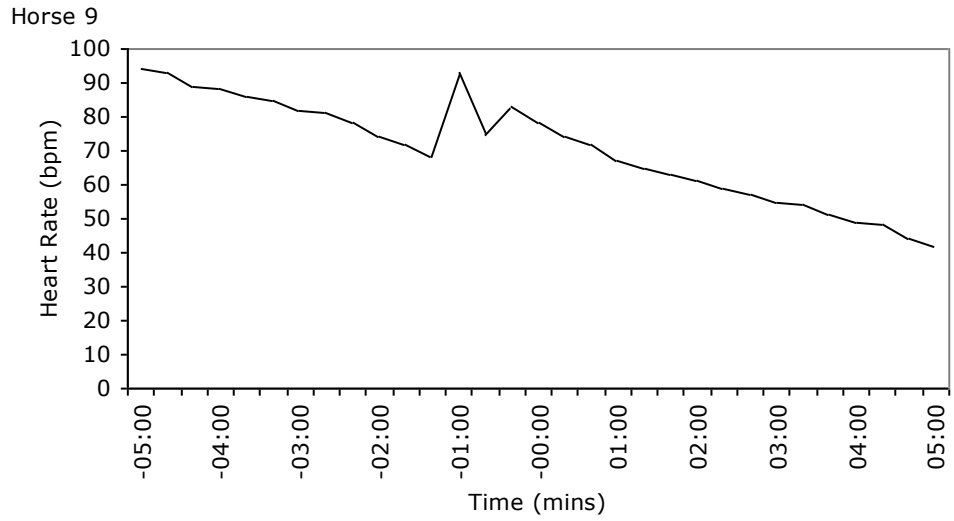


Horse 7

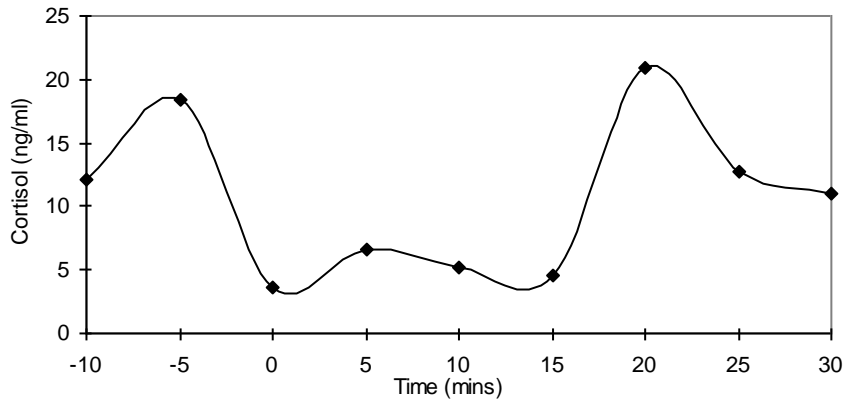


Horse 8

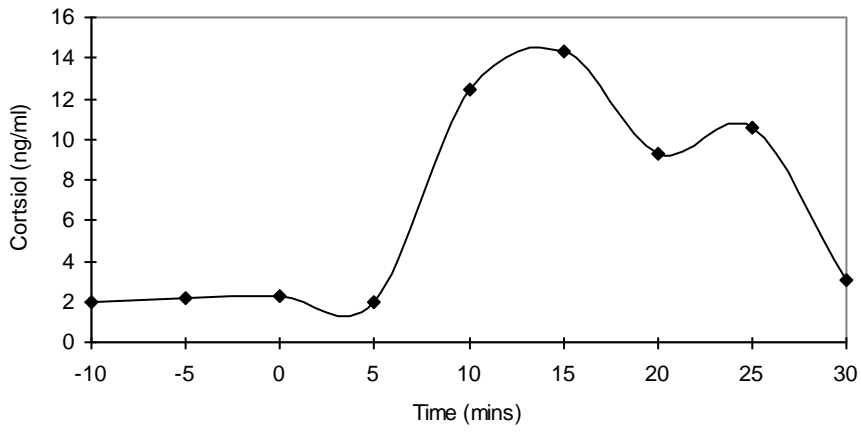




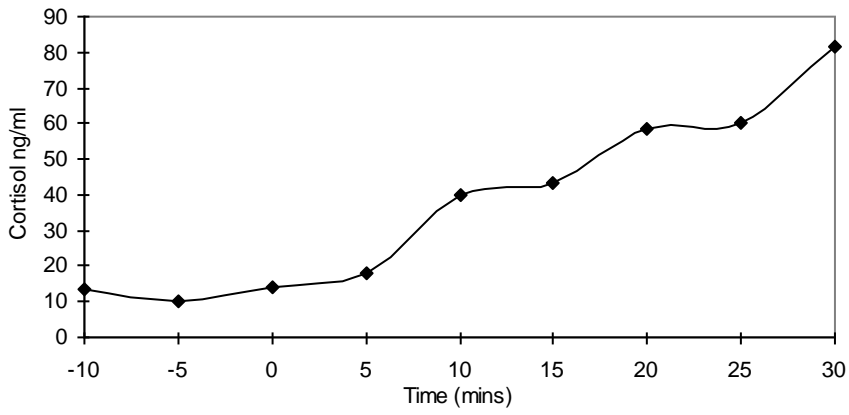
Horse 2

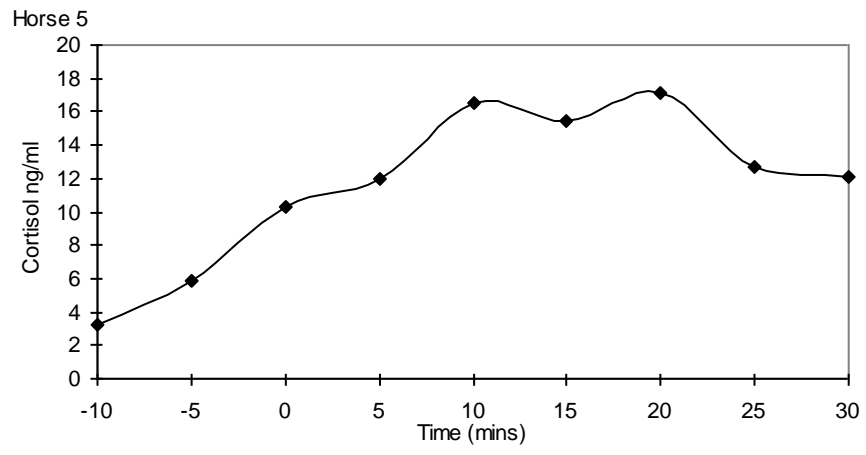


Horse 3



Horse 4





Preliminary study 3

Materials and methods

Study horse and test area

A horse known to show behavioural signs of stress when clipped was chosen by the Brackenhurst equestrian centre yard manager. The horse was a ten year old gelding at the time of the study and was a medium weight cob. Sham clipping was carried out in an enclosed barn that was familiar to the horse. The test area has been described in chapter 4.2.2.

Test procedure

The horse was led the short distance from its stable by a familiar handler and tied up using a conventional head collar and lead rope in the barn. As it is possible that exercise and anticipation may have contributed to pre stress changes in physiological measures during the startle response study, the horse was allowed to acclimatise to the new environment for ten minutes and the first physiological measure (10 minutes pre sham clipping) did not start until after this period. The horse was exposed to ten minutes of sham clipping (the clippers were placed on the horse so it could feel and hear them but hair was not removed).

Physiological measures were taken every ten minutes from ten minutes pre onset of sham clipping until one hour post onset of sham clipping. The eye remained the primary anatomical area under investigation as it had been reported to be a more consistent measure of thermal change in previous work (Cook *et al.*, 2006, Stewart *et al.*, 2008) and a thermal response of the eye had been observed in four of the five study horses in the preliminary investigation into temperature output of horses in their usual environment (Appendix 5) in addition to one horse during study B (chapter 4.0). An

investigation into thermal change of ear temperature in response to a stressful situation was also included as there is evidence that ear pinna temperature alters in stressful situations in other species (Ingram *et al.*, 2002).

Data collection and analysis

Infrared thermography

Thermal images of the eyes and ears were captured every ten minutes from ten minutes pre onset of sham clipping until one hour post onset of sham clipping. Temperature was extracted and processed from each thermal image as previously discussed and a mean temperature for left and right eye and left and right ear for each time point calculated. Temperatures were then plotted against time using Excel to investigate any changes in thermal output during the study.

Salivary cortisol

Saliva was sampled every ten minutes from ten minutes pre onset of sham clipping until one hour post onset of sham clipping. Saliva was collected, analysed and processed using the method previously discussed in chapter 2. Salivary cortisol for each time point was calculated using the methods previously discussed and plotted against time to investigate any changes during the study.

Heart rate

Heart rate was collected every ten minutes from ten minutes pre onset of sham clipping until one hour post onset of sham clipping. Heart rate was collected for this one horse using a stethoscope (Medscope Ltd, UK) placed behind the point of elbow and heart rate counted for 15 seconds. Heart rate for each time point was extracted using the method previously discussed and plotted against time to investigate any changes during the study.

Core temperature

Core temperature was taken every ten minutes from ten minutes pre onset of sham clipping until one hour post onset of sham clipping using the method previously discussed in chapter 2.0. Temperatures were recorded by hand onto the horse's data sheet and transferred to an Excel spreadsheet at a later date.

Ambient temperature

Ambient temperature was recorded every ten minutes throughout the duration of the study using a wet bulb thermometer

Behavioural response

The primary purpose of this pilot work was to investigate whether a potentially distressing management practice would activate the physiological stress response and warrant a larger investigation; therefore a behavioural assessment was not included at this stage. The horse was chosen as it was already known to show behavioural signs that it found clipping to be stressful.

Results

Infrared thermography.

Eye temperature remained constant pre and post sham clipping at approximately 40°C, however at the onset of sham clipping it increased by 9.7°C (Figure 7.1). Eye temperature decreased upon removal of the sham clipping and had returned to basal level within ten minutes

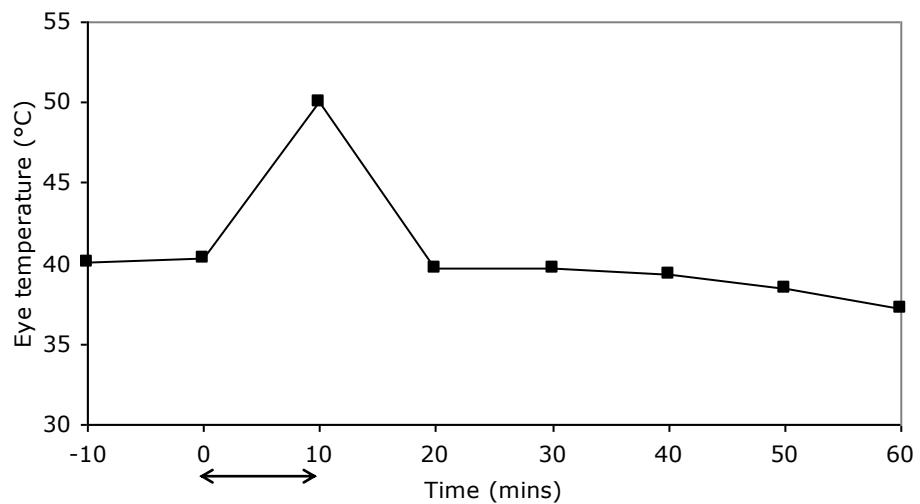


Figure 7.1. Eye temperature (°C) for one pilot horse over duration of the study. The arrow marks the time when sham clipping occurred.

Ear temperature fluctuated throughout the sampling period between 34.7°C and 39.5°C. Core temperature remained stable for the duration of the study, only fluctuating by $\pm 0.3^\circ\text{C}$. Ambient temperature increased from 16.3°C to 19.2°C throughout the duration of the study. Table 7.1 details mean eye and ear temperature in addition to core and ambient temperature for each time point.

Table 7.1 Mean eye, ear and core temperature in addition to ambient temperature for each sampling time point. Shaded rows indicate that sham clipping was being carried out.

Time	Mean eye temperature (°C)	Mean ear temperature (°C)	Core temperature (°C)	Ambient temperature (°C)
1	40.1	37.1	37.2	16.3
2	40.3	39	37.1	16.6
3	50	39.5	37.3	16.8
4	39.7	38.5	37.8	17.6
5	39.7	38	37.8	18.5
6	39.3	35.3	37.5	18.7
7	38.5	36.3	37.4	18.7
8	37.2	34.7	37.6	19.2

Salivary cortisol.

Salivary cortisol increased in response to sham clipping (Figure 7.2) from a basal level of 2.38 ng/ml to a maximum of 6.40ng/ml. Cortisol peaked twenty minutes post onset of clipping and had returned to near basal level at thirty minutes post onset of clipping.

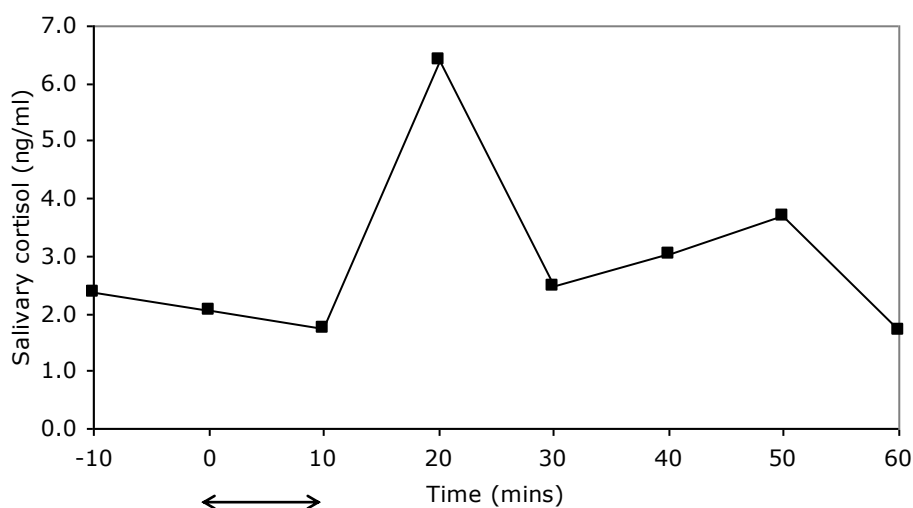


Figure 7.2 Salivary cortisol (ng/ml) for one pilot horse over duration of the study. The arrow marks the time when sham clipping occurred.

Heart rate

Heart rate increased in response to sham clipping and mirrored the increase in eye temperature. There was also a second increase in heart rate after the stressor had been removed. Figure 7.3 details the increase in heart rate and eye temperature.

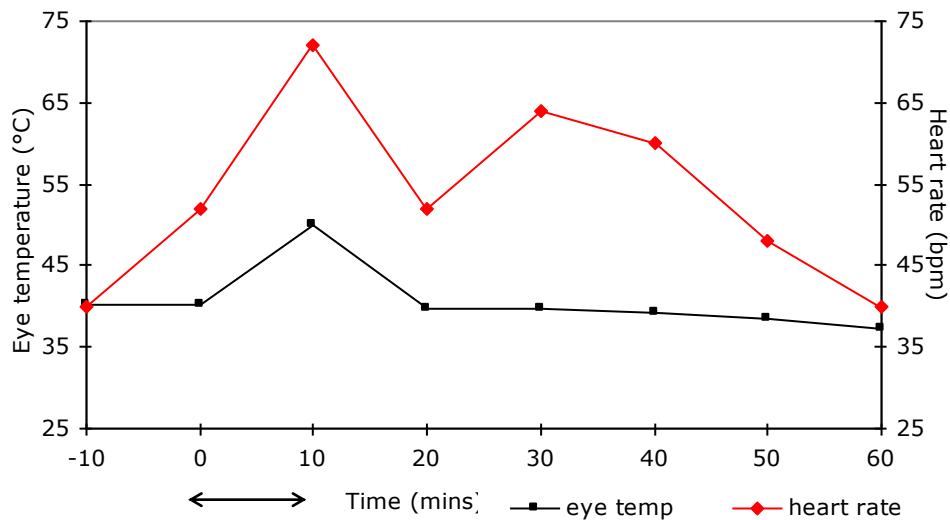


Figure 7.3 Eye temperature (°C) and heart rate (bpm) for one pilot horse for the duration of the study. The arrow marks the time when sham clipping occurred.

Relationship between eye temperature and salivary cortisol

The rapid increase in eye temperature was followed by an increase in salivary cortisol with a lag time of ten minutes between the peak in eye temperature and the peak in cortisol. Both measures had returned to basal level by the end of the study period. Figure 7.4 details the change in eye temperature and salivary cortisol over the duration of the study period.

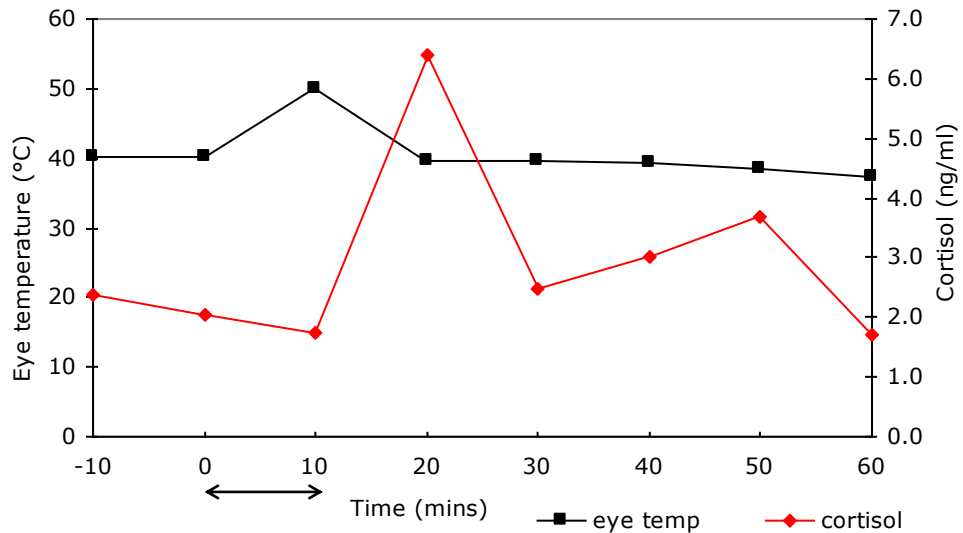


Figure 7.4 Eye temperature (°C) and salivary cortisol (ng/ml) for one pilot horse over the duration of the study period. The arrow marks the time when sham clipping occurred.

Discussion

An increase in eye temperature in response to sham clipping was evident in the study horse. No initial decrease in eye temperature was recorded, however as this has only been reported within seconds of the presentation of a stressor (Levine *et al.*, 2001, Nakayama *et al.*, 2004, Stewart *et al.*, 2008) it is possible that any decrease in eye temperature was not captured due to the timing parameters of the thermal images. Ear temperature fluctuated throughout the study. This is possibly due to the ear being an outer extremity and more prone to temperature fluctuations due to environmental temperature changes. Ambient temperature was measured using a wet bulb thermometer for this preliminary study and readings were not accurate enough to ascertain whether changes in ambient temperature directly affected ear temperature. This small study suggests eye temperature to be a more consistent measure of thermal change in response to stress than ear temperature and supports existing findings (Schafer *et al.*, 2003, Stewart *et al.*, 2008). Core temperature remained constant, which is to be expected due to mechanisms that are in place to maintain internal homeostasis (Cymbaluk and Christison, 1990). Ambient temperature increased over the duration of

the study, however whilst eye temperature increased and then subsequently decreased, ambient temperature continued to increase. This, in addition to the results of the preliminary study into the effects of distance on accuracy of IRT (Appendix 4), suggests that change in eye temperature was not associated with ambient temperature.

Heart rate increased at the onset of sham clipping and mirrored the increase in eye temperature. As heart rate is activated by the SNS this could suggest changes in eye temperature are also associated with the activation of the sympathetic nervous system in response to a stressor. However, heart rate declined and then increased again post sham clipping and this increase was not mirrored by a rise in eye temperature. The increase in heart rate post sham clipping may not have been stress related and could have been caused by movement of the study horse either physically around the point where it was tied up or possibly due to respiration sinus arrhythmia (the cyclic change in heart rate driven by the inhalation and exhalation of breathing).

Salivary cortisol increased over the duration of the study with maximum values recorded twenty minutes post onset of clipping. This is consistent with findings of past work (Colborn *et al.*, 1991, Shanahan, 2003, Stewart *et al.*, 2007) and consistent with the hormonal response of the horses during the startle response study.

Conclusion

This small preliminary study revealed an increase in eye temperature in addition to an increase in salivary cortisol in response to sham clipping however insufficient data was available to allow statistical testing as only one horse was used. The increase in both eye temperature and salivary cortisol warranted further investigation using a larger study into thermal and hormonal response of the eye to a short term husbandry practice.

Appendix 8

Individual physiological data for chapter 5

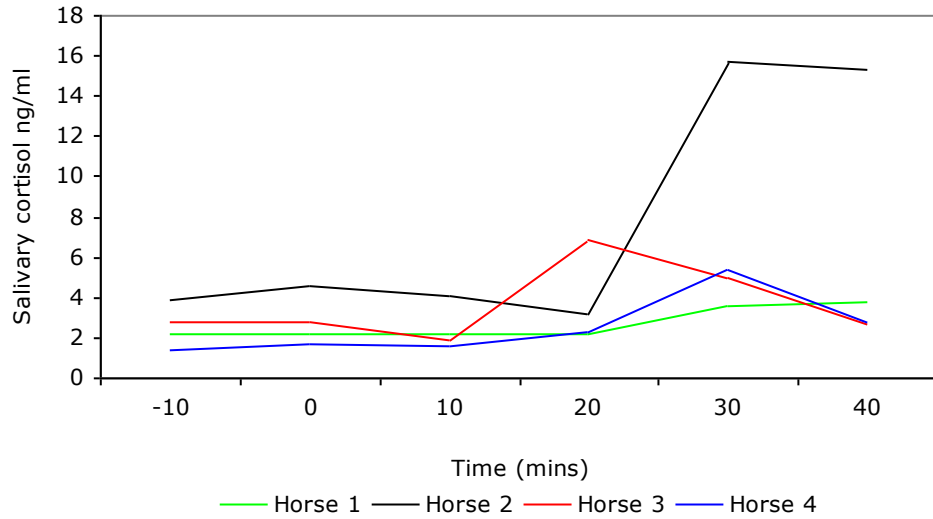


Figure 8.1 Salivary cortisol profiles of four individual horses during study C displaying differing basal values.

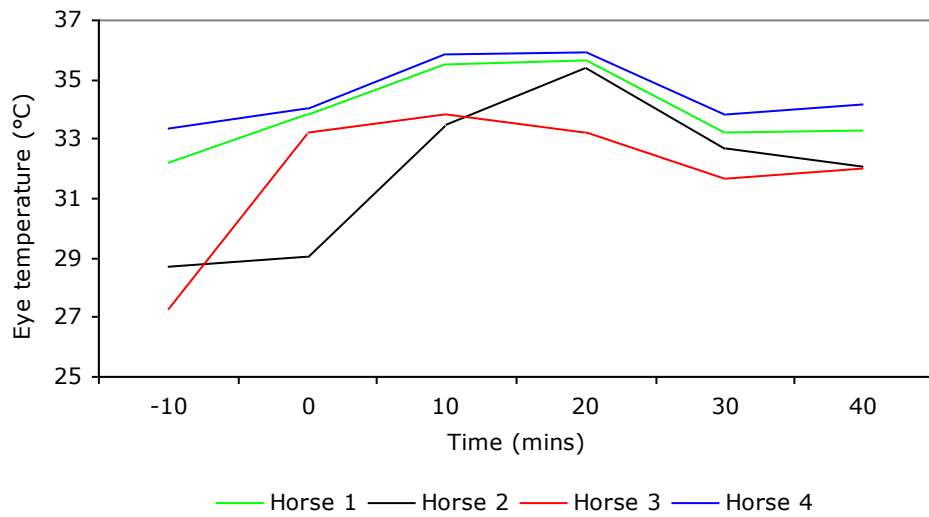


Figure 8.2 Eye temperature profiles of four individual horses during study C displaying differing basal values.

Appendix 9

Extraction and assay protocol used by Chester Zoo

Wet Weight Shaking Extraction

Pre Extraction Preparation

- Prepare an excel spreadsheet to make labels with sample numbers for each faecal sample and extract (*see label making protocol*). Print labels.
- Label 12x75mm polyethylene sample storage vials with labels.
- Next - sort frozen faecal samples into boxes by animal and then by date

Day 1

- Take boxes of frozen faecal samples out of freezer. Spread faecal samples out on trays in order (*animal then date*) and let thaw for a few hours
- Label extraction vials (*small vials with black tops*) with a Sharpie pen putting the sample number on both sides.
- When samples are thawed - weigh 0.5 g of wet faecal sample into small weigh boats then and transfer into extraction vials. *As pockets of hormones can be found in faecal samples mix samples well - use a combination of mixing with the weighing tool and crushing the bag between your fingers. Mark any unusual consistency or debris on sheet.*
 - At the same time fill small polyethylene sample storage vial labeled with 'faecal sample' with remainder of mixed sample. Cap the vials. *These are for storage, they do not need to be processed any further - place in freezer when you are finished with all your extractions.*
- Add 0.5 ml Milli-Q water and 4.5 ml methanol to every extraction vial. *If monitoring extraction efficiency add appropriate amount of endogenous hormone to each tube (follow extraction efficiency protocol)*
- Cap the vials. Vortex each tube until sample is well mixed (until all faecal material is freely mixing in the solution) ~10 seconds
- Place extraction vials in order in boxes and place on rotator - agitate overnight.

Day 2

- Remove extraction vials from rotator in the morning
- Vortex each sample
- Remove green buckets from the centrifuge - Remove caps and place extraction vials in green buckets in order. Centrifuge extraction vials for 20 minutes at 1800rpm (*you only need to turn the timer to 20 - everything else is already set*)
- While tubes are spinning label a set of glass tubes (16mm x125mm) with sample numbers on both sides which are listed in the excel extraction sheet.
- Pour off supernatant into corresponding # glass extraction tubes (16mm x125mm) and dry down supernatant in warm water bath under air in fume cupboard. Reconstitute in 1 ml methanol. Vortex briefly, sonicate, covered, for 15 minutes.
- Store 1ml extracts in the set of polyethylene sample storage vials labeled 'faecal extracts'

PROTOCOL FOR CORTICOSTERONE (CC) EIA

DAY 1

Plate coating

- use NUNC Maxisorb plates
- CC antibody working dilution is 1:15,000

- add 33.3 uL antibody stock (1:100, -20°C) to 5 mL coating buffer in a glass beaker
- add 50 uL per well antibody solution
- **do not coat column 1** - start at A2 and go down each column
- pipet all solutions in this order
- tap plates gently to ensure that coating solution covers well bottom
- label, cover with acetate plate sealer and leave overnight at 4°C
- Plates are **not ready to use the following day (day 2)**, but can be used on **day 3, 4 or 5**

DAY 3

Standards

- standard values are 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8 and 3.9 pg corticosterone/well
- dilute standard stock (20 ng/mL or 1000 pg/well, -20°C) serially 2-fold using 200ul assay buffer

Samples/controls

- dilute samples in EIA buffer to the appropriate dilution
- Use prepared Corticosterone C1 and C2 neat in assay

Control Plate

- Run an additional plate, with standards, C1 and C2 as normal
- Use a single pooled sample at the appropriate dilution across remaining wells (samples 1-26)
- Run plate as normal

HRP

- CC-HRP working dilution is 1:70,000
- add 7.14 uL stock (1:100, 4°C) to 5 mL EIA buffer in a glass beaker

RUNNING THE PLATE

Plate washing

- purge the plate washer
- wash the plates five times with wash solution
- blot the plates on paper towel to remove excess wash solution
- run plate immediately

Plate loading

- add 50 uL standard, sample, or control per well in duplicate as quickly and accurately as possible
- Immediately add 50 uL per well of diluted CC-HRP
- cover the plates, label with the time and **incubate at RT for 2 hours in the dark**

Plate washing

- wash the plates 5 times with wash solution and blot dry
- plates are fairly stable at this point and can be left **in the dark** until all plates are washed

Substrate

- Substrate buffer must be at **room temperature** before use
- prepare substrate **immediately** before use
- combine (40 uL H₂O₂, 125 uL ABTS and 12.5 mL RT citrate buffer) = substrate
- add 100 uL substrate to all wells
- cover/incubate at **RT in the dark**

Appendix 10

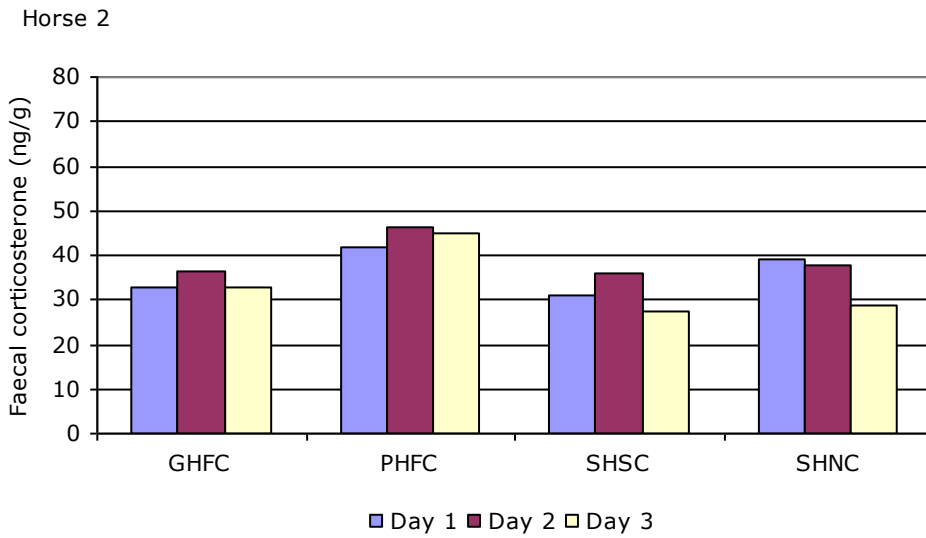
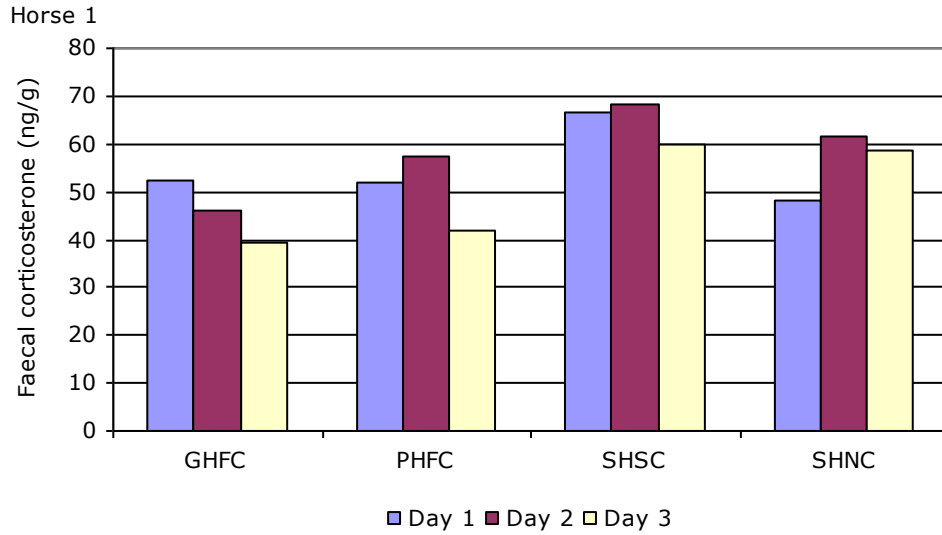
Raw data from faecal glucocorticoid analysis

Visi			Blackberry			Beau			Tosca		
Sample	pg/well	ng/g	Sample	pg/well	ng/g	Sample	pg/well	ng/g	Sample	pg/well	ng/g
Monday 1	131.256	52.50	Monday 1	98.102	39.24	Monday 1	60.881	24.35	Monday 1	79.607	31.84
Tuesday 1	115.661	46.26	Tuesday 1	94.695	37.88	Tuesday 1	61.29	24.52	Tuesday 1	83.547	33.42
Wednesday 1	98.895	39.56	Wednesday 1	72.278	28.91	Wednesday 1	59.329	23.73	Wednesday 1	77.962	31.18
Monday 2	129.327	51.73	Monday 2	81.759	32.70	Monday 2	58.918	23.57	Monday 2	64.244	25.70
Tuesday 2	143.498	57.40	Tuesday 2	91.561	36.62	Tuesday 2	68.191	27.28	Tuesday 2	67.144	26.86
Wednesday 2	104.805	41.92	Wednesday 2	82.251	32.90	Wednesday 2	75.52	30.21	Wednesday 2	76.08	30.43
Monday 3	120.166	48.07	Monday 3	77.734	31.09	Monday 3	68.191	27.28	Monday 3	66.97	26.79
Tuesday 3	153.83	61.53	Tuesday 3	89.37	35.75	Tuesday 3	58.885	23.55	Tuesday 3	65.973	26.39
Wednesday 3	146.68	58.67	Wednesday 3	68.848	27.54	Wednesday 3	113.097	45.24	Thursday 3	69.587	27.83
Monday 4	166.115	66.45	Monday 4	104.016	41.61	Monday 4	83	33.20	Monday 4	58.87	23.55
Tuesday 4	170.868	68.35	Tuesday 4	115.872	46.35	Tuesday 4	59.91	23.96	Tuesday 4	68.026	27.21
Wednesday 4	149.809	59.92	Thursday 4	112.743	45.10	Wednesday 4	64.017	25.61	Wednesday 4	55.71	22.28

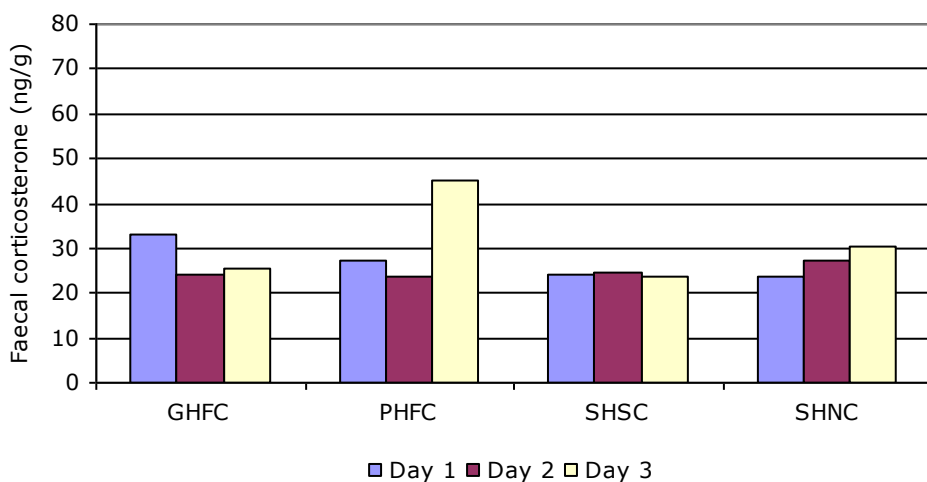
Del			Ellie/Libby			Ernie			Woody		
Sample	pg/well	ng/g	Sample	pg/well	ng/g	Sample	pg/well	ng/g	Sample	pg/well	ng/g
Monday 1	65.428	26.17	Tuesday 1 (Ellie)	65.657	26.26	Monday 1	67.237	26.89	Monday 1	55.089	22.04
Tuesday 1	70.65	28.26	Wednesday 1 (Ellie)	66.853	26.74	Tuesday 1	64.882	25.95	Tuesday 1	74.931	29.97
Wednesday 1	54.622	21.85	Monday 2 (Ellie)	88.996	35.60	Wednesday 1	75.666	30.27	Wednesday 1	65.804	26.32
Monday 2	73.554	29.42	Tuesday 2 (Ellie)	75.271	30.11	Monday 2	61.359	24.54	Monday 2	71.913	28.77
Tuesday 2	62.107	24.84	Wednesday 2 (Ellie)	61.583	24.63	Tuesday 2	53.636	21.45	Tuesday 2	69.255	27.70
Wednesday 2	67.545	27.02	Monday 3 (Ellie)	47.23	18.89	Wednesday 2	65.149	26.06	Wednesday 2	76.283	30.51
Monday 3	65.771	26.31	Tuesday 3 (Ellie)	50.183	20.07	Monday 3	79.608	31.84	Monday 3	85.03	34.01
Tuesday 3	90.179	36.07	Wednesday 3 (Ellie)	49.799	19.92	Tuesday 3	62.94	25.18	Tuesday 3	98.937	39.57
Wednesday 3	79.368	31.75	Monday 4 (Libby)	106.63	42.65	Wednesday 3	65.276	26.11	Wednesday 3	89.32	35.73
Monday 4	90.685	36.27	Tuesday 4 - am (Libby)	72.329	28.93	Monday 4	72.256	28.90	Monday 4	94.331	37.73
Tuesday 4	106.088	42.44	Tuesday 4 - pm (Libby)	103.326	41.33	Tuesday 4	68.044	27.22	Tuesday 4	94.104	37.64
Wednesday 4	111.203	44.48	Wednesday 4 (Libby)	91.208	36.48	Wednesday 4	71.626	28.65	Wednesday 4	91.16	36.46

Appendix 11

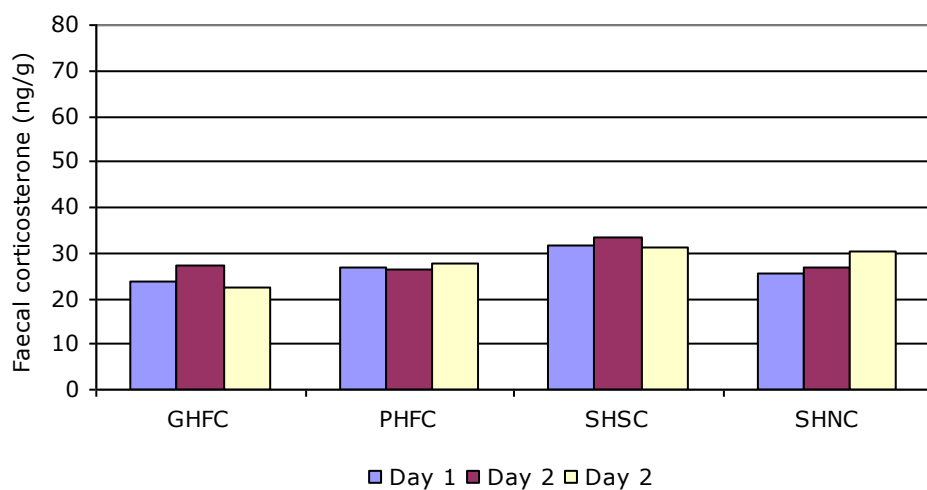
Individual faecal corticosterone data



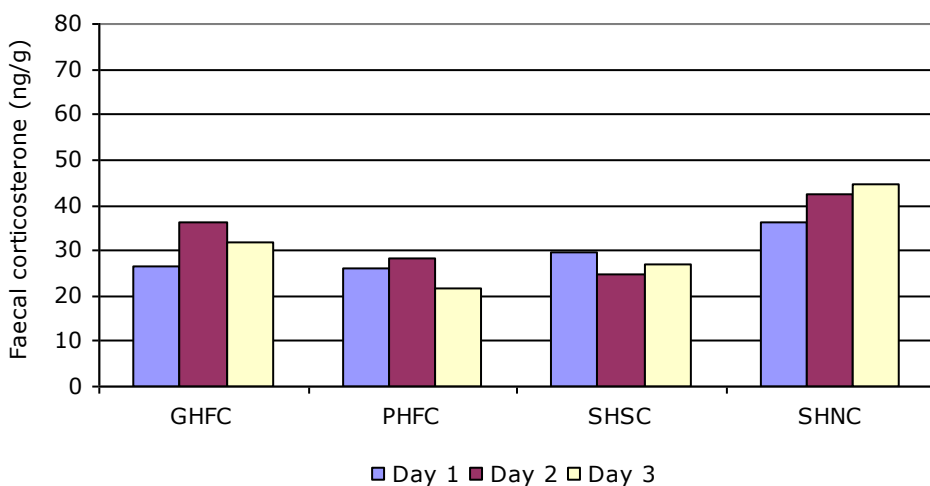
Horse 3



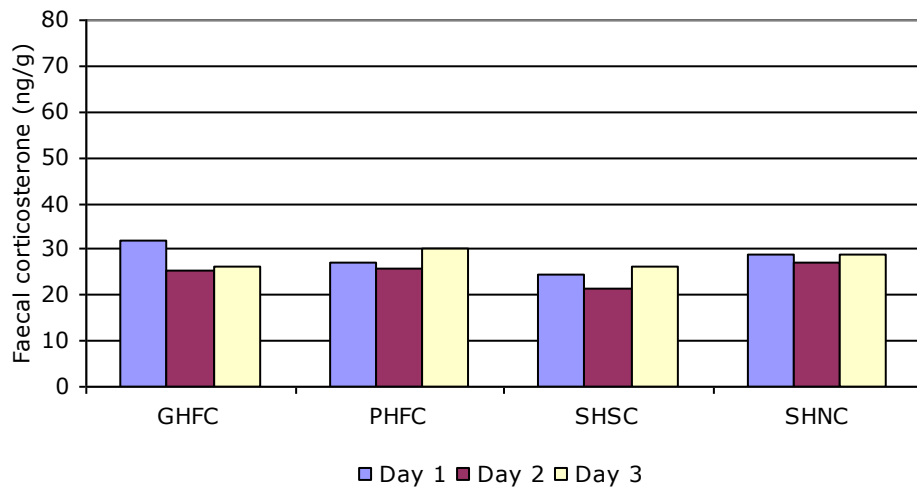
Horse 4



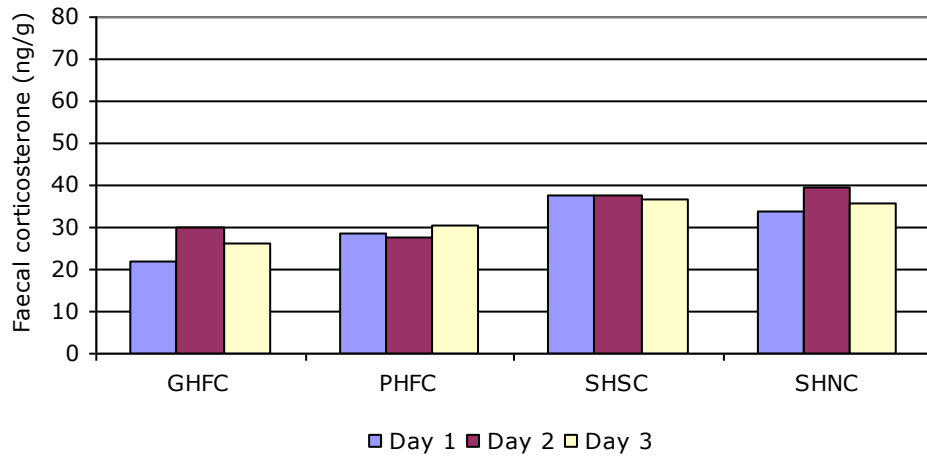
Horse 5



Horse 6



Horse 7



Appendix 12

Ethical approval and risk assessments

Ethical Review of Post-Graduate Work in ARES – April 2008

Please complete this form for the practical work undertaken that involves interaction with:

- Other people (outside the group)
- Animals
- The natural environment

1 PhD

2 Principal Investigator: Kelly Yarnell

3 List the practical activities giving a BRIEF description of practical work (making reference to standard methods where appropriate):

For pilot studies:

- Clipping horses (standard method necessary for welfare of horse)
- Acoustic startle testing (horses)
- Isolation study (horses)
- Monitoring of equine temperature using thermal camera (non-invasive) during routine stable management.

Further studies.

- Transportation of horses (standard procedure)
- Saliva sampling using cotton bud from inside cheek (horse familiar with having mouth handled)
- Monitoring of heart rate using polar heart rate monitor (horse familiar with procedure)
- Monitoring of thermal output using thermal camera (non contact and non invasive)
- Behavioural observations (non contact)

4 Please identify which of the following may be relevant to the practical work undertaken:

Legislation/Best Practice	Relevant? (indicate if yes)
Animal & Scientific Procedures Act	Yes
Animal Welfare Act	Yes
Animal Transportation	Yes
Control of pests (DEFRA)	
Countryside & Rights of Way Act	
Criminal Records Bureau check	
Data Protection Act	
Designated Areas	
Human health	
Human psychology	
Protected species	
Wildlife & Countryside Act	
Local legislation/ requirements overseas	

Ethical Review of Scientific Procedures involving Animals

School of Animal, Rural and Environmental Sciences

Nottingham Trent University

Studies involving animals are currently being undertaken in the School of Animal, Rural and Environmental Sciences by some members of the following groups:

- academic staff
- postgraduate students
- undergraduate students

The principal aims of all studies carried out in the School are:

- to increase animal welfare by furthering knowledge and understanding;
- to further education of students with regard to scientific method and the importance of ethical considerations.

The School of Animal, Rural and Environmental Sciences at Nottingham Trent University is committed to promoting the welfare of animals as outlined in the Animal Welfare Act 2006. These include :

- (a) its need for a suitable environment,
- (b) its need for a suitable diet,
- (c) its need to be able to exhibit normal behaviour patterns,
- (d) any need it has to be housed with, or apart from, other animals, and
- (e) its need to be protected from pain, suffering, injury and disease.

No study will be considered acceptable for application to an animal at the Brackenhurst site if it may have the effect of causing that animal pain, suffering, distress or lasting harm.

No procedure will be undertaken which requires a Home Office Licence under the Animals (Scientific Procedures) Act 1986, unless this procedure has been submitted in advance for approval by the College of Science and Technology Ethics Review Committee (Animals) and a licence has been obtained.

Before commencement of any study involving animals, an Ethical Review form must be completed and submitted to the School Research Committee. The project shall not commence until such approval has been obtained.

The remit of the School Research Committee with regard to Ethical Review is:

1. To set up and maintain ethical review processes.
2. To assess proposals in detail and to challenge, where necessary, the appropriateness of the animal use.
3. To maintain a balance between the interests of science and animal welfare.
4. To review ongoing projects on an annual basis.
5. To ensure that any study is planned so that the health and well-being of the animals is maintained.

Ethical Review Form for Scientific Procedures (Animals)
School of Animal, Rural and Environmental Sciences
Nottingham Trent University

- 1. Name of Applicant**
Kelly Burton
- 2. Position (eg. BSc year 3 Animal Science)**
PhD Yr 2 (Equine)
- 3. Contact details (email/telephone)**
Kelly.burton@ntu.ac.uk 07843082964
- 4. Purpose of project application (eg. BSc year 3 dissertation, PhD thesis, or paper for publication)**
PhD study Thermal and hormonal circadian rhythm of horses
- 5. Name of supervisor/member of staff responsible**
Carol Hall
- 6. Details of planned method/procedure, including explanation of how the provisions of the Animal Welfare Act 2006 will be maintained. Use a separate sheet if necessary.**

Thermal Images and salivary swabs (salivettes) will be taken of horses along with their core temperature. *(Rectal Digital Thermometer)*
IRT is totally non-invasive and non-contact and all horses are familiar with sampling of saliva. HR and rectal temperature are routine veterinary procedures that all study horses are familiar with.
All testing will be carried out in the horses' familiar environment with suitable floor surface, lighting and equipment to prevent injury to the horse and horses will not be removed from con-specific animals.
All horses are in good health and all handlers are experienced and competent. Throughout the course of the study horses will be monitored and will be excluded from the study at any sign of ill health.

A risk assessment has been completed.

Statement of ethical review

I have read the Ethical Review Procedure of the School of Animal, Rural and Environmental Sciences and agree to abide by it and the Animal Welfare Act 2006. I understand that studies will not be allowed to take place until ethical approval has been obtained, and that permission will be withdrawn and the study cancelled if there is a breach of these conditions.

Signatures:

Applicant Kelly Burton Date 11.3.09
Supervisor Carol Hall Date 16.3.09

Risk Rev

Ethical Review Form for Scientific Procedures (Animals)
School of Animal, Rural and Environmental Sciences
Nottingham Trent University

- 1. Name of Applicant**
Kelly Burton
- 2. Position (eg. BSc year 3 Animal Science)**
PhD Yr 2 (Equine)
- 3. Contact details (email/telephone)**
Kelly.burton@ntu.ac.uk 07843082964
- 4. Purpose of project application (eg. BSc year 3 dissertation, PhD thesis, or paper for publication)**
PhD study (Clipping)
- 5. Name of supervisor/member of staff responsible**
Carol Hall
- 6. Details of planned method/procedure, including explanation of how the provisions of the Animal Welfare Act 2006 will be maintained. Use a separate sheet if necessary.**

Stethoscope → Clipping horses whilst measuring physiological variables including salivary cortisol, HR and thermal output. (thermal camera, digital thermometer for rectal temp., swabbed via salivettes).
Clipping is a routine procedure that is necessary for the welfare of the horse. All horses have been clipped before and are all familiar with the heart rate monitors, sampling of saliva and thermal imaging. All are non-invasive procedures.
All equipment has been safety checked and clipping will be carried out in a well lit area with suitable flooring to prevent injury to the horse.
All horses are in good health and all handlers are experienced and competent.

A risk assessment has been completed.

Statement of ethical review

I have read the Ethical Review Procedure of the School of Animal, Rural and Environmental Sciences and agree to abide by it and the Animal Welfare Act 2006. I understand that studies will not be allowed to take place until ethical approval has been obtained, and that permission will be withdrawn and the study cancelled if there is a breach of these conditions.

Signatures:

Applicant..... *K. Burton* Date..... *2.10.08*
Supervisor..... *Carol Hall* Date..... *2.10.08*

c:\jill\nturesadmin\ares-ibs\updated ethics form 2006.doc

02/10/08

Ethical Review Form for Scientific Procedures (Animals)
School of Animal, Rural and Environmental Sciences
Nottingham Trent University

1. Name of Applicant

Kelly Burton

2. Position (eg. BSc year 3 Animal Science)

PHD Yr 3 (Equine)

3. Contact details (email/telephone)

Kelly.burton@ntu.ac.uk 07843032964

4. Purpose of project application (eg. BSc year 3 dissertation, PhD thesis, or paper for publication)

PhD study. The effects of housing design on physiological indicators of distress in the horse

5. Name of supervisor/member of staff responsible

Carol Hall

6. Details of planned method/procedure, including explanation of how the provisions of the Animal Welfare Act 2006 will be maintained. Use a separate sheet if necessary.

Thermal images and salivary swabs (salivettes) will be taken of horses along with their core temperature.

IRT is totally non-invasive and non-contact and all horses are familiar with sampling of saliva. HR and rectal temperature are routine veterinary procedures that all study horses are familiar with.

All testing will be carried out in the horses' familiar environment with suitable floor surface, lighting and equipment to prevent injury to the horse and horses will not be removed from con-specific animals.

All horses are in good health and all handlers are experienced and competent. Throughout the course of the study horses will be monitored and will be excluded from the study at any sign of ill health.

A risk assessment has been completed.

Statement of ethical review

I have read the Ethical Review Procedure of the School of Animal, Rural and Environmental Sciences and agree to abide by it and the Animal Welfare Act 2006. I understand that studies will not be allowed to take place until ethical approval has been obtained, and that permission will be withdrawn and the study canceled if there is a breach of these conditions.

Signatures:

Applicant... K. Burton Date... 1.8.09

Supervisor..... Date.....

Risk assessments

PHD Risk Assessment

Activity: PhD study Circadian Rhythm
April/July/Sept/Jan 08/09.

Method/Equipment: APH

Area: EQUINE

Activity	Hazard	Control	Risk
Horse handling	kicked/bitten / stood on by horse	Correct handling techniques have been taught and applied. PPE - Hat and gloves to be worn.	4
Walking Horse in Yard	Horse or handler may slip trip or fall	Suitable footwear should be worn. Ensure surface being worked on is firm, non-slippery and level. Correct handling techniques taught and applied.	4
Sampling saliva/rectal temp/IRT	Bitten	Technique carried out by competent person on horse familiar with process	4
Electrical equipment on yard (computers, camera)	Trip on wires/ electric shock	Ensure all equipment secure on solid surface, all wires safely out of reach of horses and walkways, electrical equipment safety checked.	3

NB Risk = Severity x Likelihood
Severity: 3 - Death or Serious Injury, 1 - Minor or no injury, 2 - Everything else
Likelihood: 3 - Certain, 1 - Almost never, 2 - Everything else.

Assessment completed on: 26.02.09

Assessment completed by: Kelly Burton

School/Department: ARES

Area: EQUINE

Activity	Hazard	Control	Risk
Horse handling	Kicked/bitten /stood on by horse	Correct handling techniques have been taught and applied. PPE - Hat and gloves to be worn.	4
Taking horses rectal temperature/Heart Rate/SR/thermal image and salivary cortisol	Kicked/bitten /stood on by horse	Hat, Gloves and protective footwear worn Horse to wear bridle with head collar and held in enclosed safe space. Training in correct methods carried out. Horse familiar with sampling techniques.	4
Catching horses in field	Kicked/bitten /stood on by horse	Hat, Gloves and protective footwear worn Correct equipment used.	4
Clipping horses	Kicked/bitten /stood on by horse Trip on wires/ electric shock	See measures above plus horse familiar with clipping and person who clips is experienced and competent.	3

NB Risk = Severity x Likelihood
 Severity: 3 - Death or Serious Injury, 1 - Minor or no injury, 2 - Everything else
 Likelihood: 3 - Certain, 1 - Almost never, 2 - Everything else

Assessment completed on: 10.06.09 by Kelly Burton

Kelly Burton

NTU Risk Assessment

Activity: PhD Pilot Study - clipping area

School/Department: ARES

Area: EQUINE

Activity	Hazard	Control	Risk
Horse handling	Kicked, bitten, stood on	Correct handling techniques have been taught and applied. PPE - Hat and gloves to be worn.	4
Walking Horse in Yard	Horse or handler may slip trip or fall	Suitable footwear should be worn. Ensure surface being worked on is firm, non-slippery and level. Correct handling techniques taught and applied.	4
Clipping nervous horse	Kicked, bitten, stood on	Correct handling techniques have been taught and applied. PPE - Hat and gloves to be worn.	4
Electrical equipment on yard	Tripped/shock	Ensure all equipment secure on solid surface, all wires out of reach of walkways and horses. Equipment safety checked. Waterproof sockets on yard used	3
Rectal temp/saliva sampling/HR/IRT	Kicked, bitten, stood on	Hat, gloves and protective footwear worn. Horse to wear bridle and held in enclosed safe space. Training in sampling methods complete. Horse familiar with methods	

NB Risk = Severity x Likelihood
 Severity: 3 - Death or Serious Injury, 1 - Minor or no injury, 2 - Everything else
 Likelihood: 3 - Certain, 1 - Almost never, 2 - Everything else.

Assessment completed on: 01.12.08

Assessment completed by (signature) *[Signature]*

NTU Risk Assessment

Activity: Phd Clipping Study OCT 08

School/Department: ARES

Area: EQUINE

Activity	Hazard	Control	Risk
Horse handling	Kicked/bitten /stood on by horse	Correct handling techniques have been taught and applied. PPE – Hat and gloves to be worn.	4
Walking Horse in Yard	Horse or handler may slip trip or fall	Suitable footwear should be worn. Ensure surface being worked on is firm, non-slippery and level. Correct handling techniques taught and applied.	4
Taking horses Rectal temperature/Heart rate/RW/thermal image and salivary cortisol	Kicked/bitten /stood on by horse	Hat, gloves and protective footwear worn Horse to wear bridle with head collar and held in enclosed safe space. Training in correct methods carried out. Horse familiar with sampling techniques.	4
Electrical equipment (clippers) on yard	Trip on wires/ electric shock	Ensure all equipment secure on solid surface, all wires safely out of reach of horses and walkways, electrical equipment safety checked	3
Clipping horses	Kicked/bitten /stood on by horse Trip on wires/ electric shock	See measures above plus horse familiar with clipping and person who clips is experienced and competent.	4

NB Risk – Severity x Likelihood
 Severity: 3 – Death or Serious Injury, 1 – Minor or no injury, 2 – Everything else
 Likelihood: 3 – Certain, 1 – Almost never, 2 – Everything else

Assessment completed on: 03.10.08 by Kelly Burton

NITU Risk Assessment

Activity: PhD study 22.05.09.
NOVA OBJECT.

School/Department: ARES

Area: EQUINE

Activity	Hazard	Control	Risk
Horse handling	Kicked/bitten /stood on by horse	Correct handling techniques have been taught and applied. PPE - Hat and gloves to be worn.	4
Walking Horse in Yard	Horse or handler may slip trip or fall	Suitable footwear should be worn. Ensure surface being worked on is firm, non-slippery and level. Correct handling techniques taught and applied.	4
Presenting horse with novel object	Kicked Bitten stood on	Hat, gloves and protective footwear worn Horse to wear bridle with head collar and held in enclosed safe space.	4
Sampling saliva	Bitten	Technique carried out by competent person on horse familiar with process	4
Electrical equipment on yard (computers, camera)	Trip on wires/ electric shock	Ensure all equipment secure on solid surface, all wires safely out of reach of horses and walkways, electrical equipment safety checked.	3

MB Risk = Severity x Likelihood
Severity: 3 - Death or Serious Injury, 1 - Minor or no Injury, 2 - Everything else
Likelihood: 3 - Certain, 1 - Almost never, 2 - Everything else.

Assessment completed on: 16.05.08

Assessment completed by: Kelly Burton

NTU Risk Assessment

Activity: PhD pilot Study
School Thermal camera.

School/Department: ARES

Area: EQUINE

Activity	Hazard	Control	Risk
Horse handling	Kicked, bitten, stood on	Correct handling techniques have been taught and applied. PPE - Hat and gloves to be worn.	4
Walking horse in yard	Horse or handler may slip trip or fall	Suitable footwear should be worn. Ensure surface being worked on is firm, non-slippery and level. Correct handling techniques taught and applied.	4

NB Risk = Severity x Likelihood
Severity: 3 - Death or Serious Injury, 1 - Minor or no injury, 2 - Everything else
Likelihood: 3 - Certain, 1 - Almost never, 2 - Everything else.

Assessment completed on: 07.10.07

Assessment completed by (signature): 

