Blink rate assessment as an indicator of brain activity in populations of domestic and semi feral native pony breeds

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ABSTRACT

Spontaneous Bink Rate (SBR) has been used as an inferred measure of dopamine (DA) transmission in humans for a number of decades, in order to learn more about dopamine related disorders such as Schizophrenia and Parkinson's disease. More recently, SBR has been correlated with both stress levels, emotional state and welfare, cognitive indicators and stereotypic behaviours in domestic horses (Roberts *et al* 2016).

However, in order for SBR to be used effectively in this species for diagnostic and research purposes, it is critical to first establish accurate base line values. Generating this data became a primary objective for this work. In addition, in order to 1) validate SBR quantification methods that can be applied to free-roaming horses and 2) to ascertain the potential effects of domestication, both semi-feral and domestic herds from various UK breeds were sought as research subjects.

A total of 128 individuals were studied between September 2019 and October 2022, with animals recruited from free living semi-feral herds with a minimum of human contact and domestic populations kept under traditional management regimes with a mixture of stabled and turnout livery. The study group consisted of UK breeds including New Forest, Exmoor, Shetland, Welsh section A and Eriskay ponies.

Full blinks, partial blinks and eyelid flutters were measured for 15 seconds on 10 separate but consecutive occasions for each horse. Irrespective of breed, domestic horses exhibited significantly lower full blink values (mean 0.974 ± 0.6215 s.d) than semi-feral equivalents (mean 1.678 ± 0.855 s.d). However, domestic animals displayed higher SBR (mean 9.813 ± 3.890 s.d) than semi-feral counterparts (mean 8.1625 ± 3.274 s.d) for partial blinks. The baseline values for full blinks from domestic horses can now be used to inform future research. However, further investigation needs to be carried out to verify the partial blinks with respect for comparison of the results, to verify that partial blinks can be used for dopamine tone, welfare and stress level indication.

At breed level, domestic Eriskay ponies exhibited lower full blinks compared to the semi-feral equivalents. With reference to partial blinks, New Forest semi-feral horses recorded significantly higher values than domestic animals of this breed. Finally, no significant effects were observed, where season or time of day were concerned.

The data obtained in this research during the 15 seconds collection time, show significant results that are present for semi-feral herds. This procedure could be utilised as a non-invasive welfare tool for monitoring dopamine levels and therefore stress and welfare during annual procedures for “drifting” or group penning during medical monitoring or sales for any semi-feral living herds.

DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of the University of Gloucestershire and is original except where indicated by specific reference in the text. No part of the thesis has been submitted as part of any other academic award. The thesis has not been present to any other education institution in the in the United Kingdom or overseas.

My views expressed in the thesis are those of the author and in no way represent those of either University.

Susan Katherine Anne Marie Martin

Susan Katherine Ann Marie Martin Date: 28th September 2023

This project was granted ethical approval by the Ethics Committee at the Royal Agricultural University prior to data collection. Ethics review number 20233127.

Director of Studies.

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I would like to take this opportunity to thank Dr Andrew Hemmings for encouraging me in the beginning to carry out an independent research project and to facilitate the idea of doing a Masters by Research. This certainly would not have been possible without help and encouragement from Dr Simon Daniels and Prof. Meriel Moore-Coyler, and needless to say my colleagues from the lab covering my absence too enabled the data collection periods.

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ABBREVIATIONS

Abbreviation	Definition
ABPM	Average blinks per minute
ACTH	Adrenocorticotrophic Hormone
ATP	Adenosine triphosphate
BC	Before Christ
BIR	Behavioural Transition
bp	Base Pair
BP	Before Present
cAMP	Cyclic Adenosine Monophosphate
CNS	Central Nervous System
CRF	Corticotropin Releasing Hormone
DA	Dopamine
DAT	Dopamine Transporter Protein
DNA	Deoxyribonucleic Acid
ELA	Early life adversity
GAS	General Adaption Syndrome
HPA	Hypothalamic-pituitary adrenergic axis
HR	Heart Rate
Kg	Kilograms
KYA	Thousand Years Ago
Mg	Milligrams
Mins	Minutes
MLP	Mesolimbic dopaminergic pathway
MPTP	Methyyl-4-phenyl-1-1,2,3,6-tetrahydropyridine
MYA	Million Years Ago
mMCO	Mid-Miocene Climate Optimum
mtDNA	Mitochondrial Deoxyribonucleic Acid
NAc	Nucleus Accumbens
nM	Nano Meters
NWSL	New World Stilted Leg
OFC	Orbitofrontal Cortex
OR	Operant Response
PCA	Principal component Analysis
PFC	Prefrontal Cortex
PVN	Paraventricular Nucleus
SBR	Spontaneous Blink Rate
SM	Susan Martin
SN	Substantia Nigra
SNC	Sympathetic Nervous System
STN	Subthalamic Nucleus
VTA	Ventral Tegmental Area

CHAPTER 1: INTRODUCTION.

When humans came to the British Isle about 100 thousand years ago, the wild ponies were a valuable source of food and the skins and fat used for winter survival (Bello *et al*. 2021). The process of domestication continued with the introduction of selective breeding from animals imported along the international trading routes, individuals were harnessed, ridden during battles and used for transportation of goods whilst trading. The more isolated herds that were not on significant trading routes remained true to breed, such as those found in Scotland and Exmoor (Speed & Etherington 1953).The eleven distinctive British native equine breeds have evolved to survive in the environments in which they inhabit, each one retaining distinct attributes enabling them to thrive in the harsh environment (Winton *et al*. 2020). These natives have been utilised for a variety of breeding purposes and exhibit an abundance of genetic diversity (Winton *et al*. 2020). These genetically important herds can still be seen today within their original environments, with the breed societies understanding the importance of maintaining the pure stock.

Modern day issues of Global warming are high on Government's agendas at present and an increase in returning areas of intensive farming to more species diverse natural environments. Areas of rewilding are becoming more numerous, assisting in the removal of carbon dioxide from the atmosphere (Schmitz *et al*, 2020). Environmental issues are greatly influenced by landowners and the human dimension is an all-controlling starting point to make the decision to allow and control the adaption of farming areas back to a more "wild" natural environment. The need for intensification of farming and other human activities have led to the degrading of ecosystems all over the planet leading to a reduction in biodiversity. The UN Ecosystem Restoration and the post 2020 global biodiversity framework has initiated the opportunity to rebuild the biodiversity ecosystem by rewilding initiatives. The rewilding initiatives have allowed the reestablishment of herds of native British pony breeds which are semi-feral with minimal human contact.

To enable management strategies designed to optimise the welfare of these herds robust baseline indicators must be established. To establish what is "normal" behaviour for individual breeds of semi-feral *Equids* it is important to understand what environmental conditions and stimuli affect the welfare of both the individual and herd. Traditionally, the

monitoring of welfare on animals has recorded both subjective and objective factors and are historically unreliable and unreproducible due to the subjective nature of the recorder (Harvey *et al* 2022). It is therefore important that a reproducible scientific approach is taken to the monitoring of animal's welfare, using a combination of both whole-animal measures and more generalised frameworks such as the 5-domain model (Harvey *et al*, 2022).

The Five Domain Model (Mellor, D.J. &, Reid, C.S.W 1994) or the welfare Decision Support System (Bracke *et al* 2002, Bracke & Spruijt *et al* 2020) is centred on the establishment of baseline readings for three domains (1) Nutrition; (2) Physical environment and (3) Health. These are reported to have an effect on the animal's internal stability and through this unbalancing of internal homeostasis and brain processing, leading to negative behaviours associated with the next domain (4) Behavioural interactions. These survival behaviours that are known to have an affect on the welfare of the individual, known as the "survival critical affects" need to be observed and recorded, alongside the individuals goal seeking behaviour associated with their environment or due to the human contact. During this initial monitoring process environmental considerations can be recorded for the baseline levels. These four primary domains interlink to produce both negative and positive outcomes affecting the fifth and final domain 'Welfare'. Any of the indicators in groups 1-4 that are used to infer welfare of individual animal and groups of animals have to be reproducible and scientific and demonstrate a link between the functional/physical impact of the animal and the mental experiences, therefore effecting the welfare of the individual or group.

Research carried out by Harvey *et al* (2023) used a range of negative mental experiences including thirst, hunger, temperature extremes, localised pain and fear amongst other stimuli and positive experiences including pleasure associated with drinking, mastication and social interaction, these experiences have been validated in wild free roaming horses to show a correlation between a measurable indicator and a physical/ functional impact on the horse.

These baseline recording can then be used to infer a welfare baseline score on the semi-feral herds within this study and used as a comparison with the domestic subjects in conjunction with blink rate recordings obtained in this research to assess the effects of domestication on British Native Equid Breeds studied in this research.

The semi-feral herds in this research are monitored annually and are usually corralled or 'drifted' into group pens for identification marking, health checks and administration of veterinary treatments such as anthelmintics.

During this process the semi-feral herds are naturally put under duress and suffer from a variety of stressors such as feed restriction, heat from over-crowding and restricted movement during penning and drifting (Weeks *et al* 2011). From a welfare measurement standpoint, current frameworks such as the 5 domains model provide a reasonable starting point from which to base assessments of sensory inputs that register imbalances or disruptions in the internal physical/functional state of individual, these are classified as survival-critical negative effects. These include thirst, hunger, pain, breathlessness, weakness and sickness all of which semi-feral Equids can potential be affected by. These affects are designated as survival-critical because they are associated with essential components of genetically embedded mechanisms that individuals require for their survival. The second category in domain 5, situation-related negative effects, are related to experiences generated by brain due to the processing of sensory inputs that mainly originate from outside the body and reflect the animal's perception of its external circumstances, i.e., its situation including frustration, boredom and loneliness amongst others. The effects of domain 5 can be a combination of factors from both categories mentioned. More recently in the monitoring of welfare of a species, researchers have also recorded the positive effects of stimuli that occur in the first four domains that lead to welfare enhancement (Mellor *et al*. 2020).

New born semi-feral *Equids* also have the potential for external stress induced neurogenesis through early life adversity (ELA) biological activities are heightened to enable the individual to respond appropriately to the environmental stressor, shaping the neuronal circuits as a part of the stress defence mechanism, leading to apoptosis of mature neurons, activation of the immune response and prevalence of neurobiological processes (Harvey *et al*. 2023). These factors are brought about by the Hypothalamus-pituitary-adrenal axis (HPA) and neurotransmitters secreted by the central nervous system including dopamine, norepinephrine and serotonin. These early life stressors are reported to play a part in the neurodevelopment and stress responses of the adults in rodents and should be considered in *Equid* studies as a welfare factor in semi-feral herds (Seung Hyun & Eui-Man 2024).

In this regard, welfare monitoring of free-living populations such as those in this research, has relied upon measurement of animal based indicators such as cortisol metabolites in faeces and hair samples (Nunez *et al.* 2014, Medill *et al.* 2023). Whilst cortisol and its metabolites are relatively easy to assay in the laboratory, levels of this hormone have been found to vary according to factors such as time of day (Irvine & Alexander 1994) and non-stress related arousal (Pell & McGreevy, 1999). In addition, cortisol is a product of the adrenal cortex, released as a consequence of sympathetic nervous system activation during activity and exercise levels (Borstel *et al.* 2017) as occurs in the drifting process of gathering and herding animals into the enclosures. In this respect, cortisol is not a direct indicator of CNS activity.

Therefore, more robust stress measurement techniques are required, that provide information in respect of central activation of stress circuitry. In this regard, quantification of Spontaneous Eye Blink Rate (SBR) has been used historically as an inferred indicator of the stress neurotransmitter dopamine (Mott *et al* 2020, Lellakova *et al* 2021, Roberts *et al* 2016) and therefore assessing the animals emotional state and welfare through blink rate has been shown to correlate with dopamine levels. Comparisons of SBR in the domestic horse have been monitored before and during a low-level stress event and compared to heart rate variability and cortisol levels in the saliva. In all subjects tested by Mott (2020) the initial startle response was observed in the SBR recordings, immediately following the exposure to sham clipping, corresponding to the heart rate variability and salivary cortisol. Following on from the first minute the SBR either returned to base levels for non-reactive horses whilst the reactive horses remained elevated. Mott reported that SBR is a reliable non-invasive measure of stress in the horse, but the initial startle response must be accounted for when using this parameter.

It has been shown by Willmore *et al* (2022) that chronic stress can have a lasting adverse effect in some susceptible individuals whilst others are not affected by the same stressor, leading to anxiety and/or depression. These differences are notable in the intrinsic properties of the mesolimbic dopamine (DA) neurons following the stressful stimuli. Behaviour in mice were simultaneously monitored alongside the neural activity in the nucleus accumbens projections (NAc), which is known to signal reward response (Parker *et al* 2016), and the tail striatum (TS) that signals threat (Tsutsui-Kimura *et al* 2022). During

stress it was revealed that susceptible and resilient mice showed different behavioural strategies and showed distinctive activity patterns in the DA terminals in the NAc but not in the TS. Resilient mice showed greater neural activity when adjacent to the aggressor leading to behaviour of fighting back compared to susceptible mice that showed an increase in neural activity at the onset of attack and again at the onset of fleeing. Results obtained in this research showed a link between DA neural activity, resilience and resilience associated behaviour during the exposure to stress stimuli (Willmore *et al*. 2022) The understanding of biological factors that bias individuals to either being susceptible or resilient to stress is important in the welfare of individuals within groups.

The dopaminergic pathway is responsible for the regulation of reward related behaviour, which is ultimately affected by stress, control of dopamine levels and dopaminergic neurotransmission within the mesolimbic pathway. Adaption to behavioural responses in relation to stress stimuli occur through the changes in the mesolimbic dopaminergic neurotransmission enabling the optimal process for coping with the stressful situation. Numerous studies have been conducted which indicate that stress is a critical factor in the development of psychopathologies within animal species, but from an evolutionary perspective it is the non-adaptive behaviour due to a cognitive or emotional distress due to an inability to process stressful stimuli, which may lead to despair or depression (Hammen, 2005). Exposure to acute stress has been shown to increase reward sensitivity, with the inclusion of reward related neural connections, in comparison to chronic stress exposure which dampened reward sensitivity increased the lack of motivation and induced a reduction in pleasure experienced (Baik 2020). The resultant changes in the dopaminergic neurotransmission through changes in the A9 cell group located in the nigrostriatal pathway of the mid-brain, can alter the behavioural responses to stressful stimuli received or anticipated, leading to avoidance or aversion, which in turn negatively regulate the dopaminergic response and therefore the reward process. Previous studies have shown that modulation of the reward system is beneficial to achieve the optimal process for coping with aversive or detrimental events, and therefore the regulation of dopamine is critical to the ability of the individual to cope with the pathophysiology of stress related behaviour (Bloomfield *et al.* 2019). Evidence has also shown that Dopaminergic neurons are also altered by the prominent and arousing changes in the environment that may not be

associated with rewards, such as surprise and arousal, both of which have been shown to excite the Dopaminergic neurons. Initial studies carried out by Karson (1983) produced a 4-fold increase in blink rate in rhesus monkeys following the subcutaneous administration of apomorphine (0.36mg/kg), which is a potent dopamine agonist, similar results were also recorded by Casey *et al* (1980) with a dose rate of 0.25mg/kg. On the other hand, dopamine antagonist (sulpiride), eliminated the apomorphine induced blink rate increase, by blocking the D_2 dopamine receptors when given 3hrs before the administration of apomorphine at a dose rate of 20mg/kg. Karson concluded that these data support the hypothesis that dopamine increases spontaneous eye blinking.

In this regard, quantification of Spontaneous Eye Blink Rate (SBR) has been used historically as an inferred indicator of the stress neurotransmitter dopamine (Mott *et al* 2020, Lellakova *et al* 2021, Roberts *et al* 2016) and therefore assessing the animals emotional state and welfare. Comparisons of SBR in the domestic horse have been monitored before and during a low-level stress event and compared to heart rate variability and cortisol levels in the saliva. In all subjects tested by Mott (2020) the initial startle response was observed in the SBR recordings, immediately following the exposure to sham clipping, corresponding to the heart rate variability and salivary cortisol. Following on from the first minute the SBR either returned to base levels for non-reactive horses whilst the reactive horses remained elevated. Mott reported that SBR is a reliable non-invasive measure of stress in the horse, but the initial startle response must be accounted for when using this parameter.

SBR has been used historically as an indicator of stress and has recently been used for comparing invasive monitoring systems for *Equids* before and after intensive police training. Stressor's examined compared blood sampling, saliva collecting and police training whilst recording blink rates for full and half blinks in comparison to cortisol levels in saliva. Results obtained by Lellakova *et al* (2021) showed that blink rate monitoring corresponds with cortisol levels collected in saliva and is a valid measure as an inferred indicator of the stress neurotransmitter dopamine.

Dopamine is known to regulate reward related behaviour within the mesolimbic dopaminergic pathway (MLP) and stress affects dopamine levels and neuronal activity in the mesolimbic dopamine system. These induced changes in mesolimbic dopamine

neurotransmission are important for behavioural activity to enable coping mechanisms for stress, adaptation to environmental dangers. Harmful adverse stressful stimuli may negatively regulate the dopaminergic reward pathway disturbing reward sensitivity potentially leading to stress-induced depression (Baik, 2020). The dopamine and reward association are also complicated by the fact that the mesolimbic dopamine system is excited by adverse stressful stimuli potentially leading to chronic depression and in some cases leading on to behavioural changes in response to reward, characterising similar responses to those seen in humans (McEwen, 1998). Acute stress can lead to an increase in reward sensitivity to allow successful reward related neural connections in the mesolimbic dopamine pathway. In comparison chronic stress has been shown to result in blunted reward sensitivity, resulting in the loss of pleasure and a lack in motivation (Ironside *et al* 2018). Changes that occur in the mesolimbic dopaminergic neurotransmission can modify or alter the behavioural response to different environmental stimuli that are associated with reward anticipation, whilst stressful events cause avoidance behaviour leading to a negative regulation in the dopaminergic response. Evidence from human studies have shown that this process of modulation is necessary for monitoring and selection of behavioural mechanisms for coping with stressful events. Further research into the interconnection of the Ventral tegmental area NAc pathway in stress induced depression and the association with the reward system modulation is still unclear and requires further research to clarify the activity of the neurons and individual pathways leading to alteration in behaviour and responses to stress stimuli (Baik 2020). The release and metabolism of dopamine especially in the MLP changes in response to the stressful stimuli, with dopamine being inhibited or enhanced depending on the intensity, duration and avoidance of the stressor. Novel short lasting mild or moderate stressors initiate an inverted U-shaped curve of dopamine release, in comparison to intense chronic unpredictable stressors have an inhibitory effect on dopamine release induced by prolonged uncontrollable unavoidable stress (Cabib & Puglisi-Allegra 2012, Marinelli 2007). Results obtained from research carried out by Holly & Miczek (2016) showed a 125-250% increase in DA levels in the NAc following exposure to an acute immobilization stress for 10-240mins and a large increase was also recorded in the PFC, in comparison psychological stress induced by predator odour exposure also induced increases in DA levels in both the NAc and PFC but minimal increases were recorded in the striatum (Herman *et al* 1982). These short term increases in DA concentrations elite reward related

neural activity, increasing reward seeking behaviour and reward associated learning. However, a combination of feedback processes in the NAc and the VTA can excite the DAergic neurons by both reward and aversion cooperate to coordinated motivational behaviour which is beneficial to adaption to environmental changes (Baik 2020). In comparison chronic repeated stressors usually lead to depressive behaviour traits Stelly *et al.* 2020).

From an equine standpoint, oral stereotypies such as crib-biting have been linked to alterations in dopamine transmission (McBride and Hemmings 2005). Furthermore, in 2015 Roberts *et al* reported that crib-biting horses display significantly lower blink rate values suggesting that there is a link between dopamine and blink rate in this species. More recently, significant correlations have also been reported between SBR and anxiety (Roberts *et al* 2016) and impulsivity (McBride *et al* 2022), both of which are behavioural phenomena that have been linked to elevated dopamine (see McBride *et al* 2017 for review).

With reference to stress quantification, Merkies *et al* (2019) investigated fluctuations in SBR as a result of a startle stimulus, whilst Mott *et al* (2020) recorded an initial SBR reduction followed by a significant elevation when a sham clipping treatment was applied. Overall, it would appear that blink rate has been linked to dopamine transmission and has been successfully applied as an indicator of stress in the horse. However, to date SBR measurement has not been trialled in free-living populations such as the New Forest Pony. In addition, despite the recent use of SBR in the equine literature, no studies have sought to obtain baseline or 'normal' values for this species, which would be required for diagnostic accuracy. Therefore, the primary aims of this investigation were twofold:

1) **To trial methods for SBR quantification in free-living herds.**
2) **To establish baseline values for both semi-feral and domestic populations, and draw comparisons between these groups such that the effect of domestication on brain function may be established.**

CHAPTER 2: LITERATURE REVIEW.

2.1 EVOLUTION OF THE HORSE

The ancestors of the modern horse such as *Hyracotherium* roamed the plains 50 million years ago, during the Eocene Period. Skeletal remains of this creature, found in Wyoming near Lake Kemmerer by J Tynsky in (2007) showed this mammal was small, about 12 inches high, the fossil had four toes on the front and three on the back leg, with an elongated skull approximately six inches long containing 44 teeth. Sharing a common ancestry with *Tapiroidea* and *Rhinocerotoidea,* it constitutes the most complete fossilised remnants found to date; the aquatic location led to the high preservation quality of this specimen (Solounias & Semprebon 2002).

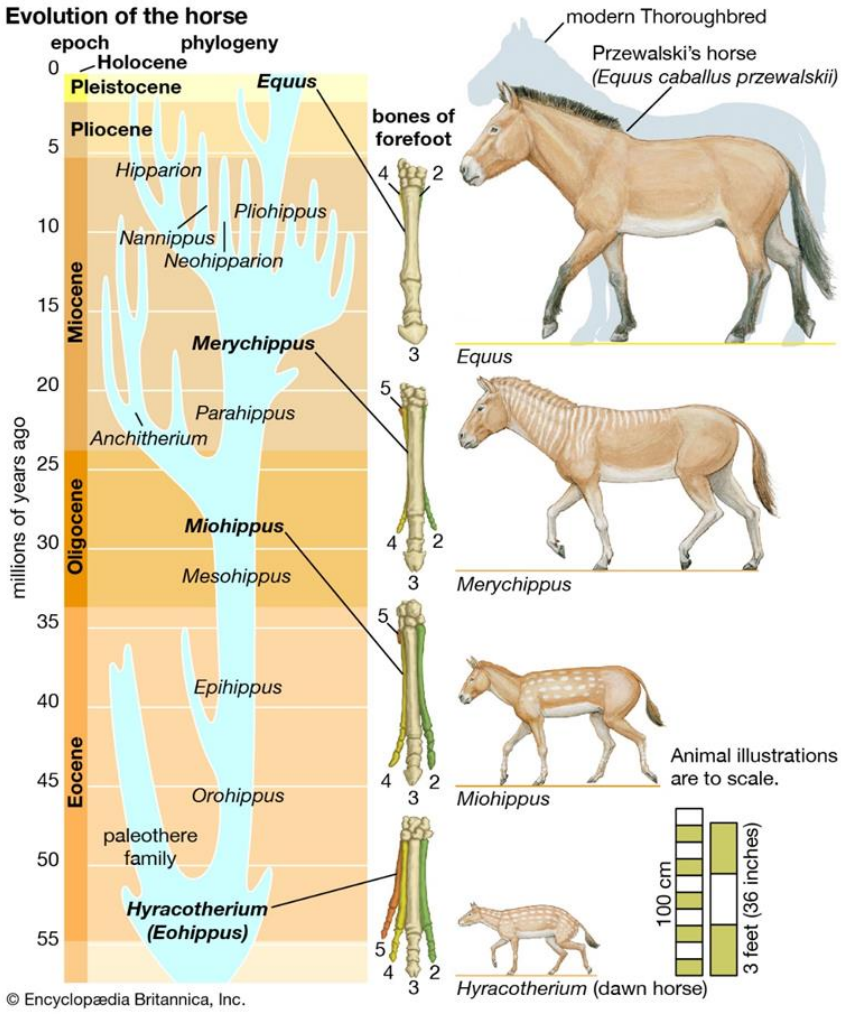


Figure 1: The descendants of Hyracotherium were grouped by cladistic characteristics based on the similarities taken from fossilised remains, paleontological records and geographical locations.

Fossil records indicate little morphological changes during the Eocene (55-34 MYA) and Oligocene (34-23 MYA), but during the mid-Miocene (18-15MYA) climatic changes referred to as the mid-Miocene Climatic Optimum (mMCO) drove further evolutionary adaptations, such as larger long-legged physiology and accompanying changes to dental morphology (MacFadden 2005), indicating the transition towards a grazing animal rather than a browser. Along with the afore mentioned morphological changes, MacFadden also reports evidence for the migration of the larger animal to distinct geographical regions. At the end of the Miocene period (5 MYA) there is evidence of 19 distinct genera, which are represented by fossilised remains found in North America, New and Old Worlds, including Astrohippus, Calippus, Cormohipparion, Dinohippus, Hippidion, Neohipparion, Plesippus, Pliohippus, Pseudhipparion (MacFadden & Hulbert 1988). The following 5 million years led to the anatomical features of the *Equus* species that are still characteristic of the modern specimens today, including fused digits, increased body size and the change to highly crowned molars for grazing of silica rich grasses (Mihlbachler *et al* 2011).

2.2 ARRIVAL OF EQUUS SPECIES INTO BRITAIN.

The first wild ponies migrated across Europe from Alaska between 100, 000, and 200,000 years ago, across the Doggerland marshy plain that is now the English Channel (Fromowitz 2016). Their colonisation within the British Isle was very successful and they became widely distributed all over the Island (Warmuth *et al* 2012). The earliest discovery of British wild horse remains date back to 8411BC (Sommer *et al* 2011). Following extensive studies carried out by Speed & Etherington (1952) who compared bones and teeth from modern Exmoor ponies, Przewalski's horse bones from the late Pleistocene 102,000 – 6,500Ma and ponies from Celtic graves 2,250Ma, it was concluded that there were two types of horses from Northern Europe which migrated to Britain. One was similar to the Przewalski's horse *Equus ferus przewalskii* adapted to the cold climate of the Steppe, a large area of flat grasslands in Eastern Europe. The second type was of a smaller horse *Equus ferus ferus tarpan*, the bones of similar specimens were excavated from sites in the Mendip Hills (Speed & Ehterington 1952) these ponies were thought to have migrated through America to Siberia during the Pleistocene and then to central and Northern Europe via Asia Minor (Turkey). Following studies of the lower jaws, particularly deeply rooted molars and premolars, which were set in a distinctive radial formation, it was difficult to distinguish ancient specimens and modern Exmoor pony remains due to the angle of the teeth formation in the jaw (Speed 1951, Speed & Etherington 1952, 1953, Ebhardt 1962).

Equus ferus bones and teeth collected from Gough's cave in Somerset show evidence of being used as knapping tools by Magdalenian hunters 23,000-14,000 (BP). Gough's cave is located in the Mendip Hills 30m above sea level, in a location between low-level marshes

and the flood plains of the Somerset levels and the upper high plateaus of the Mendip Hills at 260m above modern sea levels (Jacobi, 2004). These caves have been shown to be used as short lived, seasonal multi-activity camps, their location acted as a funnel for driving and trapping horses moving between the two seasonal grazing areas (Bello *et al.* 2021)

When humans came to the British Isle about 100 thousand years ago, the wild ponies were a valuable source of food and the skins and fat used for winter survival. During this period, there was a reduction in numbers, followed by the Mesolithic period, which lead to changes to the lowland environments. The open grazing areas became only available on the mountain habitats and the surviving populations migrated to these isolated areas (Jacobi, 2004).

During the formation of the British Channel 5,000 – 8,000 years ago, the migration of further wild equids ceased and the pony populations became isolated, reducing genetic variation with the British Equids, until variations were reintroduced under the control of humans. The equine herds became further isolated from each other as man started to cultivate lowland areas, dividing the land into farms and smallholdings preventing natural migration through grazing limitations. This promoted the isolated herds to develop in different ways and to adapt to their environments.

The intervention from humans and the mixing of genetic influences from *Equids* transferred from outside of the British Isles occurred at the start of the trading routes established across Britain and Europe. The introduction of genetic variation influenced by the locality to trade routes across the British Isles is witnessed through the analysis of the isolated herds on Exmoor and in Scotland, with Exmoor ponies, Eriskay and Shetland ponies remaining true to form (Speed & Etherington 1935).

2.3 THE ORIGINS AND GENETIC DIVERSITY OF EQUUS SPECIES IN BRITAIN.

During the Ice age 11,6000BC the sea level was significantly lower due to the ice cap formation enabling a direct migration route from Alaska to Asia via the Bering land bridge and also from continental Europe to Britain via the strip of land called the Doggerland. Initial radiocarbon evidence indicated that British native ponies were thought to have originated through a descendant of the wild horse *Equus ferus*, which existed in Europe during the late Pleistocene age (2,580,000 to 11,700 years ago).

The eleven distinctive British native equine breeds (Exmoor, Welsh, Dale, Fell, Shetland, Dartmoor, New Forest, Eriskay, highland, Connemara, Suffolk Punch) have evolved to survive in the environments in which they inhabit, each one retaining distinct attributes

enabling them to thrive in the harsh environment. These natives have been utilised for a variety of breeding purposes and contain an abundance of genetic material.

The Exmoor native breed is considered to be the most primitive horse breed of Great Britain, greatly influenced by the wild Northern horses that inhabited France and Britain from the Late Pleistocene age and other imported breeds, and a direct descendant of *Equus przewalskii* (Mohr, 1971). Studies by Speed (1951); Speed & Etherington (1953) and Ebhardt (1962) quantitatively looked at the bones and teeth from Exmoor ponies and compared them to Przewalskii horses, modern European horses and fossils found throughout northwest Europe from the Late Pleistocene period. These studies identified two horse types, using x-ray data, one larger northern horse that was adapted to cold climates and the second was a smaller northern type, remains of which were found in excavation sites on the Mendip Hills. These three data sets showed that the samples exhibited deep lower jaws, with deep set molars and premolars set in a radial pattern (Ebhardt, 1962). The mitochondrial DNA analysis also indicates a tight cluster with low variability, unlike other native British breeds that show a greater variability in maternal DNA originating from a mixture of pre-domestic horse populations (Jansen *et al*. 2002).

Detailed analysis of genetic microsatellite and mtDNA carried out by Winton *et al*. (2020) on a large dataset of British native pony breeds has revealed a complex genetic history and interrelationship with each other. Using samples that were collect from breeds located in their native origin and previously published data comprising of 485 animals and 450 microsatellites. Results indicate that the native ponies show high levels of genetic diversity, with some reduction shown in isolated small populations.

Working with 11 recognized native ponies, Welsh ponies A, B, C, and D, Fell, Dales, Highland, Eriskay, Shetland, Dartmoor, Exmoor, New Forest, Connemara, Carneddau and the Kerry Bog Pony. The molecular genetic analysis carried out enabled Winton *et al* (2020) to study the origins of the DNA, diversity and the interrelationship between the native species. Results obtained from the genetic analysis enabled Winton to collate the data and predict the genetic relationship of the native ponies and the diversity of maternal and paternal influences. The New Forest ponies showed the highest overall diversity with the lowest heterozygote excess, showing a low level of inbreeding, compared to Welsh section C and Carneddau ponies which had the lowest diversity and the highest inbreeding coefficience. Winton's *et al*. results showed that heterozygosity for the British and Irish native ponies is relatively high and comparable with other European pony breeds including semi-feral ponies (Luis *et al*. 2007, Rendo *et al* 2012). Results indicate mitochondrial diversity was very high with representation in all of the global haplotypes. Evidence of haplotype sharing across samples, indicating crossbreeding historically between British native breeds, although breed specific mtDNA was evident especially in Fells and Welsh ponies. This would suggest that stallions were brought in to service local herds, as evident from the distinct differences

between maternal and paternal histories of the breeds, with paternal crossover evident with non-native breeds including Arab and Thoroughbreds.

Results obtained from the haplogroup typing show the presence of Welsh ponies in the rare J group at position A40 node, indicating historical links to the ancient European and Asian sample from 60 to 80 KYA, with the haplogroup J-K split occurring 20KYA. Results obtained for the Welsh ponies suggest the divergence of the historical lineage during the periglacial period at the end of the glacial period, 100,000-25,000 years ago.

Shetland ponies studied showed evidence of restricted diversity from other British natives although autosomal and mitochondrial analysis has clusters associated with Nordic breeds, suggesting a Scandinavian origin (Petersen *et al*, 2013).

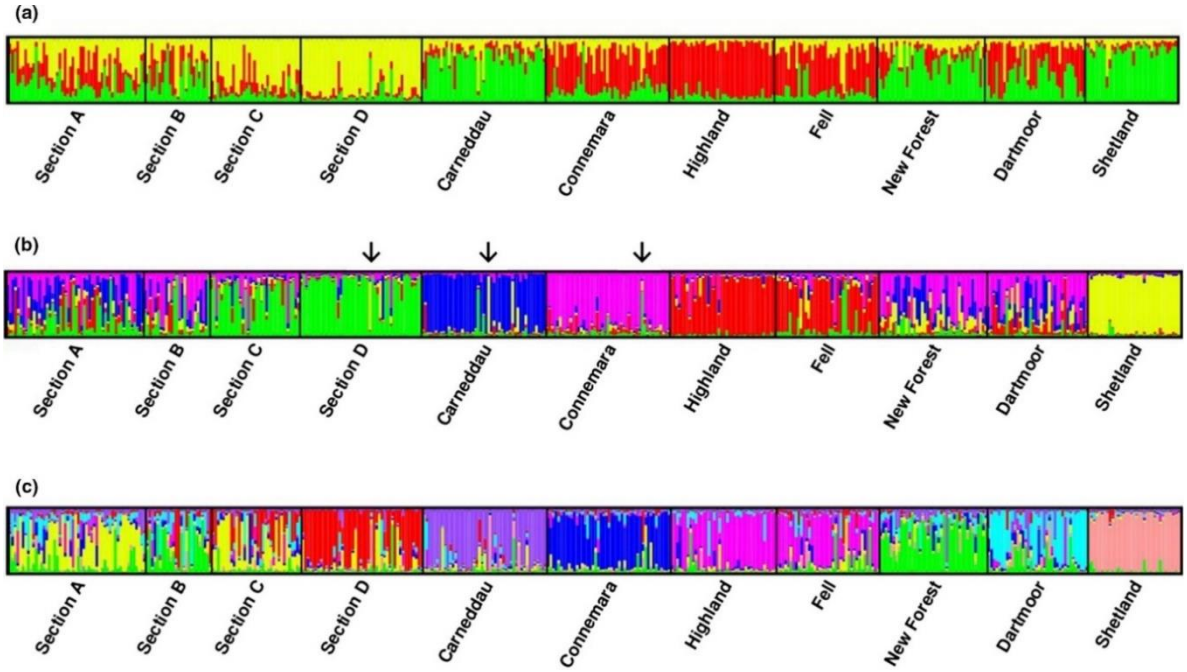


Figure 2: Consensus plots for six independent analysis runs for 12 SSR loci of 11 population bands for (a) K=3, (b) K=5 and (c) K=8. Individual vertical bands depict single animals within a population, indicating the degree of genotype mixtures between breeds according to allele frequency.

↓ Animals within a breed assigned to another cluster according to the allele frequency profile. (Winton et al 2020)

The results obtained from Winton *et al* molecular studies are valuable for the future conservation of the breeds demonstrate that specific breeding programmes should be maintained or developed to sustain the inherent genetic diversity.

2.4 DOMESTICATION OF THE HORSE

The Oxford dictionary defines domestication as follows; “the process of making a wild animal used to living with or working for humans”. Genetic analysis of samples collated indicates the earliest evidence of horse husbandry to date is at the Botai camps in the North of Kazakhstan and date back to 5500Bp, approximately 2000 years earlier than evidence of domestic horse in Europe (Outram et al. 2012).

Through intensive fieldwork and genetic DNA analysis from 63,529 bone fragments, evidence has provided three distinct areas for horse domestication at the Botai site, where the data presented below show that horses were selectively bred for domestic use in 4000BC at these locations. Genomic analysis, through DNA sequencing of bone fragments revealed that horses at the Botai camps were similar to the domestic horses within Kazakhstan during the Bronze Age 3300BC to 1200BC, but significantly different from wild horses from the Eneolithic period (2500-2200BC). The difference in bone structures, third metacarpal and proximal phalanx length of the forelimb, revealed at the Botai sites, compared to those found more widespread within Northern Kazakhstan, indicated that horses were selectively bred, from the wild horse population to enhance advantageous features ie. finer body morphology and leg length for speed and endurance, along with DNA analysis of coat colouration between corralled remains and feral ancestral skeletons. Management for the promotion of general health, increasing fertility, enabled the control of breeding and domestication. These animals were then utilised, for transportation, evidenced by tooth damage indicating that horses were harnessed and receiving bit damage (Outram *et al*. 2012) and nutritional supplementation via consumption of both milk and meat, for humans. Pottery from the site was also analysed for lipids, using $\delta^{13}C$ and δD fatty acid analysis, results showing residues of mare milk fats within the fragments, suggesting that milk was being extracted, stored and utilised by the human inhabitants.

In addition, analysis of the Botai sites also revealed a shift from hunting and gathering of mixed animals from the wild, to an intensification of domestically reared horsemeat, indicated by controlled slaughter techniques at settlement sites and limitation of transportation of body part material (Outram *et al* 2009). This change in meat rearing practice no doubt came about due to lifestyle transitions from a nomadic existence to a more sedentary settlement. In support of this notion, a corral like area of soil was discovered at the site, along with increased phosphorus and sodium concentrations. Together, these findings strongly point towards the confinement and intensive rearing of horses for human use. This evidence was confirmed at another site 100km west of Krasnyi Yar in Kazakhstan, which revealed close-set posts and a palisade trench (Gaunitz *et al*.2018).

Following extensive DNA sequencing and Principal component analysis (PCA) carried out by Guanitz *et al*, samples including bone fragments from Botai and 22 from Eurasia from

5000MYA, along with published genomes from 18 ancient and 28 modern horses were used to explore genetic evidence of captive breeding, through DNA sequencing analysis. Included in these samples were 3 wild archaic samples (42,800 to 5100 years ago), 7 Przewalski's horses and 78 domesticated horses (25 Eneolithic, 5 from Borly4, 7 bronze age, 18 Iron Age, 1 Parthian, 1 Roman, 3 post Roman) and 22 modern horses from 18 different breeds. The results obtained indicate that Przewalski's horses and the Botai Eneolithic samples group together to the exclusion of all other samples. Phylogenetic results from this study indicate that domestic horses do not descend from a single evolutionary ancestor, as would be expected if they originated from the Botai specimens alone. The Przewalski's horses in the dataset were revealed as direct descendants of Borly4 and both are descendants of the Botai horses. The results obtained indicate that the Przewalski horses are not wild horses but feral descendants from Botai horses, which were not retained as a domestic line but returned to feral state sometime after 5000 years ago (Orlando 2020).

All other domesticated samples cluster into a second monophyletic group which include samples from 4000 years ago, with limited cross over with Botai or Borly4 specimens occurring up to 1000 years ago, indicated by the occurrence of both matrilines and patrilines in the domestic gene flow (Gaunitz 2018). The limited genetic cross over from Botai species suggests that there was another source of genetic input to the domestic *Equus* species within a large geographical area including Western Europe, Turkey, Iran and Central Asia (Old World) within the last 3,000 MYA. The earliest domesticated *Equus* remains excavated in Hungary radiocarbon dated at ~4100-year Bp, have little genetic material from the Botai line (Gaunitz 2018). There are numerous clusters of fossilized specimens from Russia, Romania and Georgia also showing limited Botai influences, which suggests another line existed within the Old World referred to as the "DOM2" line (Schubert *et al*. 2014, Librado *et al.* 2017).

2.5 CHANGING ROLE OF THE EQUID POPULATION IN THE MODERN WORLD.

During development of the relationship between humans and horses, their usefulness for transportation and carrying of loads was identified. From the data collated from the Botai camps in the North of Kazakhstan, showing evidence of large numbers of Equids contained in corals and the use of Equids as a form of transportation of both humans and loads the horse has been utilised for its strength and ability to carry weights and to move at speed greater than a human running.

During the period from 1870 to 1910 the number of equids used in agriculture increase in Great Britain, the use of horses in agriculture changed at the start of the first world war when the domestic horse population was commandeered for military service for both

ridden and carriage work. Following the end of the World War I in the 1920's the horse numbers in agriculture and transportation decline due to the invention of the combustion engine, which replaced the use of the horse for land cultivation. As a result of the development of mechanical cultivation techniques the role of the horse changed within Britain and throughout the developing world. After World War II the horse's role became one of recreation and sport within the developed world, but remains a means of transportation and of agricultural importance in developing countries, or in landscapes that are lacking in roads (Crossman & Walsh, 2011). Estimates to date suggest there are 850,000 horses in Britain, with an estimated 374,000 horse owning households (Data from the National Equestrian Survey 2019), with the United States having the highest population of equids at 18% of the world's population.

However, the importance of the equid population within today society has developed into a sports and leisure activity within the wealthier countries. In Britain 14,000 horses are currently in professional training, with 10,353 horse races being held by the British Horse racing Authority in 2021, with the total equestrian sector in the UK worth £4.7billion (PetKeen, 2023). The British Horse Society records show that there are 1.8 million regular horse riders in Britain (2023), with 44.6% of horse owners competing in equine disciplines as amateurs.

2.5.1 INTERIM SUMMARY OF EVOLUTION FROM HISTORICAL PALAEONTOLOGY TO EQUIDS IN BRITAIN TODAY.

The study of evolution and migration of the equid species traditionally has been through the study of fossil remains comparing the morphology, geographic location and association with human remains. With the development of DNA recovery from ancient fossilised fragments and identification of satellite markers on the genetic material, it has been possible to trace the relationship, dispersal and inheritance of the equine gene pool through their evolution with a great deal of accuracy. This research has identified the origins of the ancestors of the modern equine species, their dispersal from the New World in America, to the Old World via the Bering Land Bridge and into Europe and from Europe into the British Isles via the Doggerland ice bridge. This migration across America and the establishment of human societies alongside the equine populations were beneficial in the survival of the equid species by provision of feed and protection from predators. The domestication and selective breeding enabled the equine species to be utilised as a domestic animal providing meat, milk and transport. The use of the equine species by human populations has changed from transport for migration, at speed during battle, as a beast of burden for transporting goods along trading routes and as farming assistance to a form of leisure, sport and pleasure.

The process of domestication has developed from corralling the animals in pens, using the animals for milk and meat, keeping them localised to the nomadic humans and restricting their breeding abilities, to the modern-day livery yards with restricted grazing, stabling and high energy feeds. This change in lifestyle for the equine species in modern day society may

not always be beneficial and advantageous for the welfare of the individual. Horses in traditional liveries are showing indications of stress and habitual behaviours, which are displayed as stereotypic behaviours.

2.6 THE EFFECTS OF DOMESTICATION ON THE BRAIN.

Results obtained from comparative studies of wild and domestic populations, have reported consistent patterns of brain reduction in domestication of a wide range of species when compared to the wild counterpart, correlated with a reduction in cranial capacity (Balcarcel *et al*. 2021). Research has sited this reduction in brain size in many populations post domestication and has led to the hypothesis of 'domestication Syndrome' (Wright *et al*. 2020), phenotypic traits which characterise domesticated species but do not show in the wild relatives. These traits have to be identified as a result of domestication but not as a result of selective breeding, as with the split between chickens that are selectively bred for egg production or broiler meat production (Zuidhof *et al*. 2014). Modern domesticated horses have been reported to have a 14-16% reduction in brain size compared to Przewalski wild (Rohrs & Edinger, 1993) although these results need to be considered in the light of changes increase in body size in modern domesticated equids compared to historical fossilised remains. The changes seen in brain size as a consequence of domestication differ between species with a reduction in hippocampus and other limbic systems decreased in dogs and sheep (Kruska 1988), with an increase in cerebellum and cerebral hemispheres in domesticated birds (Henriksen *et al.* 2016). Changes that occur are also dependant on the evolutionary age of the brain structure, and the cytoarchitectural arrangements of the nerve elements in the specific areas, the hippocampus with a high-density neural area, processing a high level of neural activity, a decrease of size will lead to a decrease in functional activity within the hippocampal region. The reduction in the limbic system activity will lead to changes in the domesticated species indicative of domestication, with an attenuation in aggressive behaviour or behaviour associated with survival instincts (Kruska, 1988). Historically, the changes in behaviour and temperament during the process of domestication has been one of the primary drivers and has been reported by the research carried out on Silver Foxes (*Urocyon cinereoargenteus*) which have been selectively bred for tameness, with a corresponding reduction in wariness and low reactivity to humans (Trut, 1999). The behavioural changes associated with domestication of Equids can be witnessed in feral animals that have returned to a semi wild existence. This process of domestication in reverse is under scientific scrutiny as a model for understanding the permanence and nature of the changes that occur with domestication (Price, 2002) and the process that occurs through continuing generations, when individuals become adapted to their free-living environment and emancipated from human's direct care and control (McKnight 1964). To

date, the potential reversal of the domestic neuro-phenotype has not been investigated in horse populations and is certainly an area that warrants further study.

2.7 BRAIN AND BEHAVIOUR: A FOCUS ON DOPAMINE.

Historically research has investigated the level of dopamine and behavioural traits in other species, including humans (Grall-Bronnec *et al*. 2018) but little has been done in horses until Roberts *et al* (2018) and McBride *et al*. (2022) investigated behavioural traits and dopamine pathways, receptor cells and their effect on Equine behavioural traits. Increasing evidence is being reported for the association between dopamine and behavioural dysfunction in domestic animals which is influenced by management practices and domestication procedures. Early life stress during weaning and environmental restriction for normal behaviour traits are shown to have significant influence on the development of stereotypic behaviours (Waters *et al.* 2002, Latham 2005) and these behaviours are linked to the disturbance in regulation of the basal ganglia DA pathway, a reduction in impulse control and reduced sympathovagal tone between the increase in sympathetic nervous system over the parasympathetic nervous system, giving rise the "fight or flight" stress response behaviour. This can also be exaggerated by the high fat and carbohydrate feeds that are found in concentrates used in domestic livery yard facilities, giving rise to a reduction in impulsive control behavioural mechanisms (Volkow *et al.* 2017). Research carried out by McBride *et al.* (2022) concluded that there appears to be a link between compulsive (an irresistible urge) and impulsive (instant gratification) behaviour, but there also appears to be neurophysiological differences between them. However, the link seen in humans between central DA tone and impulsivity also appears to be present in Equids (McBride *et al*. 2022).

2.7.1. DOPAMINE RECEPTOR SUBTYPES

Following in depth research in the field of gene cloning in the late 1980's it has led to a greater understanding of location and function of five distinct DA receptor subtypes (D_1-D_5) within the CNS. The use of pharmacological manipulation has enabled the role of D_1 and D_2 to be categorised, D_1 being a receptor that stimulates adenylyl cyclase, that catalyses the conversion of ATP to cAMP. This then initiates a series of enzymatic reactions leading to a phosphorylation cascade, activating multiple proteins that regulate both the rate and force of cardiac contraction, ready for the flight or fight reaction seen during the stress process. In comparison the Dopamine D_2 receptor is an autoreceptor, regulating the activity of dopamine neurons, synthesis release and uptake of DA, the D_2 receptor acts as an inhibitory control mechanism, and therefore inhibit the stress reaction of flight (Ford, 2014). D_1 receptor is most widespread and expressed at higher levels than any of the other receptors, as shown by the occurrence of D_1 mRNA which has been found in striatum, nucleus

accumbens and the olfactory tubercle, but also occur in the limbic system, hypothalamus and thalamus. The D_1 receptor protein is expressed in the endopeducular nucleus and the substantia nigra pars reticulata but no mRNA is present, suggesting that their presence is in projections only.

Dopamine receptors D_1 and D_5 share a high homology in their transmembrane domain and is coupled to the stimulation of DA, unlike receptor D_2 which acts as the inhibitory control point. These two receptors' types do not contain introns as compared to the second group which show the presence of introns. Following intensive gene cloning research, homology has been identified between D_1 and D_5 and then the three other receptors D_2, D_3 and D_4 have shown high homology in the transmembrane sequences, indicating the origin for the two groups from two different processes of evolution, the first through a process of gene amplification giving rise to two similar receptor genes D_1 and D_5, then the second group originating through a process of speciation and genetic polymorphism for the group contain D_2, D_3 and D_4 (Missale *et al*. 1998) .

The D_5 receptor is in comparison poorly expressed in rat brains and is mainly restricted to the hippocampus, parafascicular nucleus of the thalamus and the lateral mamillary nucleus, all of these areas which the D_1 receptor is poorly expressed. But, D_5 has a similar function as described by the D_1 receptor for the activation of adenylyl cyclase, in comparison D_2, D_3 and D_4 receptors that inhibit adenylyl cyclase and activate K+ channels inhibiting the coupling of DA to the receptors by altering the polarisation (Einhorn *et al.* 1991). Through the development of antibody research for the DA receptor subtypes it has made it possible to define the exact cellular and subcellular location of both D_1 and D_5 within the pyramidal neurons, the hippocampus and the dentate gyrus. Within the pyrimdal neurons the D_1 receptors are located dentritic spines and D_5 dendritic shafts (Bergson *et al*. 1995, Smiley *et al.* 1994).

The D_2 receptor has been located mainly in the striatum of the olfactory tubercule, in the core of the nuclueus accumbens, granular cells of the hippocampal formation, hypothalamus and septal region of the amygdala (Bouthenet *et al*. 1991). Within the ventromedial shell of the nucleus accumbens in the limbic region there are high concentrations of the D_3 receptors, with a low expression in hippocampus. High expression of D_4 receptors is found in the frontal cortex, amygdala, hippocampus, hypothalamus and messencephalon.

2.7.2 FOUR MAIN NEURAL DOPAMINE PATHWAYS OF THE BRAIN

Dopaminergic neurons are mainly located in the VTA within the substantia nigra pars compacta and the arcuate nucleus of the hypothalamus within the mid brain. The slender axons of these neurons are located in different areas of the brain, linked together into four main pathways; mesocortical, mesolimbic, nigrostriatal and tuberoinfundibular. The DA

producing cell groups are identified as group “A” cells containing catecholamines and are sub-divided into cell groups A8-A16.

- The mesolimbic neurons originate in the VTA transporting DA into the nucleus accumbens through the amygdala and the hippocampus.
- The Nigrostriatal pathway originates in the basal ganglian substantia nigra pars compacta, with the neostriatum through axon ramification in the caudate nucleus and putamen, involved in locomotion and sensorimotor networks.
- Tuberoinfundibular pathway originates in the hypothalamus and controls hypophyseal portal system.
- Mesocortical originated in the VTA sending projections to the nucleus accumbens which is involved in the adaption and motivation of new behaviour within a changing environment specifically involved in survival methods (Nicola 2007)

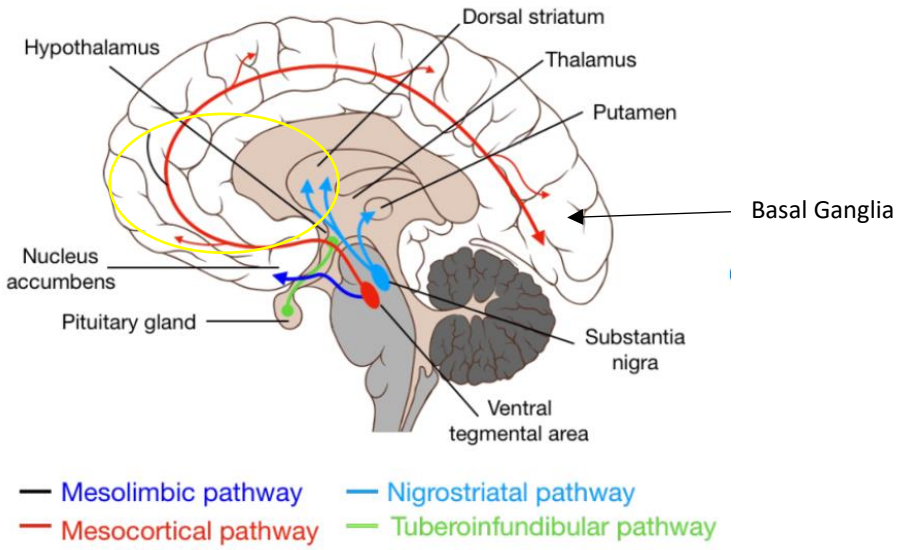


Figure 3: Dopamine pathway (Lauri Nummenmaa)

2.7.3.1 THE MESOLIMBIC PATHWAY

The mesolimbic pathway originates from the VTA and comprises of neural projections into the ventral striatum and the nucleus accumbens (NAc). This pathway is involved in reward and adaption learning behaviour, the importance of DA in effort rewarded can be seen in negative feedback symptoms often featured in schizophrenic behaviour where apathy is a common feature during learning (Hauser *et al.* 2017). The NAc is the area that primarily mediates feelings of pleasure and reward, and through a process of positive feedback

reinforces the feelings of pleasure and promoting a repetition of the behaviour. An over stimulation of this DA pathway can lead to cravings and euphoria in the human.

2.7.3.2 THE MESOCORTICAL PATHWAY

The mesocortical pathway also originate in the VTA, where the neural action potential travel to areas within the prefrontal cortex (PFC) and the orbitofrontal cortex (OFC) along cell line A10. Dopamine clearance at the PFC is slower after the excitation due to a limited number of dopamine transporter proteins (DAT), leading to elevated levels following and initiating stimuli (Seamans & Yang, 2004). This pathway can have a modulating effect through the efferent nerves on the mesolimbic dopamine concentrations (Carr & Seasack, 2000).

2.7.3.3. THE NIGROSTRIATAL PATHWAY

The dopamine projections start in the substantia nigra and lead to the dorsal stratum especially the putamen and caudate parts of the basal ganglia and are made up of projections from A9 neurons (Baik 2020). These areas are associated with the sensorimotor networks and habitual learning and motor control through the process of stimulus response and action outcome learning through goal directed behaviour (Yin & Knowlton, 2006). This pathway contains approximately 80% of the dopamine in the brain (Bridges, 2016). The neurons in this pathway stimulate purposeful movement and damage or impairment of this pathway leads to motor control impairment as seen in Parkinson's sufferers' spasms.

2.7.3.4 THE TUBEROINFUNDIBULAR PATHWAY

The final dopamine pathway originates in areas of the hypothalamus, projecting into the infundibular region of the median eminence of the hypothalamus along cell lines A12 (arcuate nucleus) & A14 (periventricular nucleus). Dopamine is released into hypothalamic-hypophyseal portal circulatory system and inhibits prolactin release affecting metabolism, sexual desire and the immune system. Inhibition of dopamine on the D_2 receptors in this pathway leads to effects on fertility, libido and bone health (Majumdar & Mangal, 2015).

2.8 DOPAMINE IN RELATION TO STRESS

Stress in a biological context may be defined as a relationship between an organism and external or internal factors that disrupt the physical, mental or emotional homeostasis that causes bodily or mental tension (Davis 2021). Although the concept of stress is a complex of multifaceted events and characteristics, horses have developed specific reactions and pathways to mitigate the detrimental effects of stress and strive to restore homeostasis (Daev 2019). These stressors activate a homeostatic mechanism through the neural pathway designed to regain physiological integrity, optimizing biological functions.

Therefore, the process of optimizing homeostasis is a constant process of neural and hormonal fluctuation to maintain biological equilibrium.

Initial research showed that stress induced regulation of the dopaminergic system showed both release and metabolism of DA, especially in the mesolimbic pathway. DA can be inhibited or enhanced on the basis of duration, intensity and avoid ability of the stressor. Depending on the stress stimuli the DA response differs, mild to moderate novel stressors that are short lasting have an activating effect of DA, whereas intense chronic or unpredictable stressors have an inhibitory effect on DA release. Research carried out by Holly & Miczek (2016) on rats showed that acute stress increases extracellular DA levels in the mesolimbic and mesocortical systems with minimal increases seen in the striatum. Stelly *et al*. (2020) continued research in this area and reported that acute or short-term stress led to a change in the DA level in the midbrain promoting reward-related neural learning. In comparison to this chronic stress is reported by Xu *et al*. 2012, Cryan *et al*. 2004 & Castagnes *et al*. (2011), to lead to depressive like behaviours in rodents.

During the application of a stressor, a nonspecific three-stage response is observed, first reported by Dr. Hans Selye (1950), as part of the General Adaption Syndrome (GAS). The initial stage causes a wave of high concentrations of catecholamines along with the activation of the cardiovascular, respiratory and locomotory systems, leading to an increase in energy consumption within the body, as seen in the flight or fight response. Within the semi-feral herds this would normally lead to the flight response, seen as a survival mechanism during evolution. During the process of domestication this initial phase maybe a passive response, by decreased mobility (napping), but an increase in starring at an object or stressor. Alongside the increase in catecholamines there is an increase in the secretion of corticotropin releasing hormone (CRF) from the paraventricular nucleus (PVN) of the hypothalamus, which mediates the release of corticotropin which in return leads to the release of adrenocortical steroids (Goldstein 2003).

If the stressor is unresolved the second resistance phase is activated through the release of glucocorticoids leading to an increase in additional energy from the metabolism of glucose sources. If the second phase leads to the individual regaining biological equilibrium the body would enter the recovery stage (Kovac *et al* 2022).

The third stage would be initiated if the normal homeostasis had not been regained within the second phase, leading to a phase of exhaustion alongside numerous pathophysiological changes (Kovac *et al* 2022).

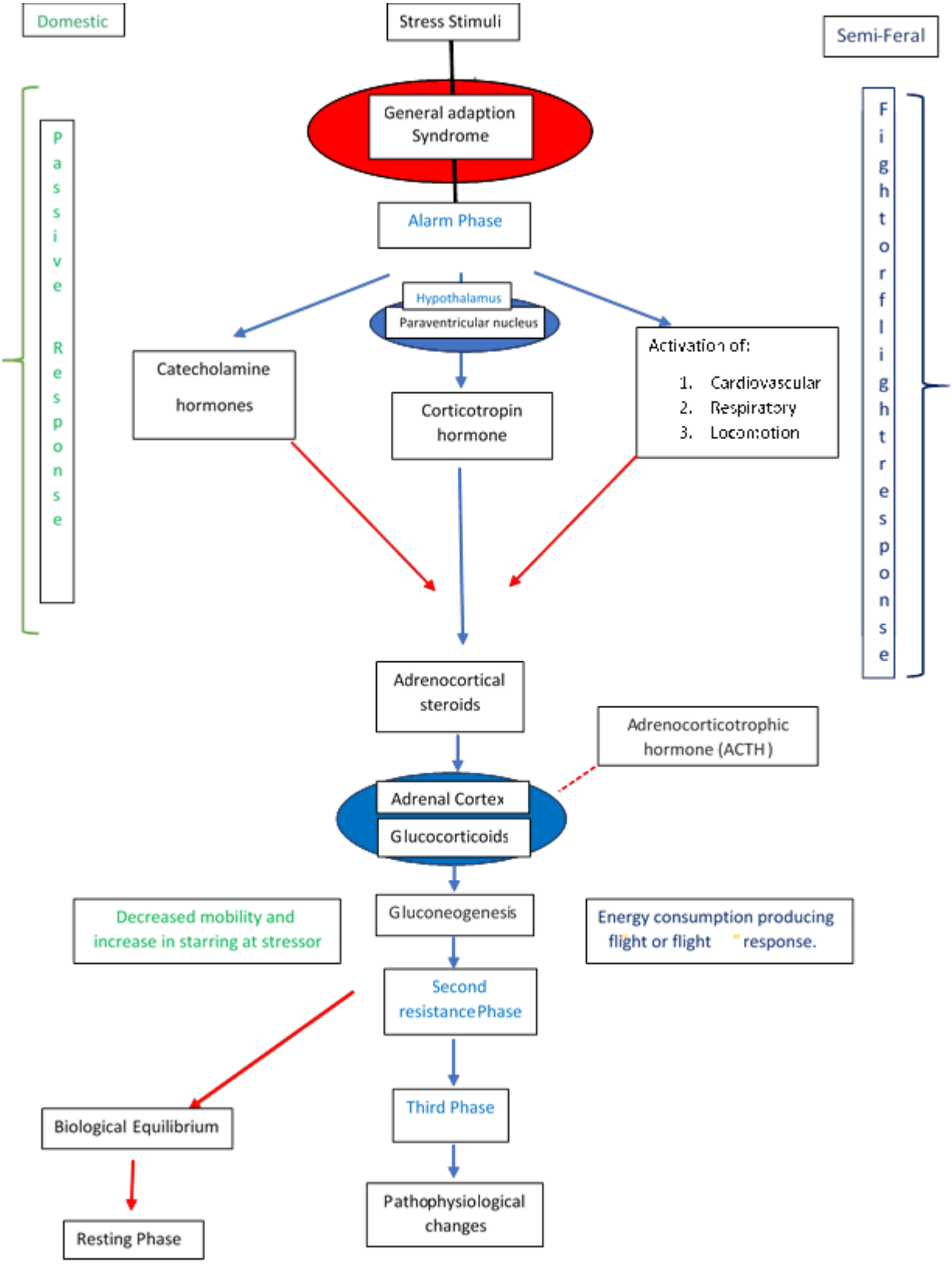


Figure 4: General adaption syndrome to re-establish biological equilibrium following a stress stimulus.

The domestication of the horse has reduced its natural flight response and increased its adaptation to human husbandry. There is scientific evidence, that the organization of equids' behaviour initially remained relatively unchanged with domestication (Christensen et al. 2002), although some of the constraints imposed on horses during the last century conflict with their naturally evolved behaviour (i.e. being social herd living and fearful with the fight and flight response) and may lead to welfare problems through causing stress (Goodwin, 2002). It is important to understand the process of domestication and the ability of the domestic horse to adapt to the environment in which it is kept, its ability to cope with the unnatural restrictions that are imposed upon it by humans and to be aware of the welfare implications these restrictions may have upon the domestic horse (Budzynska 2014). Budzynska reported that there are at least two types of coping strategies (i) proactive and (ii) reactive, enabling individuals to adapt to specific challenging situations during domestication, enhancing the probability of coping with the environment over the stress demands on the individual, both of immediate stressors and long-term challenges potentially leading loss in body weight, gastric ulcers and reproductive problems.

The reaction to stress produces a complex reaction of both neurological and endocrine reactions to produce the "fight or flight" response (Derakhshan *et al*. 2019, McBride *et al* 2017). As a result of this response being initiated a number of biological complexes take place, and are controlled by the 'primitive' part of the brain without conscious decision-making processes. The amygdala is within the control centre of the limbic system and has no direct connections from the prefrontal cortex, and numerous connections to the cortex, the "flight or fight" response is not controlled by a conscious decision (LeDoux 2007). Amygdala sends information to the hypothalamus which is fundamental to the secretion control of Adrenocorticotrophic hormone ACTH (Lacroix *et al* 2000) and this influences the secretion of both adrenal cortex and corticosterone that regulate vasoconstriction and dilation (Buijs 2000). Catecholamine hormones produced by the nerve cells such as adrenaline, noradrenaline and dopamine are produced, which facilitate immediate physical reactions in preparation of rapid muscle contraction, including heart muscle acceleration and lung action increase, and movement of the blood from the skin surface to the deeper vascular system (Merkies *et al*. 2019). This facilitates an increase in blood flow through vasodilation to the muscles and deep tissues. Inhibition of upper intestinal action, digestion in the stomach is greatly reduced or stops completely, but co-insides with an increase in liberation of nutrients particularly fats and glucose to increase availability of nutrients for energy production. There is an inhibition of the lacrimal gland production of optical tears and a reduction in salivation, accompanied by a dilation of the pupil and an increase in blood flow to the eyes.

The exposure to high levels of environmental stressors may have influenced the ancestors of the equid to be able to adapt to environmental pressures more quickly promoting evolution or contributed to the reflex defence mechanisms (Gabriel 2005).

A number of studies have researched into the stress levels experienced by horses during training (Werhahn *et al*. 2012), acute physiological stress during transportation (Ishizaka *et al* 2017) and domestic housing arrangements (Suagee-Bedore *et al.* 2021). These may be exhibited through stereotypic behaviour that maybe linked to the elevated sensitivity to dopamine and endogenous opioid levels and the dysfunction of basal ganglia input/output pathways, with increased numbers of mu opioid receptor density in both the ventral and dorsal striatal regions within the stereotypic brain (Hemmings *et al* 2018). Receptor density occurs with a genetic predisposition to some genetic breeds, but this has not been compared to British Native breeds living in semi feral conditions (Freymond *et al.* 2019).

2.9 MEASURING DOPAMINE VIA BEHAVIOURAL MEANS: A FOCUS ON *BLINK* RATE

Early research carried out and reported in 1945 by Professor Hall looked at base level of human blink rate whilst under taking both a conversation and reading from a passage. During the initial experiment the subjects were unaware that their blink rates were being recorded. The average blinks for both genders were significantly reduced whilst undertaking a reading compared to conversation. During the test 1 (reading out loud from a passage) males were seen to blink more, 29.3 average blinks per minute (abpm) compared to females 18.3abpm. Test 2 consisted of talking to the observer, 3.57 per minute for males compared to 2.58 per minute for females. From the initial research carried out Hall categorised blinks into two groups A voluntary and B reflex. Group B was then further divided into three sub categories: of which group D (described as blinks of technique or conditioned reflexes) are observed in most organism, described as brief covering of the eye that does not interrupt vision and may last for 0.4 seconds, with a complete covering of the eye only lasting 0.15 seconds.

Hall then went on to look at the blinks in group D which occur in most vertebrates, within the wild whose main objective is to survive and reproduce. These two functions are controlled by involuntary unconditioned reflexes in feral vertebrates, described as aggression and self-preservation, which are opposed to each other, but vary between species. Carnivores are usually classified as hunters and are aggressive food seekers, whereas herbivores are hunted and rely on flight reflexes for survival. Hall's initial experimentation looking at moving stimuli and stationary head, compared to stationary stimuli and moving head are reflected in these two groups. He found that if fixation on an object was maintained the blink rate was zero in more than 90% of the subjects, regardless of whether the head and object were stationary, when the object was stationary and the head was free to move or both head and object were free to move separately. Holmes in 1938 had previously described two distinct cortical ocular reflexes, one excited by extra-macular origin causes movement of the eye and the second of macular origin leads to the

object being kept in the centre of the vision by movement of the head. Using these two forms of eye/head movements separately primates and higher evolutionary species are able to receive both visual stimuli from a wide vision at low definition and in contrast focus on a small area with higher definition. Using these descriptions animals that are carnivores will normally use the fixation method for hunting whereas the herbivore is likely to use the extra-macular reflex.

Comparisons of blink rates in both herbivores and carnivores have been shown to differ significantly and fall into the two categories described by Holmes (1938). Initial blink rate records first taken from animals in Edinburgh Zoo by Blount (1927) showed Herbivores to have an average blinks per minute ranging from 2 (goat) to 22 (cow) with an average of 10.5 for 8 different species compared to the carnivores of an average of <2 per minute for the 5 species studied.

As seen in early studies a decrease in blink rate is observed in patients suffering from Parkinson's disease, a disease which is associated with a decrease in central dopaminergic activity, indicating that there may be a mediation link between dopaminergic activity and blink rate. Further research has been carried out to investigate the association between blink rate and the functional state of dopamine as a neurotransmitter and its use as a non-invasive quantifiable clinical monitor. Subsequent studies carried out by Hornykiewicz (1974) also recorded a decrease in blink rate in Parkinson's patients with decreased dopaminergic activity, but those Parkinson's patients that had levodopa-induced dyskinesia exhibited twice the mean blink rate (21 blinks/minute) compared to other Parkinson's patients in the study (11 blinks/minute). Initial studies carried out by Taylor *et al* (1999) produced a 4 fold increase in blink rate in rhesus monkeys following the subcutaneous administration of apomorphine (0.36mg/kg), which is a dopamine agonist, these results were also recorded by Casey *et al.* (1980) with a dose rate of 0.25mg/kg. Apomorphine is known to have effects on other neurotransmitter systems and further analysis of dopamine altering drugs were administered to record results.

Experimentation carried out by Karson (1983) investigated the effects of central dopaminergic agents on reading and conversations as described by Hall, whilst recording blink rates. Follow up studies were then carried out to look at groups of humans suffering from Parkinson's disease (11 blinks per minute, $p<0.002$) and Schizophrenia (31 blinks per minute), these two groups were then compared to a control group (23 blinks per minute).

Eye blink rate has been used as a correlated non-invasive tool for stress levels in humans as an indirect marker of central dopamine function within the striatum and therefore has been studied as a method for investigating stress response in horses (Karson, 1983). Blink rate has been correlated with a number of invasive physiological measurements alongside visual assessments of their behaviour. Initial analysis made by Merkies *et al.* (2019) showed the need for both behavioural and physiological analysis of behaviour, as training of domestic individuals may mask the behavioural signals but physiological indicators were still present.

However, the use of invasive recording methods in themselves may induce a stress response in some animals and therefore a non-invasive tool would be beneficial for semi-feral Equids.

Horses exhibit three types of blinking; full, partial and eye lid flutter. Analysis of blink rate and heart rate (HR) records were analysed and found to show significant correlation after the stress inducer had been activated for full $p<0.006$ and partial $p<0.0001$ (Merkies *et al*. 2019). Humans have also displayed a decrease in SBR when challenged with a stressful visual stimulus (Argiles *et al*. 2016) therefore maximising the amount of information being taken in through the visual input. Therefore, the stressor also plays a role in both the physiological and SBR response. Merkies carried out a number of experiments to investigate the eyelid response to a number of induced external stressors. Visual and social stressors were used in the study to simulate a startle response and feeding restriction causing anxiety. The results obtained from the study showed after exposure to a stressful incident or trigger, spontaneous blink rate full and partial decreased whilst eyelid flutters increase when exposed to feed restriction trigger had been applied $p<0.0001$. Other research has shown that this decrease in SBR occurs only during the first minute of a ten-minute sham clipping experiment and precedes a significant increase in SBR. This would relate to the initial fixation of a feral horse on the stressor followed by the flight response (Mott *et al*. 2020).

To understand and predict the relationship between a response to a stimulus and the change in blink rate, it is necessary to understand the neurophysiology and how it translates to cognition. The DA as discussed effects both the cortex and striatum depending on the phasic or burst release process, producing an enhanced good feeling reward (Hollerman and Schultz, 1998) or the tonic level which may suppress the neural activity by suppressing the spontaneous firing which occurs with stress stimuli (Frank 2005). As highlighted earlier both D1 and D2 receptors in the prefrontal cortex and the basal ganglia operate a modulation effect on decision making and continuous processing of cognitive representation feeding back to the cortex from the basal ganglia processing. As discussed the D1 receptors provide a “Go” signal and inhibit the D2 “No Go” signal in high DA concentrations allows the cortex to proceed with actions or behaviours the cortex is processing (Maia & Frank 2011), whereas the opposite applies with the activation of the D2 pathway in an environment of low levels of DA which inhibits the “No Go“ D2 receptors to produce a suppression and stability of the cortical response.

Therefore, SBR is a useful non-invasive measure of determining altered dopamine sensitivity within the striatum, particularly the rostral part of the ventromedial caudate and can be used as a non- invasive indicator of stress levels for health and welfare of domesticated horses. Research has compared mild stressful conditions on domestic horse using both SBR and other invasive measures through analysis of salivary cortisol and heart rate (Mott 2020) and found SBR in horses to be a valuable non-invasive technique for monitoring DA levels and therefore stress levels in horses.

2.10 FINAL CONCLUSIONS TO THE LITERATURE.

Fifty-five million years of evolution have produced a gregarious trickle feeding herbivore, who's physical and behavioural characteristics have been harnessed by humankind to provide transport, sporting attributes and companionship. However, the domestic environment imposes a range of stressors which have the potential to impact negatively on the welfare status of this species. In addition, the gradual process of domestication may select for adaptive changes in the central nervous system which to date have not been quantified. The existence of domestic and semi-feral populations of horses in the UK provide the opportunity to explore these changes, whilst the discovery of non-invasive indicators of brain activity such as SBR offer suitable metrics upon which such as study could be based. With an eye towards more comprehensive use of SBR, from an evolutionary but also a welfare standpoint, baseline or 'normal' SBR values must first be established, and methods for SBR measurement in free-living herds developed. As such, the experimental aims of this investigation are twofold:

1) To trial methods for SBR quantification in free-living herds

2) To establish baseline values for both semi-feral and domestic populations, and draw comparisons between these groups such that the effect of domestication on brain function may be investigated.

Linked to these aims the following hypotheses were used to drive the experimental work:

'There will be no significant differences in blink rate values between domestic and semi-feral herds'

'There will be no significant differences between the studied breeds'

CHAPTER 3: MATERIALS AND METHODS

3.1 SAMPLE POPULATIONS

A total of 128 individuals were studied between September 2019 and October 2022, with individuals recruited from semi-feral and domestic populations. For the purposes of this study, semi-feral animals were defined as generally free-living individuals with a bare minimum of human intervention, which ranged from annual group penning for the purposes of marking and anthelmintic administration (New Forest) to absolutely no human contact in the case of the Exmoor and Eriskay ponies. On the other hand, domestic populations were subject to what was considered normal management programmes consisting of a blend of turnout and stabled conditions (see table 1). Overall, 66% were female, and 33% were males (including geldings) although there were 2 New Forest ponies which were not assigned a gender for logistical reasons. Ages of the recruited animals ranged from 0.3 to 24 years (see table 2).

Table 1: Management Procedures for domestic and semi-feral subjects.

Management Procedures	**Domestic**	**Semi-Feral**
Hard Feed	√	x
Daily Human contact	√	x
Anthelmintic Administration	√	√ (New Forest only)
Restricted grazing by field size	√	x
Individual turnout	√	x
Access to stabling	√	x
Grooming procedures	√	x
Farrier treatment	√	x

Table 2: Study Population of all breeds and management procedures.

NA= Not assigned. *****No domestic Shetland ponies were available for this study as a comparison to the semi-feral herd.

Horse Breed	**Total Number**	**Age Range (Years)**	**Female total**	**Male total**
New Forest Semi-feral	30	1-19	30	0
New Forest Domestic	14	4-13	6	8
Eriskay Semi-feral	15	NA	8	7
Eriskay Domestic	13	1-23	7	6
Exmoor Semi-feral	9	NA	0	9
Exmoor Domestic	4	6-15	2	2

Shetland Semi-feral*	20	0.3-24	19	1
Welsh Sec A semi-feral	15	NA	15	0
Welsh Sec A Domestic	8	9-16	0	8

*No domestic Shetland ponies were available for this study as a comparison to the semi-feral herd.

3.1.1 PONY RECRUITMENT STRATAGEY

The herds were located using contacts made with various rewilding groups (Exmoors), Verderers (New Forest) and breed preservation groups (Eriskays and Shetlands), along with free roaming Semi-feral Shetlands on common land. The number of individuals varied according to those within herds and the accessibility of the herds themselves.

There was a bias towards females in the New Forest breeds, as stallions are only run for a period of six weeks during spring/summer to ensure restricted mating and foaling periods. Exmoors studied on the Mendip Hills were a herd of geldings.

Recruitment of the domestic individuals were achieved through Facebook and word of mouth through livery yards and friends.

3.2 GEOGRAPHIC LOCATION OF SAMPLE POPULATIONS

Both the domestic and semi-feral herds were located from geographically diverse areas of the British Isles (see figures 5 and 6). For precise map location areas (including longitude and latitude coordinates) please see appendix 1. The following sections present information relating to the historical background and botanical diversity of the sites occupied by the semi-feral herds. As the domestic counterparts were maintained under what is considered normal management conditions, additional detail has not been presented.

Figure 5: Location of semi-feral herds

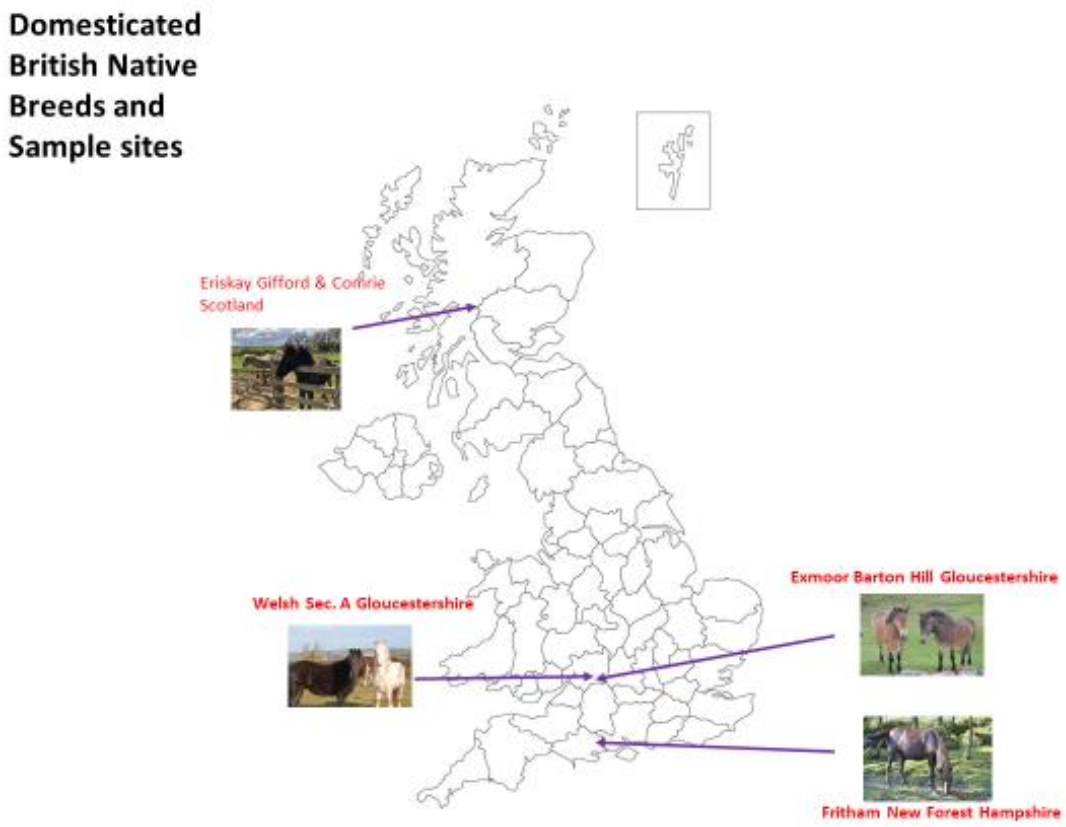


Figure 6: Location of domestic herds

3.3 RESEARCH SITES FOR SEMI-FERAL HORSES.

3.3.1 BURRINGTON HAM AND BLACKDOWN MENDIP HILLS, SOMERSET

The semi- Feral Exmoor herd studied was located on the Mendip Hills in Somerset which was once within the Royal Forest of the Mendips. It was grazed by sheep for many centuries, but the commons had become a neglected but a substantial part of Mendip landscape with the limestone gorge dividing the two common areas. The commons themselves include areas of geological, archaeological, ecological and cultural importance. However, in recent history the physical neglect of the area has damaged several of the values associated with the ancient open landscape, with the advance of shrub and bracken across the commons affecting the diverse wildlife of the site.

The site is under the Commoners Regulation (Burrington) Provisional Order Confirmation Act as recently as 1911, following the site survey the management proposals concentrated on the reintroduction of grazing and browsing stock, ponies and sheep. It was hoped by the reintroduction of browsing stock to revitalise the wildlife of the area by the control and reduction of shrubs and bracken to enhance the establishment of the open common areas.

The Burrington Commons are not a natural wilderness, they are a rich natural resource made up by a range of habitats established over millennia by man's activities including the grazing of livestock under the allocated 2417 Stint rights. Each Stint is approximately equivalent to 0.15 of a modern livestock unit, 1 Stint would allow the grazing of a single sheep, 5 stints 1 cow or 7 Stints a horse. Records suggest that the commons have changed little over the last 600 years up until modern records established in the 1950's. This is backed up by the survival of plant species on the limestone coombe which suggest a direct link to post glacial landscape. It was therefore decided by the owners of the land to reintroduce the forms of management that maintained the ancient habitats in the past. Under the Commons Registration Act of 1976, a number of stint holders lapsed, but a substantial number of Stints remain and these are now used for the grazing of the present livestock on the Burrington Commons by the Wills Family Estate.

Natural grazing has been continuous by the natural fauna including the rabbit and roe deer populations. The deer levels have been managed over history, the rabbit populations thought to be the main grazing control agents were affected badly in the 1950's by an extensive outbreak of Myxomatosis. The rabbit populations levels never fully recovered and the grazing control levels have not been re-established.

3.3.2 SHETLAND ISLE

The Shetland archipelago is located 62 miles north of mainland Scotland and the capital Lerwick is equal distance from Bergen in Norway and Aberdeen in Scotland.https://en.wikipedia.org/wiki/List_of_Shetland_islands The Shetland archipelago comprises of 300 islands and skerries, 16 are inhabited. In addition to the Shetland Mainland the larger islands are Unst, Yell and Fetlar. In the post-glacial period approximately 6200 BC, the islands were exposed to a tsunami, caused by the Storegga Slides, an immense underwater landslip off the coast of Norway (Dawson *et al* 2008) causing unusual rock formations on the coastline.

They have been inhabited since Neolithic times for several centuries, the excavations at Jarlshof near the southern end of the mainland have provided archaeological evidence of life in Shetland since the Bronze Age. The earliest colonizers were Mediterranean in the middle of the second millennium BC, followed by the Vikings ~AD800. Using a combination of blood groups, non-metrical skull variant frequencies and linguistics the closest relatives of the inhabitants were linked to those of Jaeren in Southern Norway (Jakobsen 1928, Berry & Muir, 1974).

Due to the northerly location of the Shetland Isles, there are approximately only 400 plant species, but there are species that remain in isolated ungrazed areas that were historically present from thousands of years ago. The plants that are located on the islands are adapted to extreme salinity, dryness, high winds and low levels of nutrients. The soil substrate is made up of shingle banks and shifting sandy soils on the lower shores, common species are Sea Rocket (*Cakile maritima*), Silverweed (*Potentilla anserine*), Sea Sandwort (*Honckenya peploides*), Sea Mayweed (*Tripleurospermum maritimum*), Goosegrass (*Eleusine indica*) and

the rare Oysterplants (*Tradescantia spathacea*). Further inland on the cliff tops species have adapted to poor soil, rapid drainage and exposure to the wind, by development of succulent leaves and maximising growth early in the spring. Inland the moorland and meadows are populated with wild flowers, Meadow buttercup (*Ranunculus acris*), Yellow Rattle (*Rhinanthus minor*), Devil's Bit Scabious (*Succisa pratensis*) and Autumn Hawkbit (*Leontodon hispidus*). The highest peak on the Shetland is Ronas Hill 450m high made from granite, the vegetation on this section is sparse and consists of low creeping forms of Alpine plants (https://www.shetland.org).

3.3.3 HOLY ISLE

The Holy Isle is an island in the Firth of Clyde, off of the west coast of central Scotland, located inside Lamlash Bay on the larger Isle of Arran. The island is around 3 kilometres long and around 1 kilometre wide. Its highest point is the hill Mullach Mòr. Holy Isle is currently owned by Lama Yeshe.

There is evidence of a monastery dating to the 13th century on the North end of the island, close to the current buildings of the retreat. In 1488, the Holy Isle and land in the Lamlash area was owned by John Hunter, before being passed over to the Earl of Arran in 1527. The island continued to be part of the Arran Estate in Hamilton ownership into the 20th century, although it was rented out to various people. Part of the land was passed to the Forestry Commission and the National Trust, whilst other parts went into private hands. In 1968, the UFAW (Universities Federation for Animal Welfare) was asked to advise on the husbandry and management of the island's animals, which at that time consisted of Blackface sheep and a herd of feral goats. A Field Study Centre was set up in 1969, and after the death of Stewart Huston, they were able to buy the island in 1971. During the following years they introduced 25 Soay sheep and 5 Highland cattle, and a few years later 5 Eriskay ponies. When the UFAW could no longer afford to keep the island it was sold again.

Lama Yeshe, the current owner has given the animals the chance to establish their own behaviour patterns without human interference, both within their own herd and between the different groups. They are mostly undisturbed by people, showing no fear of humans, and live harmoniously on the island with the other sheep and goats. People are encouraged not to feed or disturb the animals when visiting the island to enable them to remain in a semi-feral condition. Apart from the mammals introduced by humans, there are many birds and a wide variety of sea life.

There are 25 different species of trees growing on Holy Isle, ranging from oaks and beeches around the garden at the north end of the island, to the small willows, hawthorns, whitebeams, rock beams and hazelnut trees which are an indicator of the mineral rich soils. Aspen, elms, wild cherry, holly and 5 species of willow, grow at the north end. In the next few years there are plans to plant trees at the south side of the island as well. When the trees at the north end are tall enough not to be damaged by the horses and goats, the fences will be taken down to open up the tree areas.

Currently there are three types of large mammals living on Holy Isle, Eriskay ponies, Soay sheep and Saanen goats. Both the ponies and the sheep were introduced by the Universities Federation for Animal Welfare (UFAW) when they owned the island, the goats are believed to have been there for many centuries.

The geological structure of Holy Isle, together with the strong winds and salt-spray, limits the number of species able to thrive and survive. Most of the island's flora consists of heather, bracken and grassland, with approximately 200 identified species of flora on the island. The greatest abundance of plant species, outside of the cultivated gardens, is found in a ring just above sea level. Bearberry *(Arctostaphylos uva-ursi)*, grows on the south-east slopes where the horses and other animals graze. Other species include primroses (*Primula vulgaris*), bluebells (*Hyacinthoides non-scripta*), pale butterwort *Pinguicula lusitanica*), sea-radish (*Halophila ovalis*), quaking grass (*Briza media)*, bog pimpernel (*Anagallis tenella*), thrift (*Armeria maritima*), bog myrtle (*Myrica gale)*, wood anemone (*Anemone nemorosa)*, 14 species of sedge (*Cyperaceae sp),* sea centaury *(Centaurium littorale)*, lady's bedstraw (*Galium verum)*, two St. John's worts (*Hypericum perforatum)*, yellow pimpernel (*Lysimachia nemorum)*, wild strawberry (*Fragaria vesca*) and barren strawberry (*Waldsteinia fragarioides)* , blueberry (*Vaccinium sect. Cyanococcus*) , field pansy *(Viola tricolor var. hortensis*) , wild thyme (*Thymus serpyllum)* and corn spurrey (*Spergula arvensis)*.

(https://www.holyisle.org/the-island/wildlife-on-the-island)

3.3.4 NEW FOREST.

The New Forest today is an area of 219 square miles of unique lowland heath lying less than 1000 feet above sea level, comprising of mainly alluvial clay, gravel and sand, with areas of chalk at its north boarder. The forest soil condition is therefore poor and has had little use for agricultural purposes. During the last ice age there is evidence that vegetation of the area would have changed from tundra to native deciduous trees, mainly oak, hazel, elm and lime, with the change of occupation from hunters to primitive farmers around 5000 BC.

Due to the nutrient poor soil the primitive farmers were thought to frequently clear woodland areas to cultivate crops where the soil was able to support growth. The forested areas were cleared by fire, leaving the pervious areas to colonise with poor grass for grazing. This continuous cycle throughout the bronze age (2400-700BC) helped to create the extensive acid heath that the forest comprises of to date.

There has been little evidence of significant occupation of the area during both the Iron Age (700BC – AD43) and during the Roman occupation (AD43-410), but there is evidence of land division by means of boundary ditches which enabled the protection and grazing of livestock. There is evidence that the Romans used the poor clay soil for pottery making and charcoal for fuel. There is historical evidence of the area known as “Ytene” the land of the Jutes, an Anglo-Saxon tribe of occupation during both the Saxon and Viking periods with the Church at Brockenhurst dating back to the Saxon period. During this time people living in the area grazed their livestock including cattle, pigs and horses.

William I in 1079 designated the area which laid outside Winchester, the capital of England, as the “Nova Foresta” which meant the land was preserved for Royal hunting of deer and boar. The area was made up of large areas of open heath, lawn areas and woodland. The New Forest was subject to “Forest Law” and commoners were severely punished for any transgression, preventing them from grazing livestock or collecting fuel. In 1217 the Charter of the Forest restored some of the commoner’s rights, repealing the death penalty and brought in the Verderers by an Act of Parliament in 1877.

The role of the Verderers today is to protect and administer the New Forest’s unique agricultural communing practices, conserve its traditional landscape, wildlife, flora and fauna and cultural heritage. The Verderers Court comprises of the Official Verderer (statutory appointment made by the King), and five elected Verderers representing The Forestry Commission, DEFRA, The National Park Authority and Natural England.

During the Stuart and Tudor period 1660-18th Century the demand for timber production increased for naval construction and the use of the New Forest as a hunting ground was less significant. Enclosed areas for timber production were established, with an Act of Parliament in 1698 preventing the Pollarding of trees in the Forest. In 1851 the Deer Removal Act of Parliament cleared the deer from the enclosures and gave the Crown power to statutory enclose a total of 16,000 acres for timber production. Due to the power of the Crown, Verderers and the Agisters the New Forest’s environmental habitat has stayed much as it was in medieval times. During 1845 the New Forest gained its first railway line (Southampton to Dorcester) opening the area up for Victorian recreation. The Victorians started to landscape the New Forest by the introduction of “ornamental” trees during the period 1882-1889 a total of 3000 trees were planted, including Rhododendron species, descendants of which can still be seen today with the forest.

During the period of the two World Wars the forest was depleted of all large oak trees, 230,000 tons during World War I and 440,000 tonnes in the Second World War. To restore timber to the forest fast growing conifers were planted to replace the slower growing broad leaf trees in 1924 as directed by the Forestry commission.

During the 1950-60s to comply with regulations the Forestry Commission again tried to eliminate the broad leaf trees and replace them with conifers.

The Forestry Commission now manages the Crown Land, which has expanded the New Forest back to the area first established by William I, the unique habitat was granted National Park status in 2005 and the Forestry Commission has an obligation under the mandate to conserve the natural and cultural heritage. As part of this environmental maintenance ponies, livestock and deer graze the habitat to maintain the short grass lands and shrub size. The New Forest still remains Crown Land in the 21st Century.

3.4 GATHERING OF BLINK RATE DATA

Blink rate data was gathered between September 2019 and October 2022. Initially, the individual subject and/or herd were allowed a period of ten minutes to become accustomed to the presence of a single observer (SM) within close proximity of the herd, this allowed the subject to become accustomed to the observer's presence, helping to reduce both the initial startle effect (Mott 2020) and also the environmental affect in line with the 5 domain theory (figure 8). The observer sat on a portable stool approximately 20 meters from the chosen individual, remaining as stationary as possible during the monitoring phase, in a position to enable observation of the left eye. Bi-lateral observation would be difficult to observe due to the head anatomy of the horse, and based upon published accounts by other authors (e.g. Pickup 2017, Roberts 2018) the left eye was chosen as horses show a left eye stare bias to stressful triggers (Smith *et al*. 2016). Using a Nikon camera (D7500 SLR) with a Af-S Nikkor 55-300mm 1:4 5-5.6 GED lens to obtain a magnified view of the horses' eye, the observer recorded each full blink (Full blinks were defined as full occlusions of the eye ball by the eye-lid with both upper and lower lids meeting, resulting in total sight elimination), partial blink (Partial blinks were recorded as incomplete closures of the eye-lids, with the upper lid lowering over the part of the eye , partially eliminating sight) or eye lid Flutter (Flutters were recorded as movement of the upper eyelid through innervation of the levator palpebrae superioris muscles with no movement of the orbicularis oculi muscles (Blount 1927)), observed during a sampling period of 15 seconds during the acclimatization phase using a standard timer application on a mobile phone and to record data collection periods.

A B C

Figure 7. The recording of blink rate using "half blink", "full blink" and "eye fluttering" alongside facial expressions and behavioural attributes was used.

A. Full blinks complete closure of both eyelids with concomitant suppression of vision.

B. Patrial Blink incomplete closure of the eyelids.

C. Eyelid flutters movement of the upper eyelid through innervation of the levator palpebrae superioris muscles with no movement of the orbicularis oculi muscles (Blount 1927).

Each set of results were recorded in a tabular form in an excel spreadsheet. This counting procedure was replicated 10 times for each individual at one period to enable the calculation of representative mean values. The mean values for each collection period were then used for the statistical analysis.

With all blink rate recordings, date, time of day and weather conditions (as per Meteorological website) were recorded (see Appendix 11.3 and 11.4). Finally, using the same equipment listed above, photographs were recorded of each individual for identification and replication purposes. In the case of the New Forest pony groups, existing heat-brand markings assisted this process. In all other groups, individual coat colour and markings were used as an identification guide.

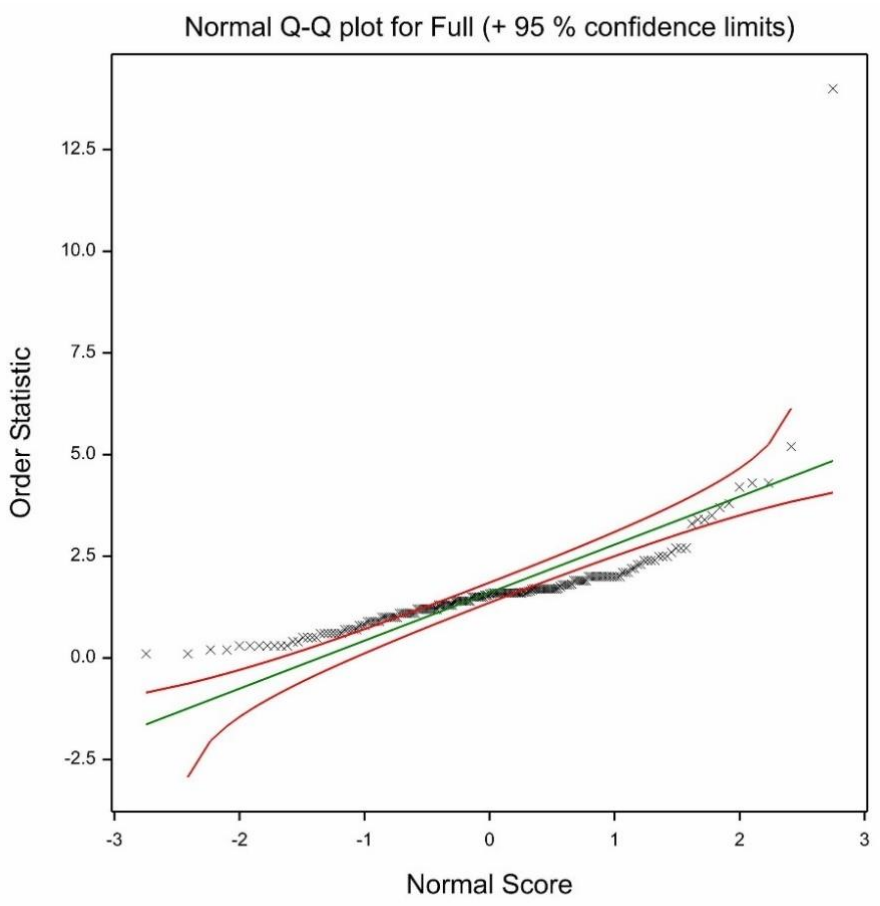
Figure 8: Spontaneous eye blink recording procedure.

Breed information of British Native ponies used in this study can be found in Appendix section 11.2.

3.5 DATA ANALYSIS.

All results were tabulated into an excel spreadsheet, recording location, owner, breed, date, time, individual identification markings and photographic record. Scores for full, partial and flutter eye blinks and an average for the ten data collection replicates and ratio of full/ partial blinks were grouped for each individual and each data collection time.

Using the mean from the data collection a Normal Q-Q plot (+95% confidence limits) was applied using Genstat (Version 22, VSNi). Tests for normal distribution were applied, to the complete data set for both full and Partial blinks. Although the data set for Partial blinks did lie within the 95% confidence limits, this was not the case for the full blink data (Figure 9).



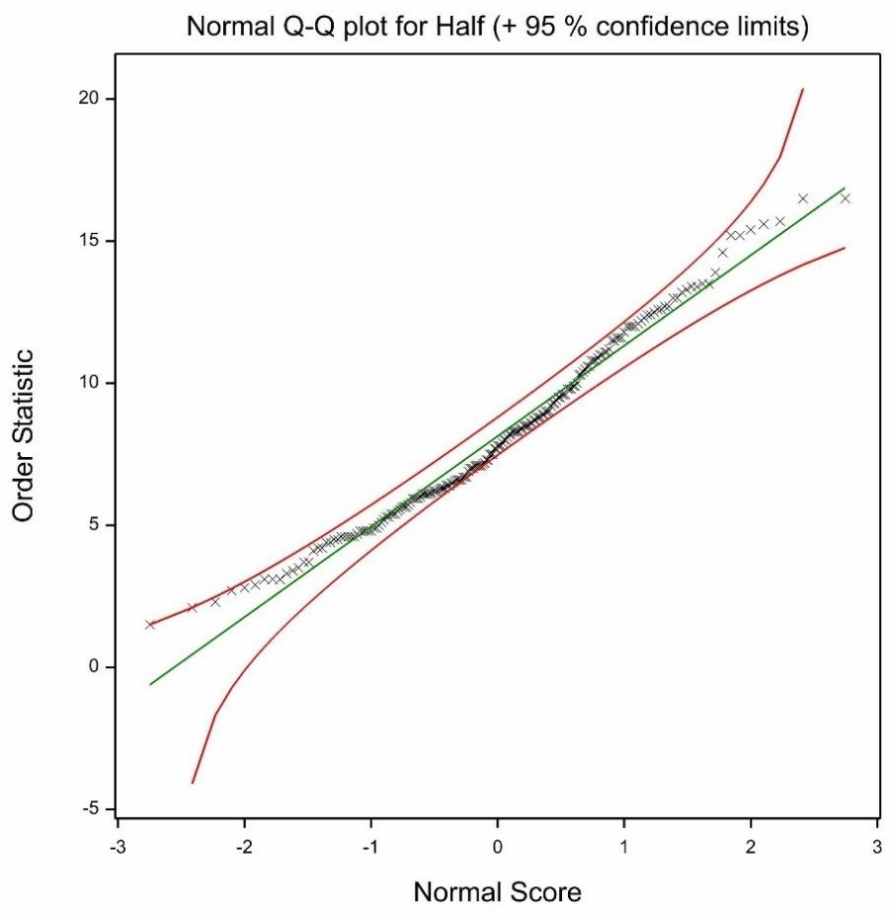


Figure 9: Analysis of the mean data using Q-Q plot (+ 95% confidence limits) showed Full blink data lay outside of the confidence limits, revealing that the data was unbalanced.

For this reason (and taking into consideration the unequal sample numbers between breeds) non-parametric statistics were selected. Where differences between two groups were tested e.g. eye blink rate between domesticated and semi feral animals irrespective of breed type then Mann-Whitney U tests were used (alpha level 0.05). Where comparisons of more than two groups were made e.g. between eye blink rate of the differing breeds, or between differing breeds including management style (domestic Vs. Semi-Feral) then a Kruskall-Wallis non-parametric one-way ANOVA was used. Where significant differences were detected (alpha level 0.05) a Mann-Whitney-U tests was used post *hoc.* To ensure differences *post hoc* were not due to type one error a Bonferroni correction was applied to correct the P value in line with the number of comparisons drawn. All analysis was undertaken using Genstat (Version 22, VSNi). Finally, more refined multivariate analyses were not attempted due to lack of studied variables linked to factors such as location, management and environment. Future research could be carried out to investigate the breed and management differences to applied startle response stimuli, using electronic recording apparatus which could be used to record the SBR.

3.6 ETHICS REVIEW

This project was granted ethical approval by the Ethics Committee at the Royal Agricultural University prior to data collection. Ethics review number 20233127.

4.0 EXPERIMENTAL RESULTS

The following hypotheses were used to drive the experimental work:

1) **'There will be no significant differences in blink rate values between domestic and semi-feral herds'**
2) **'There will be no significant differences between the studied breeds'**

The salient findings relating to these hypotheses are presented below:

4.1 DIFFERENCES IRRESPECTIVE OF BREED FOR DOMESTIC VERSES SEMI-FERAL TYPE.

Analysis via Mann-Whitney U revealed a significant difference in full blinks, between domestic and semi-feral individuals (U = 2037.0, p<0.001), with semi feral recording higher blink rates than domestic equivalents (Figure 10). Significant differences between populations were recorded for partial (U=3240.0, p<0.001) (see figure 11) and full:partial ratio (U = 2196.0, <0.001) with domestic populations recording higher blink rates for partial blink and ratio than semi-feral. (See figure 12).

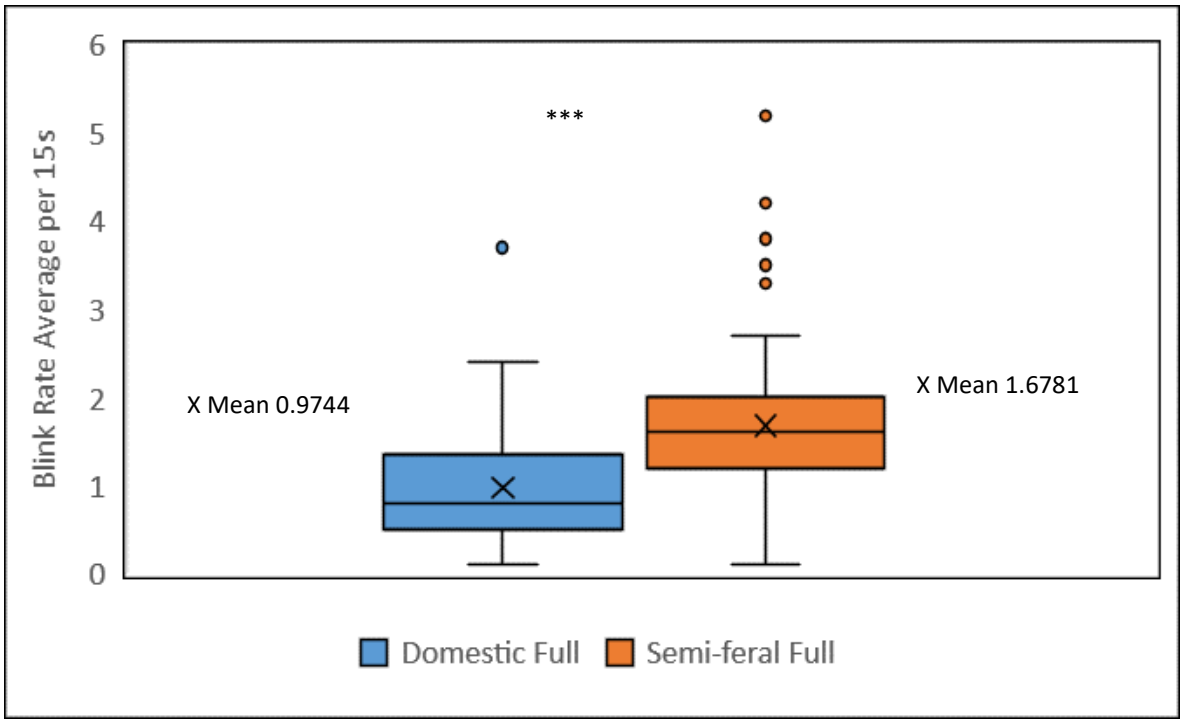


Figure 10: Comparison of Domestic versus Semi Feral irrespective of breed for full blinks (***=p<0.001). X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

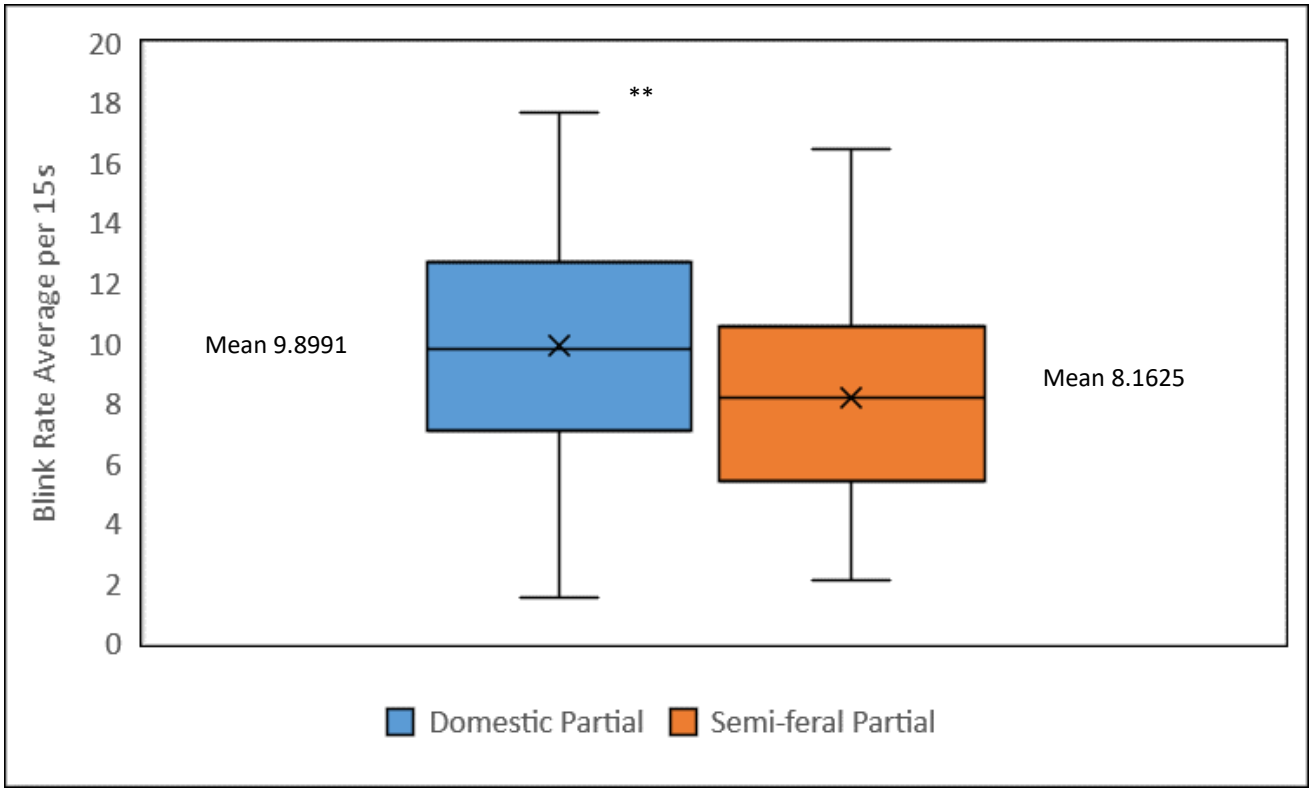


Figure 11: Comparison of Domestic versus Semi-feral irrespective of breed for partial blinks (**=p<0.002) X= mean, whisker = minimum to lower quartile / maximum to upper quartile, ** = significant results.

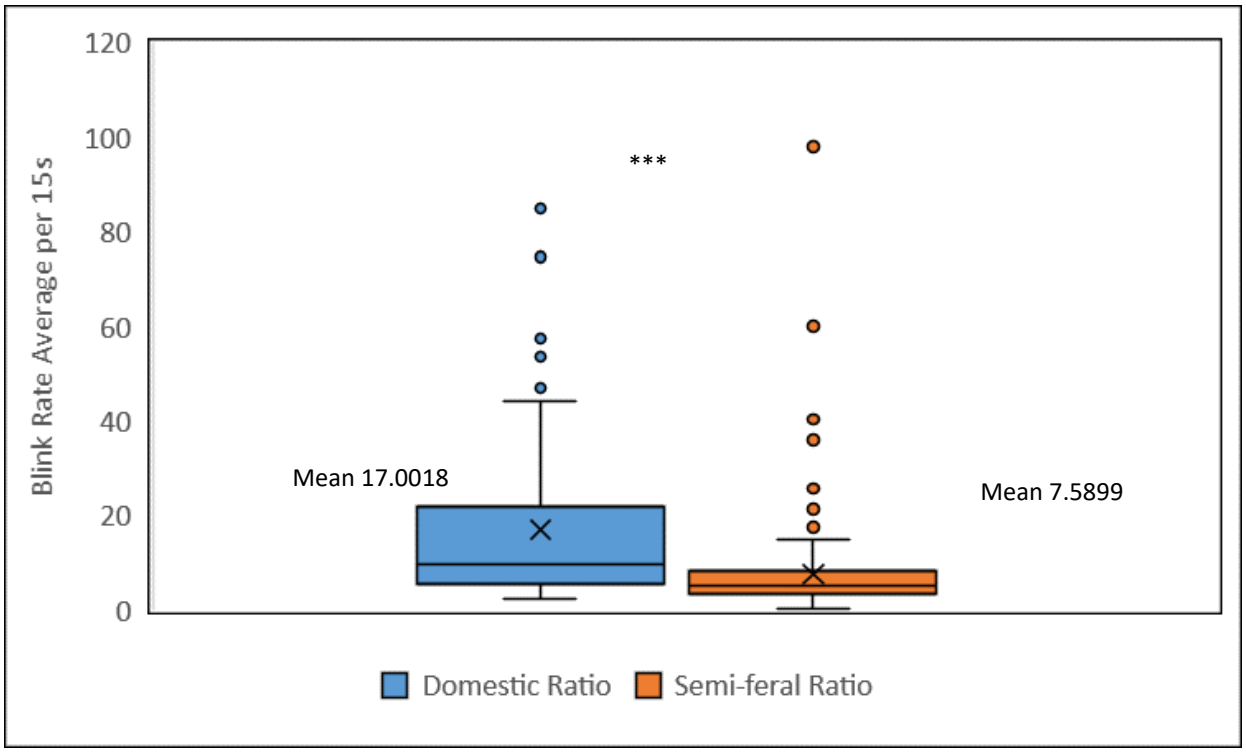


Figure 12: Comparison of Domestic versus Semi-feral irrespective of breed for blinks ratio (***=p<0.001), X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

4.2 BREED LEVEL DIFFERENCES FOR DOMESTIC VERSUS SEMI FERAL.

4.2.1 BREED LEVEL DIFFERENCES FOR FULL BLINKS FOR DOMESTIC VERSES SEMI-FERAL.

The Kruskal-Wallis one way analysis of variance uncovered a significant overall difference at breed level (H = 72.35, p<0.001). Following application of Bonferroni correction (threshold value p=0.0125) significant differences were observed between semi-feral and domestic Eriskay ponies (U=4.5, p<0.001). No other significant differences at breed level were observed (see figure 13).

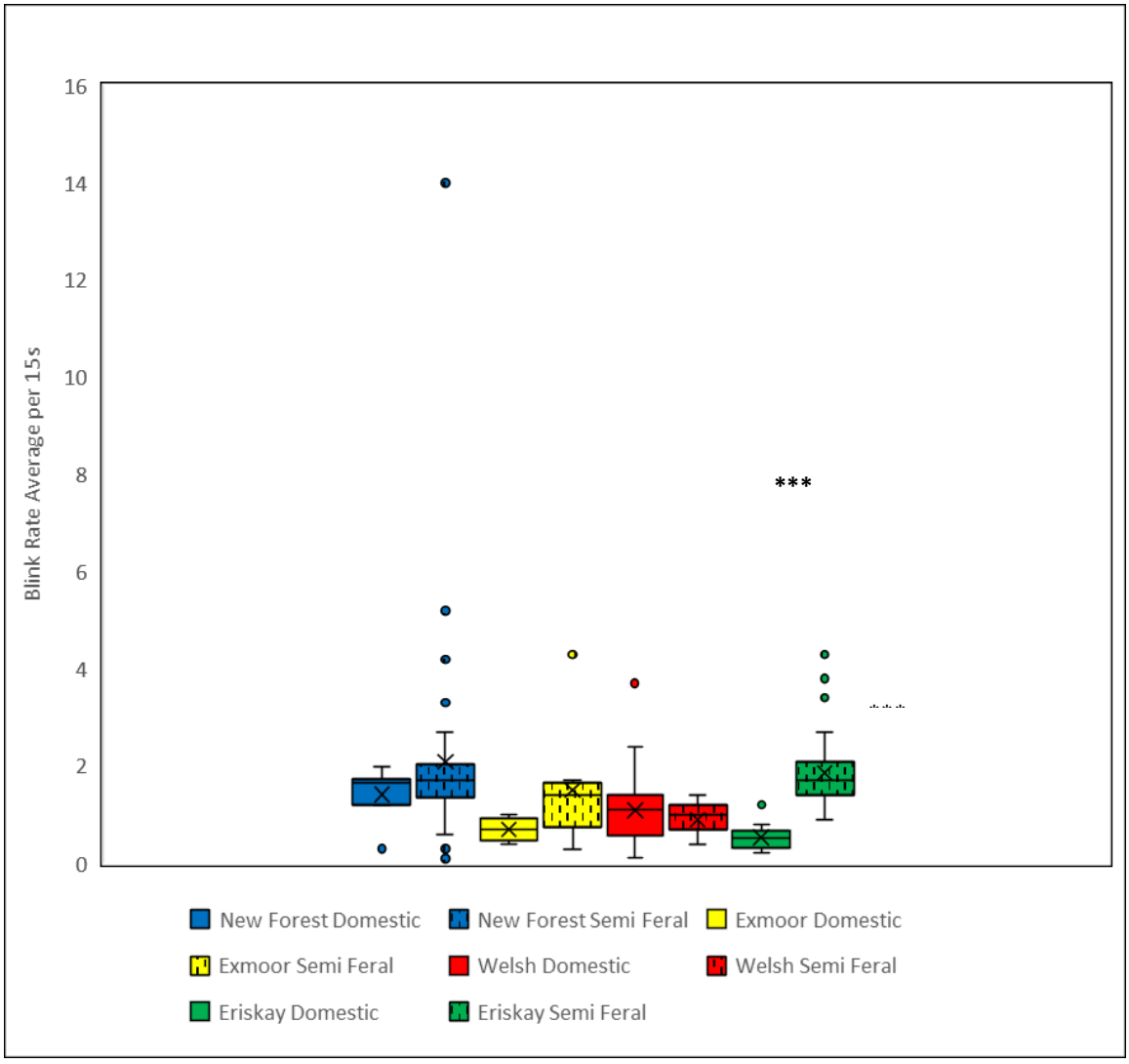


Figure 13: Breed level differences for full blinks for Domestic verses Semi-Feral (***=p<0.001). X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

4.2.2 BREED LEVEL DIFFERENCES FOR PARTIAL BLINKS FOR DOMESTIC VERSES SEMI-FERAL.

The Kruskal-Wallis one way analysis also highlighted significant differences at breed level for partial blink rates (H55.53, p<0.001). When Bonferroni correction was applied (threshold value p=0.0125), significant differences were evident between semi-feral and domestic Eriskay ponies (U=97.0, p<0.001), Exmoor ponies (U=0, p<0.01), New Forest ponies (U=127.0, p<0.001). No significant differences were observed for Welsh Section A ponies (See figure 10).

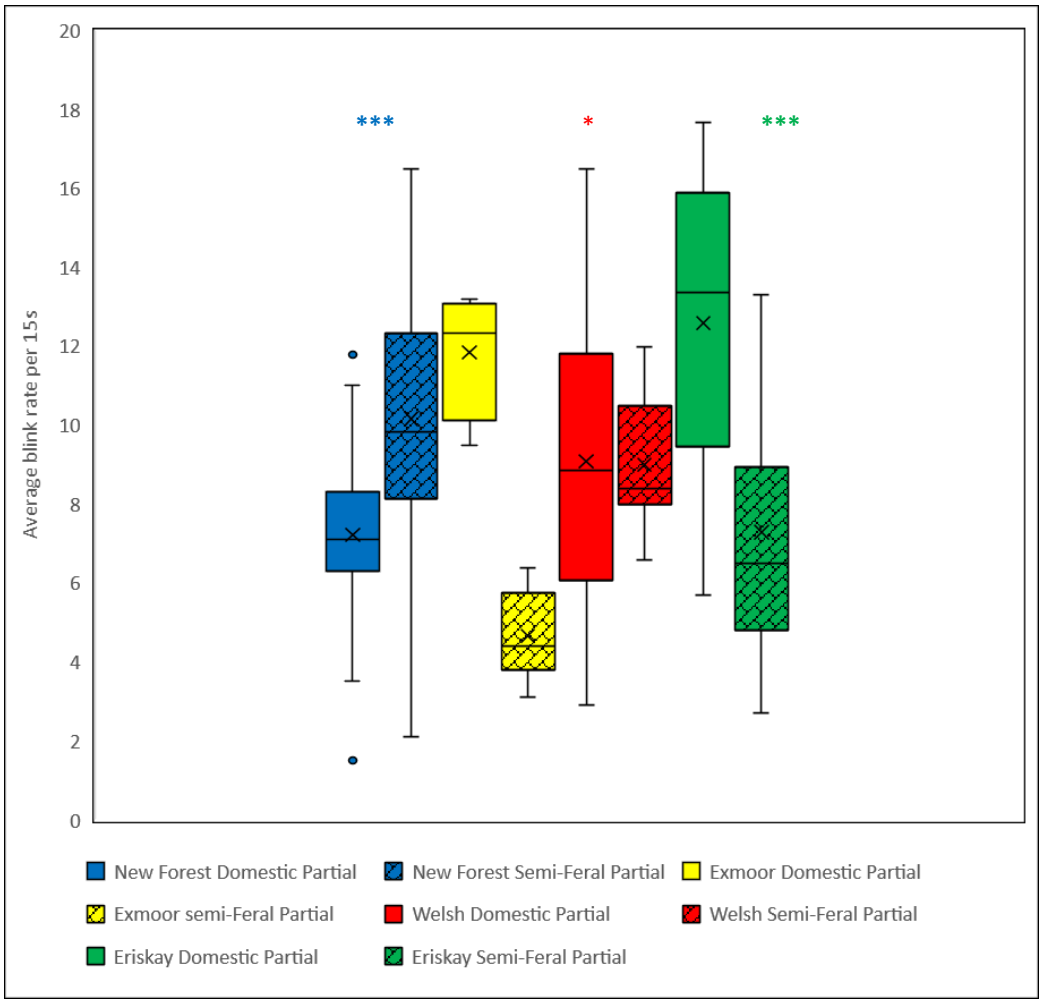


Figure 14: Breed level differences for partial Blinks for Domestic verses Semi-Feral (***=p<0.001, *=p<0.05). X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

4.2.3 BREED LEVEL DIFFERENCES FOR BLINKS RATIO FOR DOMESTIC VERSES SEMI-FERAL

The Kruskal-Wallis one way analysis also highlighted significant differences at breed level for blink ratio rates (H77.73, p<0.001). When Bonferroni correction was applied (threshold value p=0.0125) significant differences were evident between semi-feral and domestic Eriskay ponies (U=14.0, p<0.001) and Exmoor ponies (U=8.0, p<0.01). No other significant differences were observed for New Forest or Welsh Section A ponies (See Figure 11).

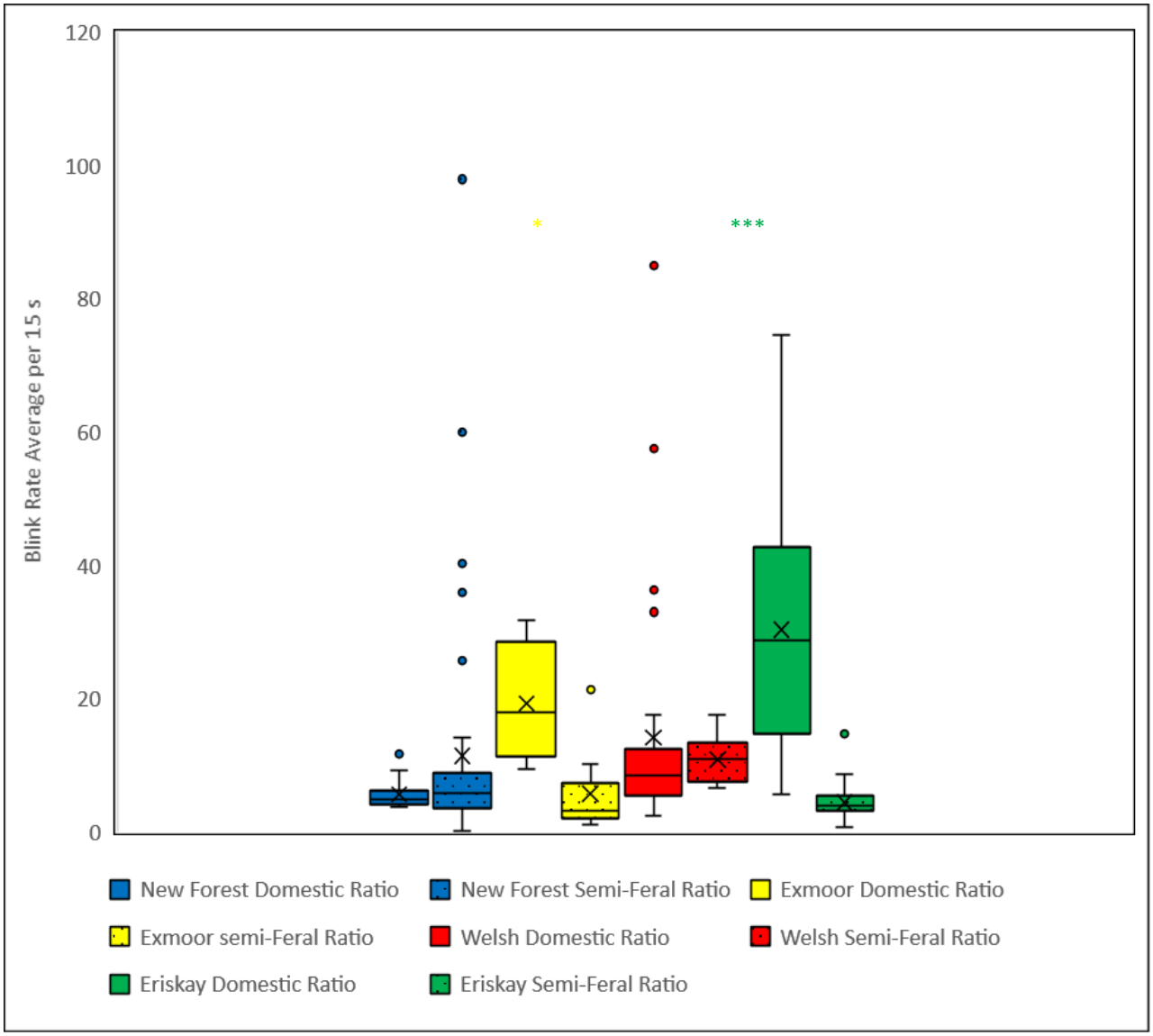


Figure 15: Breed level differences for blinks ratio for Domestic verses Semi-Feral (***=p<0.001, *=p<0.05). X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

4.3 BLINK RATE DIFFERENCES BETWEEN BREEDS IRRESPECTIVE OF DOMESTIC VERSES SEMI-FERAL.

Analysis via Kruskal-Wallis one way analysis of variance revealed a significant difference in full blinks, between native breeds (H44.25, p<0.001). Similar significant differences between breed populations were recorded for partial (H31.21, p<0.001).

4.3.1 BREED LEVEL DIFFERENCE FOR FULL BLINK.

The Mann-Whitney U analysis revealed significant differences at breed level for full blink rates (U = 1776.5, p<0.001). Bonferroni correction (threshold value p=0.005) After Bonferroni correction significant differences were evident between Eriskay and Welsh section A ponies (U=204.0, p<0.001), New Forest and Welsh ponies (U=511.5, p<0.001), Shetlands and Welsh section A ponies (U=151.5, p<0.001). No other significant differences were observed between New Forest or Welsh Section A ponies (See Figure 24).

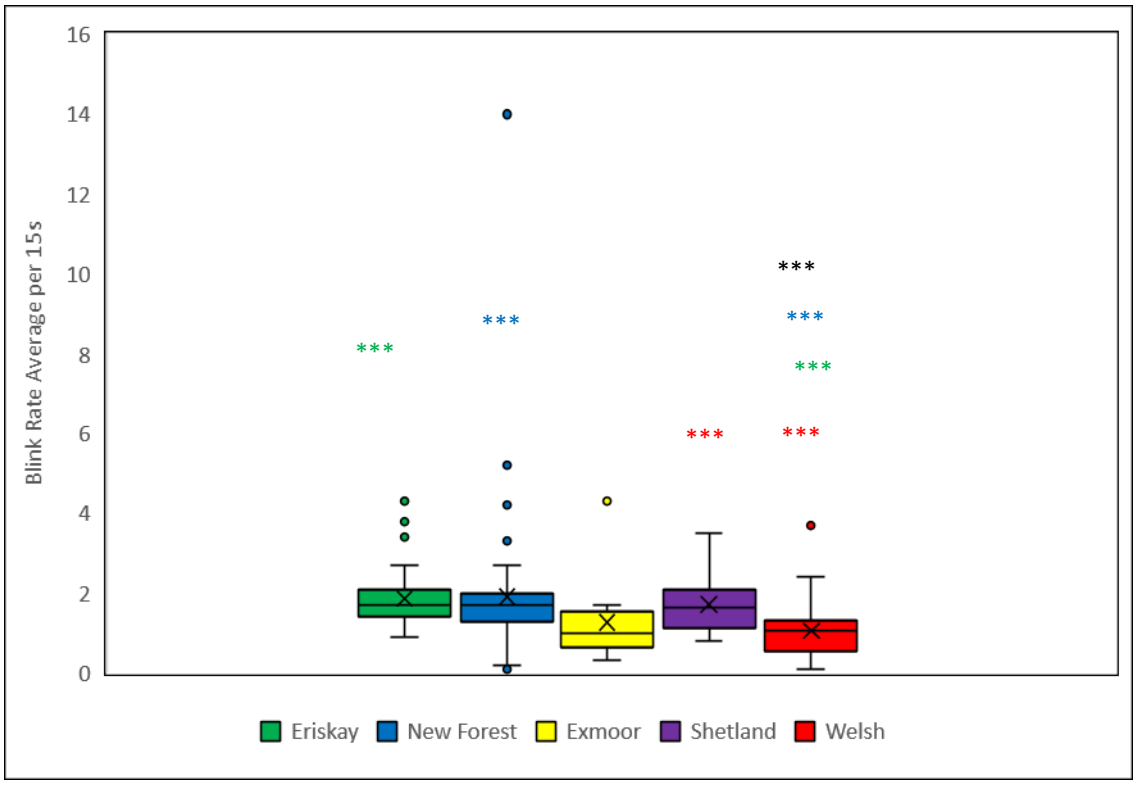


Figure 16: Breed level differences for full blinks (***=p<0.001)**.** X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

*** significant differences observed between Eriskay and Welsh section A breeds.

*** significant differences observed between Shetlands and Welsh section A breeds.

*** significant differences observed between New Forest and Welsh section A breeds.

4.3.2 BREED LEVEL DIFFERENCES FOR PARTIAL BLINKS.

The Mann-Whitney U ANOVA analysis also highlighted significant differences at breed level for partial blink rates subsequent Bonferroni corrections demonstrated significant differences (threshold value p=0.005) between Eriskay and Exmoor ponies (U=67.5, p<0.001), Eriskay and New Forest ponies (U=715.0, p<0.001), Exmoor and New Forest (U=35.5, p<0.001), Exmoor and Welsh section A ponies (U=37.0, p<0.01), New Forest and Shetland ponies (U=33.0, p<0.01). No other significant differences were observed between any other breed combinations. (See Figure 17).

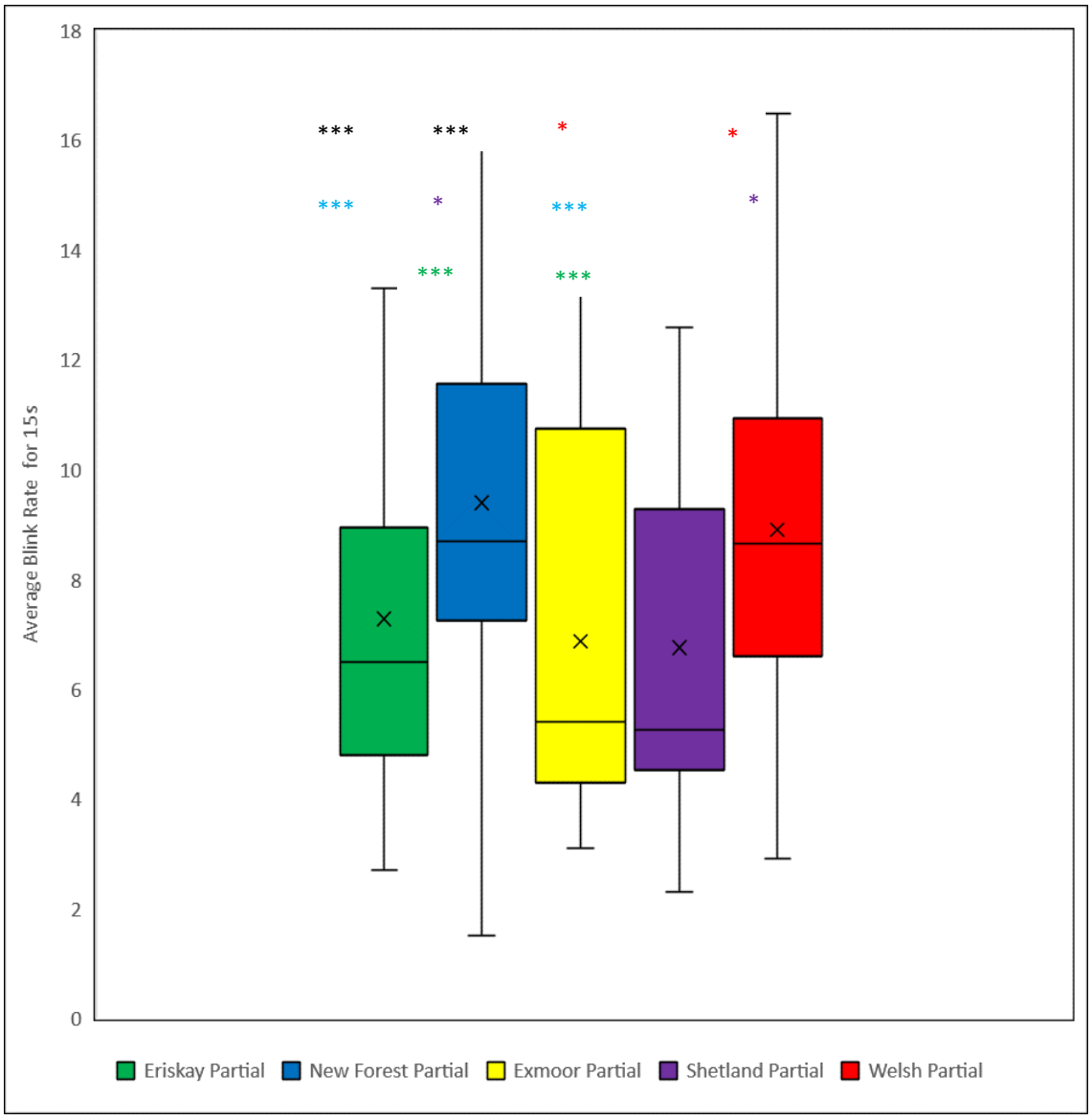


Figure 17: Breed level differences for partial blinks (***=p<0.001, **=p<0.01, *=p<0.05). X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

*** Significant differences observed between New Forest and Exmoor breeds

*** Significant differences observed between Eriskay and New Forest breeds.

*** Significant differences observed between Eriskay and Exmoor breeds.

* Significant differences observed between Exmoor and Welsh Section A breeds.

* Significant differences observed between New Forest and Shetland breeds.

4.3.3 BREED LEVEL DIFFERENCES FOR BLINK RATIO FULL:PARTIAL.

The Mann-Whitney U ANOVA analysis also highlighted significant differences at breed level for full: partial ratios blink rates (H46.92, p<0.001). When Bonferroni correction were applied (threshold value p=0.005) significant differences were evident between Eriskay and New Forest ponies (U=744.5, p<0.001), Eriskay and Welsh section A ponies (U=22.6, p<0.001), Exmoor and Welsh ponies (U=73, p,0.01), New Forest and Welsh section A ponies (U=710.5, p<0.001), Shetland and Welsh section A ponies (U=134.5, p<0.001). No other significant differences were observed for New Forest or Welsh Section A ponies (***=p<0.001). (See Figure 18)

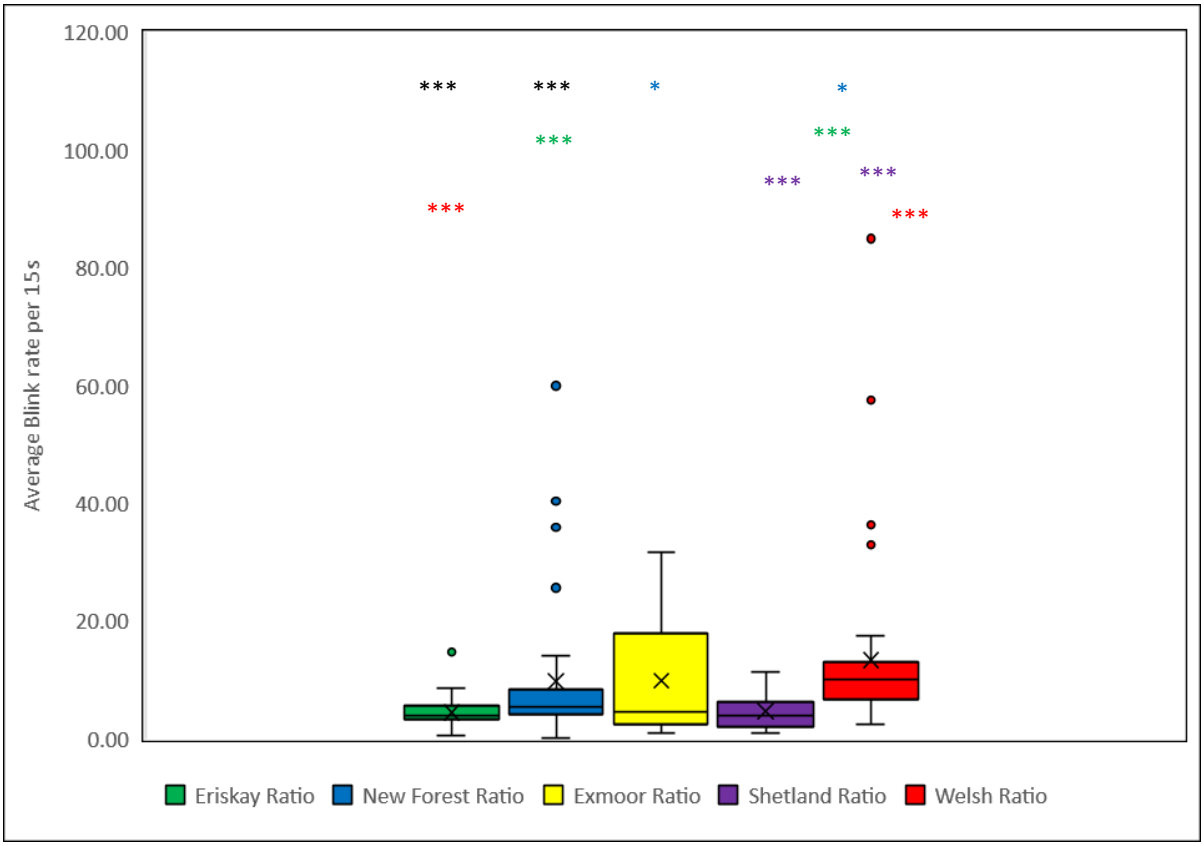


Figure 18: Breed Level Differences for blink rate ratios. (***=p<0.001, **=p<0.01). X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

*** Significant differences observed between Eriskay and New Forest breeds.

*** Significant differences observed between Eriskay and Welsh Section A breeds.

*** Significant differences observed between New Forest and Welsh Section A breeds.

*** Significant differences observed between Shetland and Welsh Section A breeds.

* Significant differences observed between Exmoor and Welsh Section A breeds.

4.4 BLINK RATE DIFFERENCES FOR GENDER IRRESPECTIVE OF BREED.

The Mann-Whitney U (Wilcoxon rank-sum) analysis showed significant differences between females and males irrespective of breed for full blink rates (U=3453.0, p<0.013). These significant results show that females have significantly more full blinks than males. But no significant difference for either partial (U=3667.0, p<0.055) or ratio full:partial (U=4229.0, p<0.666) where recorded. (See Figure 19)

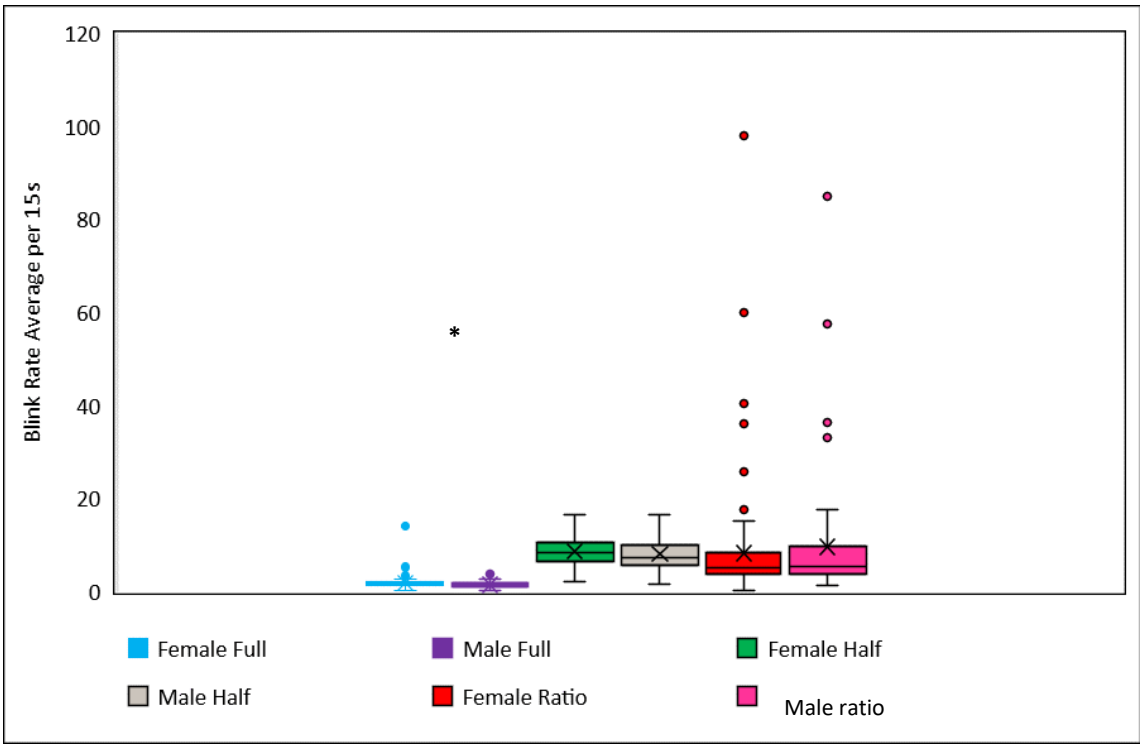


Figure 19: Gender blink rate differences irrespective of breed and Domestic and Semi-feral. (*=p<0.05). X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

4.4.1 BLINK RATE DIFFERENCES FOR GENDER WITHIN AND BETWEEN BREEDS, (*=P<0.05).

The Mann-Whitney U (Wilcoxon rank-sum) analysis showed significant differences between female Welsh section A and female Eriskay for full blink rates (U=30.5, p<0.001). Significant differences were also recorded for male Welsh section A compared to male Eriskay for full blink (U=30.5, p<0.001). Indicating both male and female Welsh section A blink less than both male and female Eriskay. Significant differences were also observed between male Welsh section A and female Eriskay for full blink (U=195.0, p<0.001) See figure 20. No other significant differences for gender between or within breeds were recorded for full blinks, partial blinks or eye lid flutter (see figure 21).

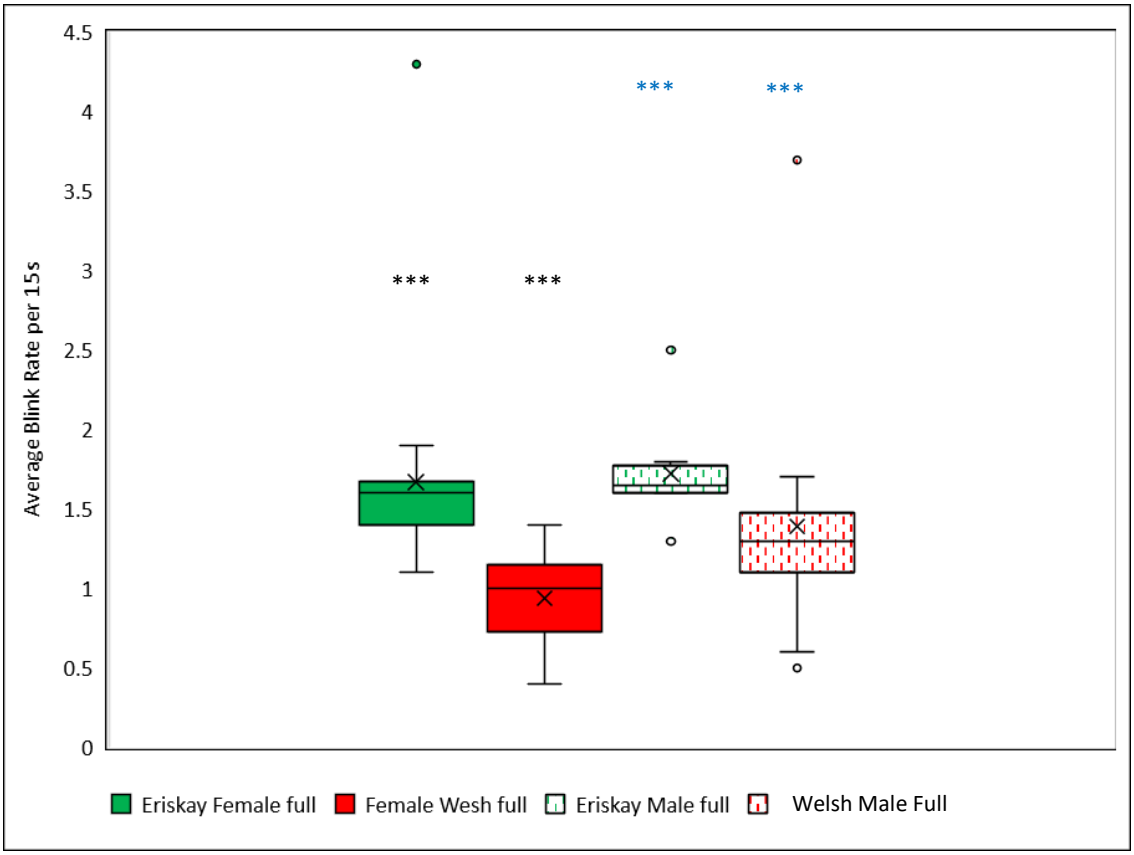


Figure20: Blink Rate differences for Gender between Eriskay and Welsh section A breeds (***=p<0.001). X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

*** Significant differences observed between female Eriskay and female Welsh Section A breeds.

*** Significant differences observed between male Eriskay and male Welsh Section A breeds.

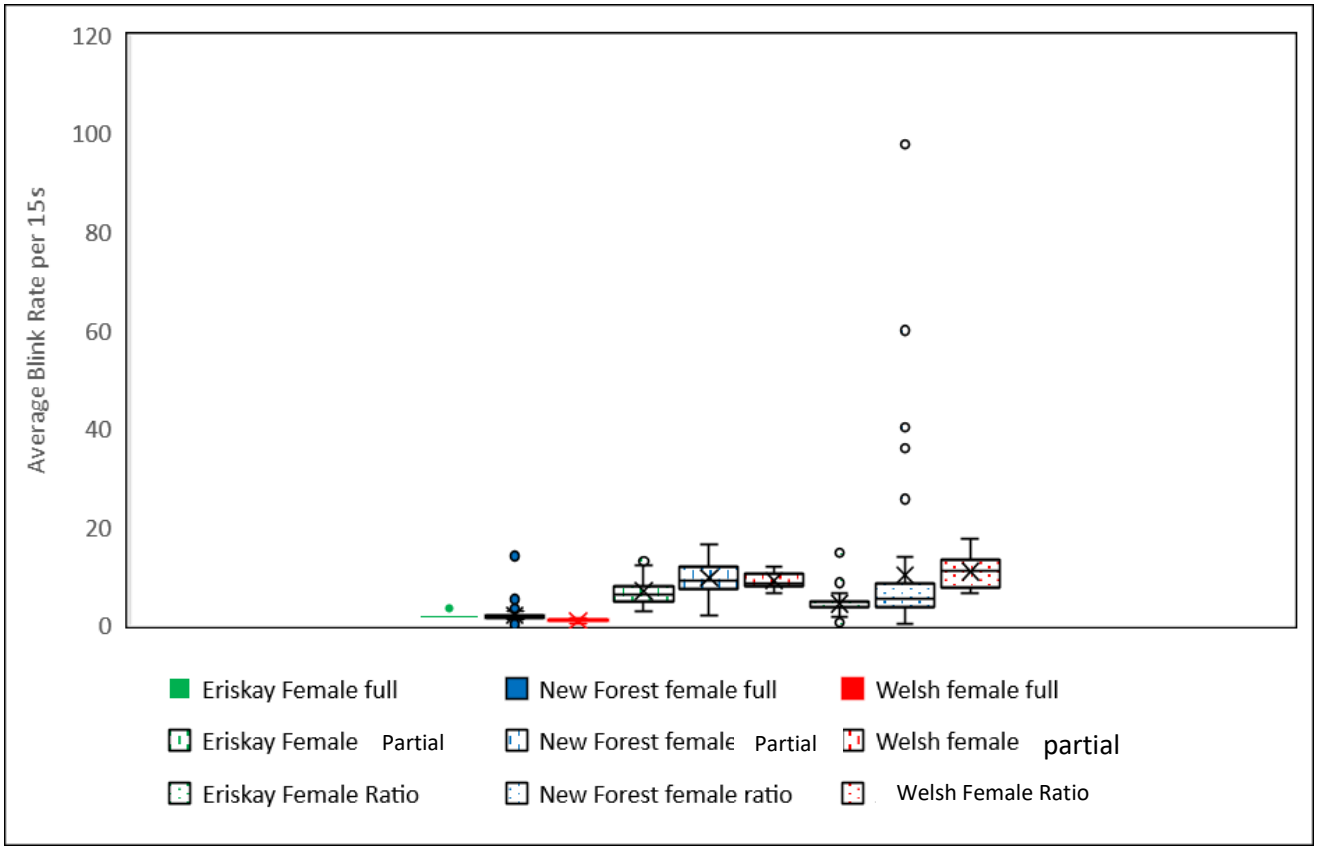


Figure 21: Blink Rate differences for females depending on breed (***=p<0.001) for full, partial and eye lid flutter. X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

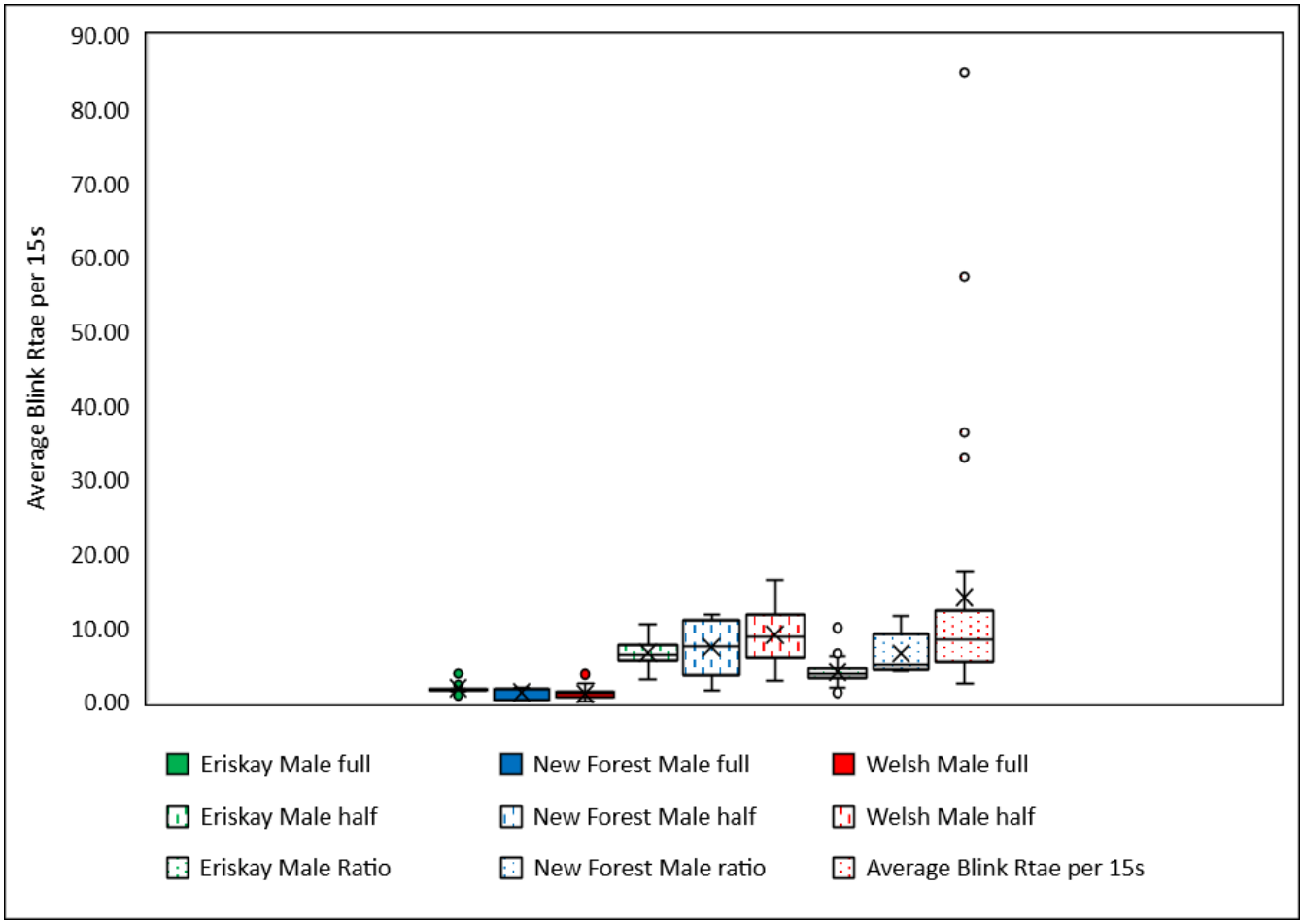


Figure 22: Blink Rate Differences for males depending on breed for full, partial and eye lid flutter. X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

4.5 BLINK RATE DIFFERENCES FOR DATA COLLECTION TIME.

4.5.1 BLINK RATE DIFFERENCES FOR TIME OF DAY IRRESPECTIVE OF BREED.

Analysis via Mann-Whitney U revealed no significant differences in full blinks ($U = 4501.5$, $p<0.635$), partial blinks ($U=4481.0$, $p<0.599$), and for ratio full:partial ($U=4456.5$, $p<0.556$) between morning (AM) and afternoon (PM) irrespective of breed and keep type (domestic or semi-feral).

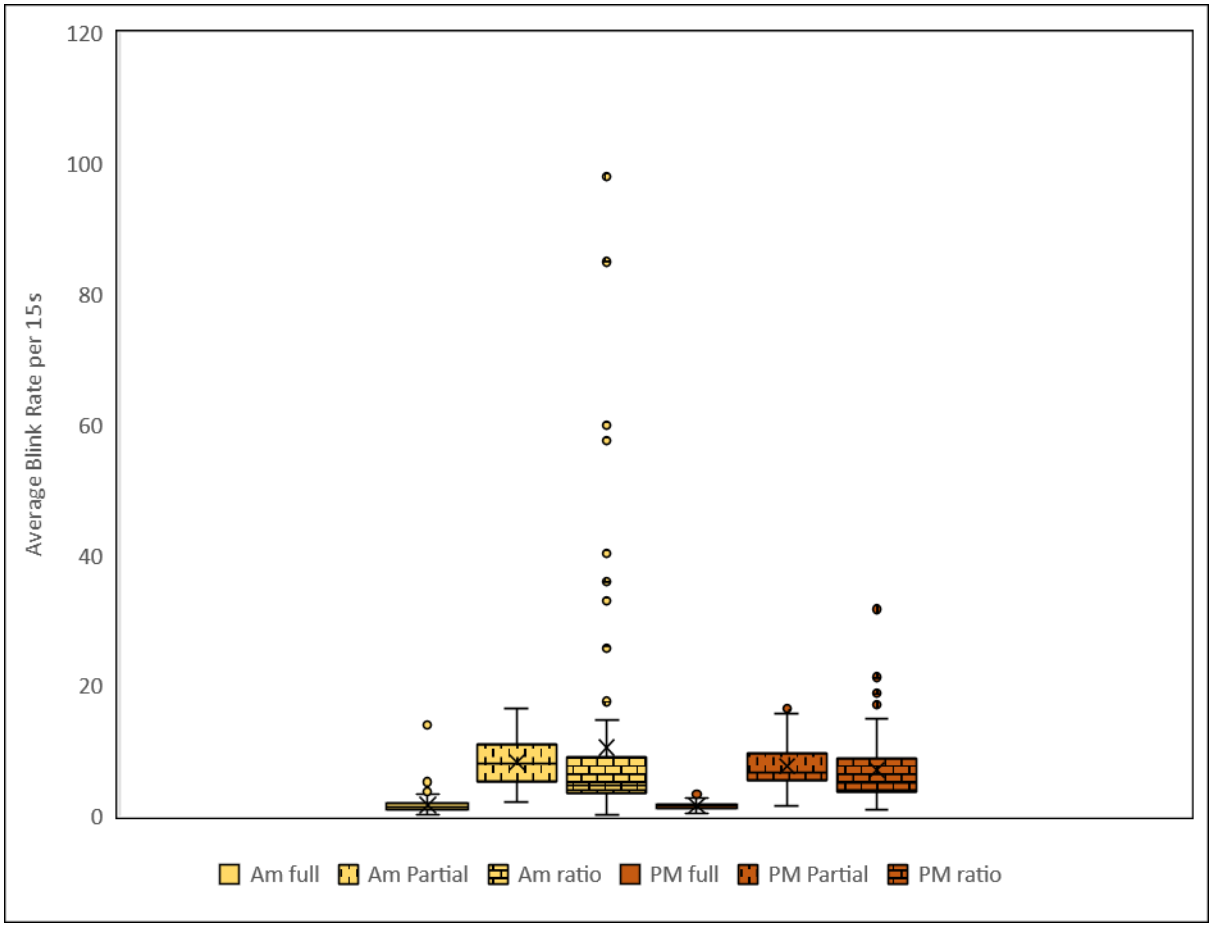


Figure 23: Blink rate differences for data collection time irrespective of breed. X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

4.5.2 BLINK RATE DIFFERENCES FOR TIME OF DAY FOR NEW FOREST PONIES.

Analysis via Wilcoxon matched-pairs test revealed no significant differences in full blinks ($T=151.5$, $p<0.776$), partial blinks ($T=127.5$, $p<0.229$), and for ratio full:partial ($T=61.0$, $p<0.487$) between morning (AM) and afternoon (PM) for the New Forest ponies.

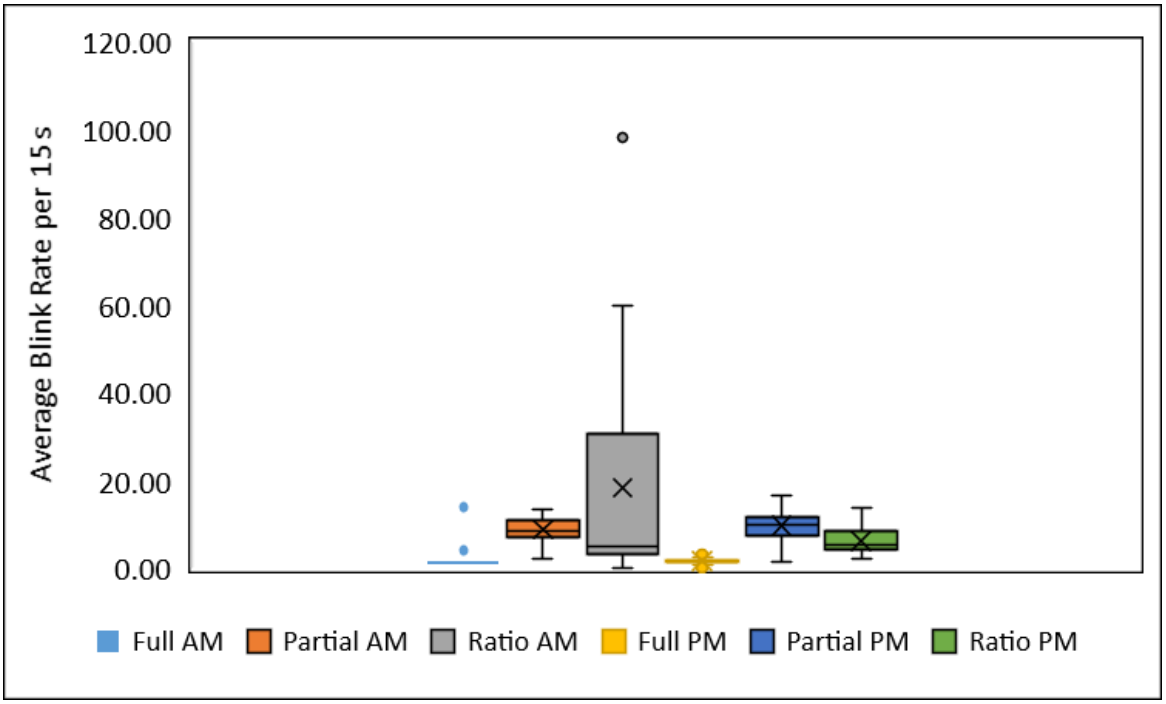


Figure 24: Blink Rate differences for New Forest Ponies dependant on data collection time (AM and PM) X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

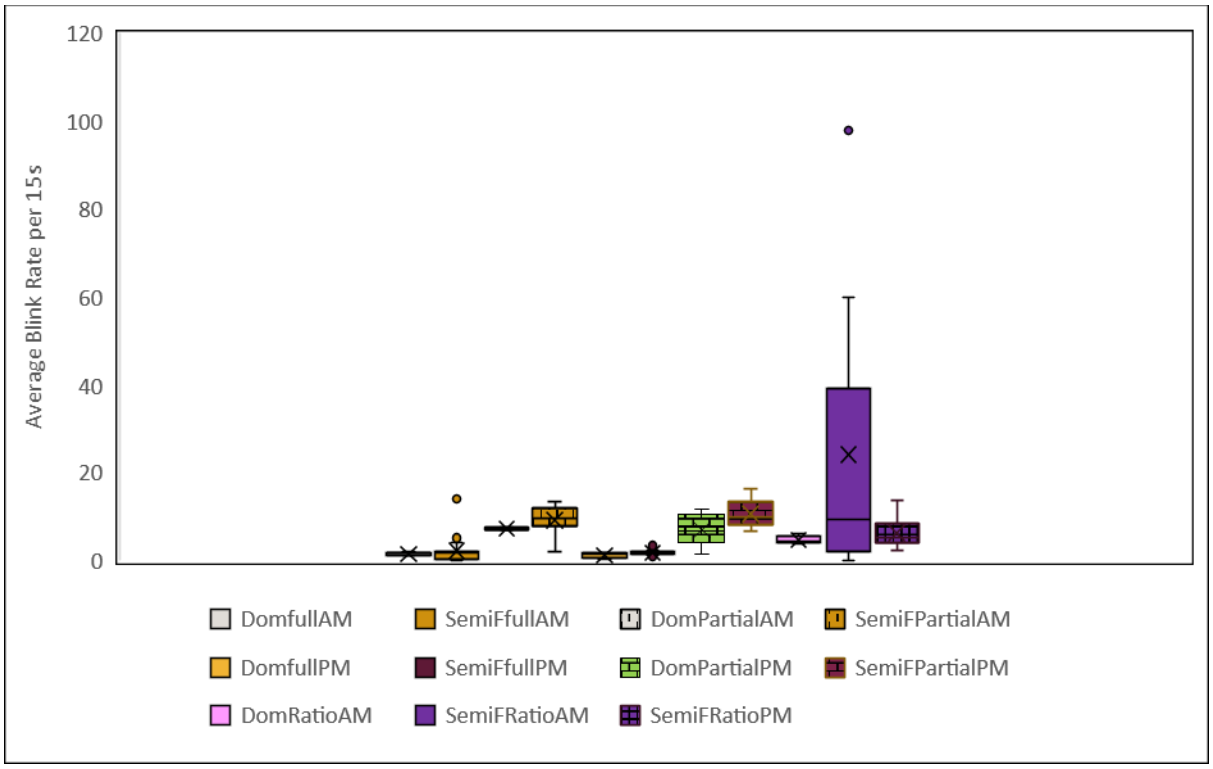


Figure 25: Comparison of blink rate differences for New Forest Ponies both Domestic and Semi-Feral dependant on collection time (AM and PM). X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

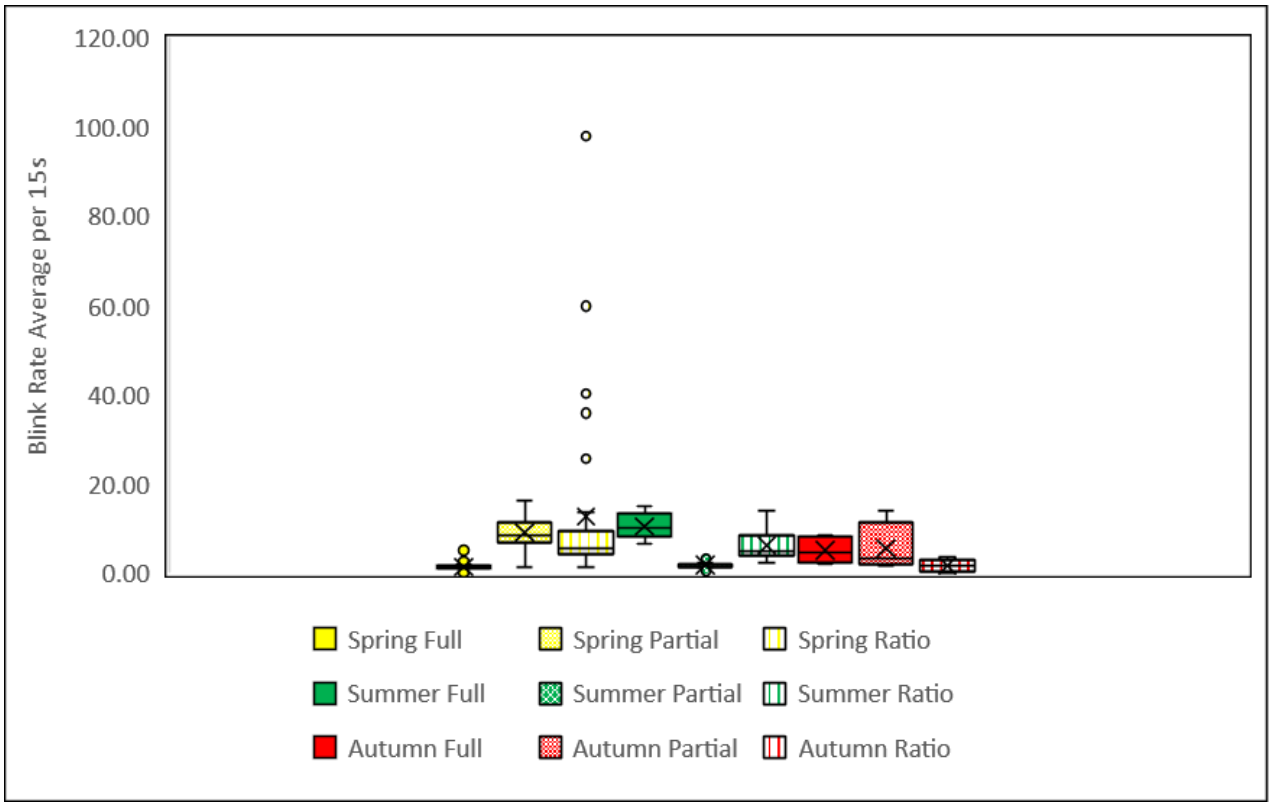


Figure 26: Blink Rate differences for seasonal data collection times. X= mean, ● = Outlying data points, whisker = minimum to lower quartile / maximum to upper quartile, *** = significant results.

6.0 EFFECTS OF WEATHER DATA ON BLINK RATE VALUES

The effects of temperature, humidity and wind-speed were plotted against blink rate values for all semi-feral groups. The charts below (figures 27, 28, 29) summarise findings from the cohort of semi-feral New Forest ponies. All other data can be found in appendix 11.3. Qualitative assessment of the plots revealed no obvious effects of any of the weather values, and so no further comparative or correlational statistical analyses were applied.

6.1 TEMPERATURE 0^C

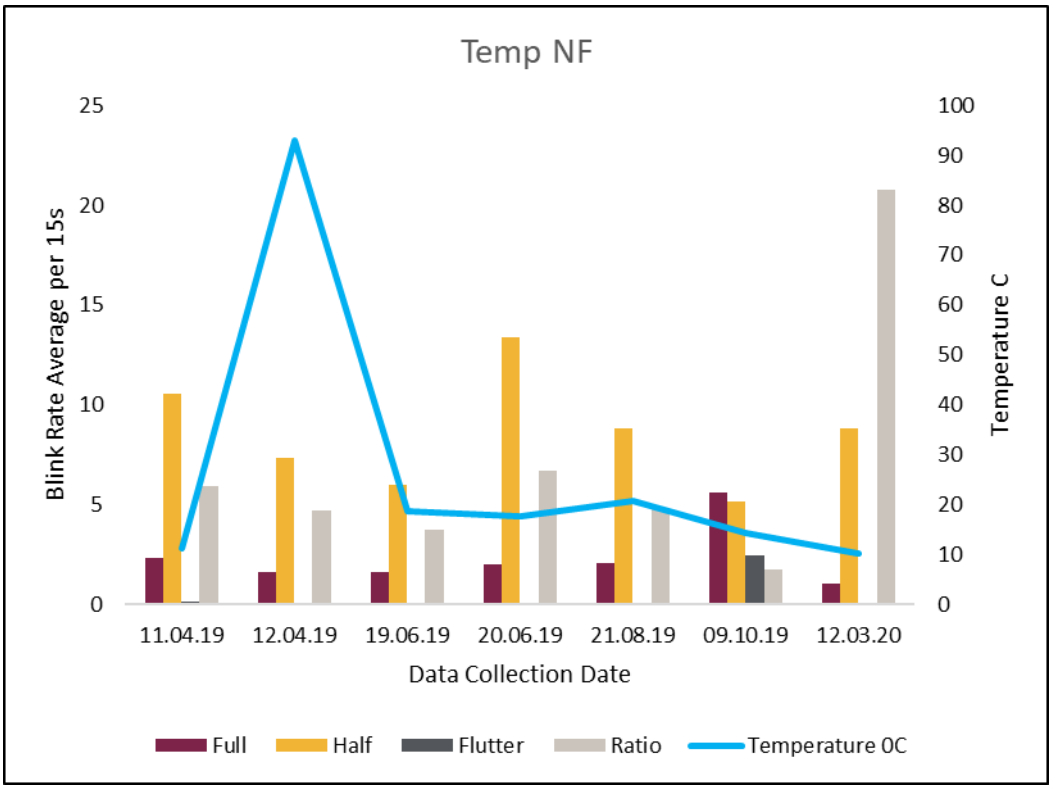


Figure 27: Temperature effects on New Forest ponies blink rate for full, partial and flutter compared to temperature climate changes during collection periods.

6.2 WIND SPEED

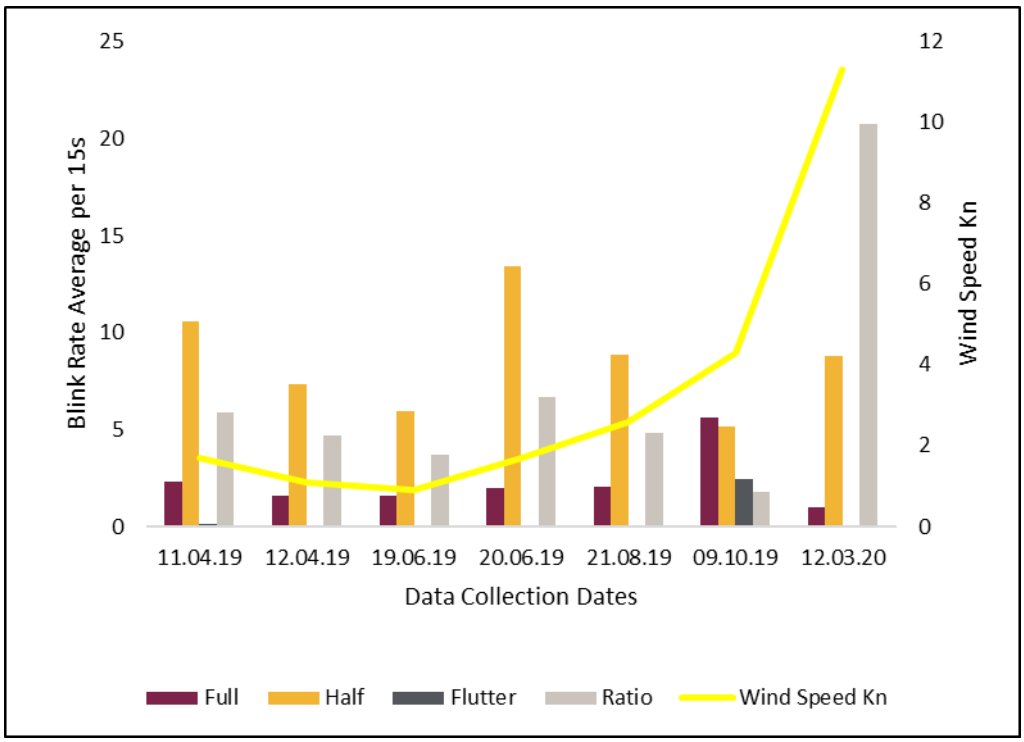


Figure 28: Wind speed effects on New Forest ponies blink rate for full, partial and flutter compared to wind speed changes during collection periods**.**

6.3 HUMIDITY %

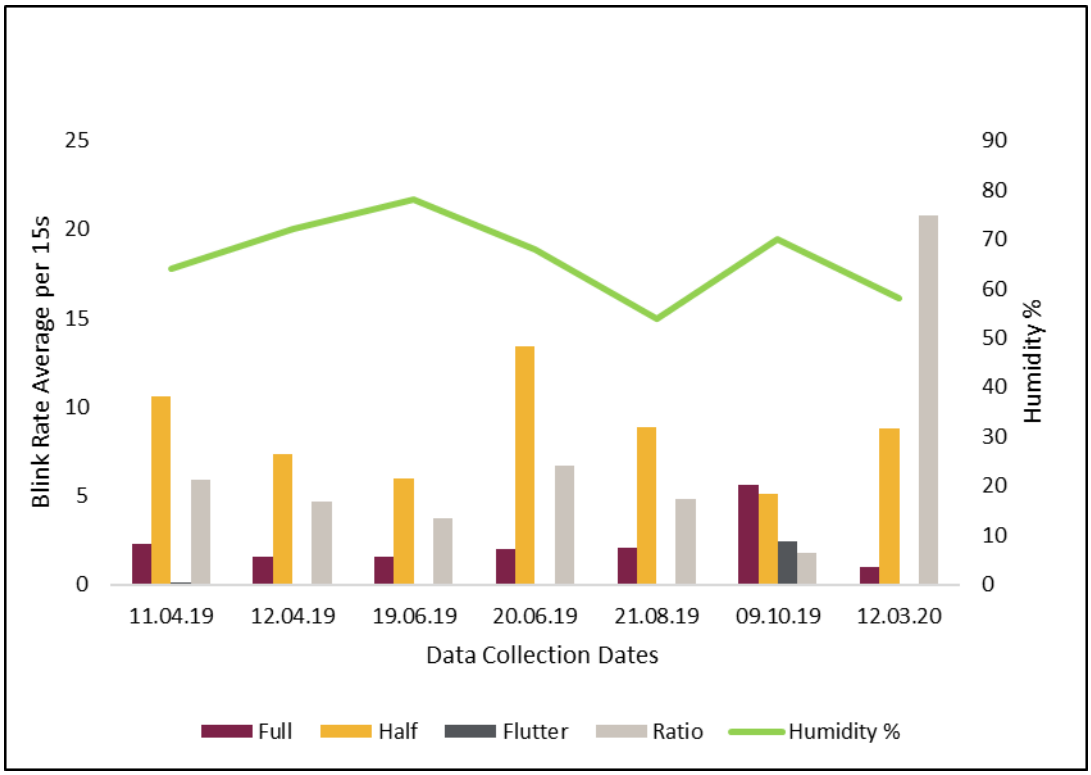


Figure 29: Humidity effects on New Forest ponies blink rate for full, partial and flutter compared to humidity changes during collection period.

7.0 DISCUSSION

This study explored Spontaneous Blink Rate as an indicator of dopamine levels in British Native breeds to ascertain if differences exist between these breeds when comparing domesticated and semi-feral subjects. No studies to date have been carried out on semi-feral equids using spontaneous blink rate as an indication of stress and therefore this measure has potential for use as a non-invasive welfare assessment tool that could be used for monitoring of semi-feral herds (Broom & Johnson 1993).

The results obtained suggest that that there are significant differences in dopaminergic activity between semi-feral and domestic individuals irrespective of breed type. These differences are also evident when comparisons are made within breed between semi-feral and domestic equivalents. Significant results have also been recorded between the British Native breeds that have been studied during this research.

On this basis, both null hypotheses relating to 1) breed and 2) domestic versus semi-feral differences may be rejected. The ramifications of these findings are considered below.

7.1 BLINK RATE DIFFERENCES BETWEEN SEMI-FERAL AND DOMESTIC HORSES.

The significant increase in full blinks, seen in the semi-feral native ponies compared to domestic counterparts may be due to the requirement of the semi-feral cohorts to be more aware of potential threats from within the herd and their environment. In this respect it could be concluded that the midbrain dopamine response to stress is more sensitive in semi-feral animals. In contrast,, Hall (1945) observed that fixation of the head on a novel stimulus resulted in a zero-blink rate in 90% of subjects, enabling the individual to gain total visual information of potential threats. Moreover, Merkies *et al* (2019) and Mott *et al* (2020) observed an initial slowing of blink rate upon initial application of a stressor. However, in both of these studies SBR then elevated beyond the pre-stress values, lending credence to the notion of a sensitised dopaminergic response to stress in the semi-feral animals featured in these studies. In other species such as mice, there exists particular strains such as the C57b, which exhibit a passive response to stress coupled with lower dopamine transmission. On the other hand, the DBA/2 strain demonstrates a more active coping response in the face of stress, alongside hypersensitive dopamine pathways (Cabib 2012). For flight species such as the horse, individuals leading a semi-feral existence may correspond to the DBA/2 murine equivalent, due to the requirement for a heightened flight response. Moreover, as these differences in coping ability are strain specific, there is obviously a strong genetic determinant of stress coping phenotypes. From an equine

standpoint, breeds such as the Eriskay Pony, who exist under challenging environmental circumstances, the DBA/2 type active coping tendency may well have been naturally selected over multiple generations. Finally, the reduction in blink rate followed by the increase in full blinks correspond to the results obtained by Holmes (1938) and Blount (1927) indicate that herbivores have a higher blink rate due to the involuntary survival mechanism of fight or flight. Overall, it would appear that semi-feral horses are more reliant on flight from danger compared to the domestic equivalents that are in comparison more reliant upon human intervention for safe living conditions, although the process of domestication inflicted upon horses also may have a detrimental effect on the individuals' well-being and welfare (Yarnell *et al* 2013, Yarnell *et al* 2015). Of course, Investigation using other external factors which could be addressed in future studies, such as increased prevalence of flying insects leading to altered SBR.

The apparent decrease in partial blink rate for the semi-feral ponies compared to the domestic ponies appears to show the opposite trend, when compared to the full blink results obtained in this research. However, results obtained from research carried out by Merkies *et al* (2019) when comparing full, partial and eye lid flutters showed that partial blinks occurred more often during the control (low stress) phase of the study and also during feed restriction treatments and least often during separation or startle test.

The results presented herein, which show a significant reduction in partial blinks for the semi-feral ponies (irrespective of the breed) correspond with the theories that animals are constantly monitoring their environment for potential threats and feeding or social opportunities. When the physical process of partial blinking occurs, the individual will lose access to the visual information. Therefore, the average mean blinks per 15 seconds for the domestic 9.8991 (39.59 per minute) and semi-feral 8.1625 (32.65 per minute) would result in the individuals loosing 300ms per blink, resulting in these two groups potentially loosing 11.88 seconds per minute and 9.795 seconds per minute respectively. Whilst blinks are necessary to keep the eyes moist and in good health the visual information is clearly reduced whilst the eye is closed (Yorzinski JL 2016). Would the semi-ferals within this study have adapted their behaviour to a more stressful environment to maximise visual input leading to a reduction their partial blink rate?

The semi-feral horses in this study will also be affected by their emotional state which will be directly attributed to their behaviour and welfare, with the approach/ avoidance phenomena playing an important part in their behaviour and experience. Elliot *et al* (2013) proposed that emotional responses in equids are based upon survival advantages and the emotional responses are either positive motivational, where the outcome is generally rewarding, or in contrast, avoidance behaviour leading the individual moving away from the negative stimulus and a negative consequence. The behaviour has also been linked to fear of the threat and therefore avoidance and or aggression and advancement towards the stimulus. These behaviour outcomes are compounding factors associated with the emotions

and therefore welfare of the individual and of the herd. Through a process of engagement with the environmental stimuli the individual builds a “bank” of stimuli associated with beneficial or detrimental outcomes. Through this process of “banking” associated outcomes, the individual and therefore herd develop evolutionary evaluations, enabling the individual to adapt and thrive through a process of reacting to environmental stimuli (Cacioppo *et al*. 1999). This may result in the increase in SBR full blinks observed by the semi-feral group within this research. The emotional wellbeing of the semi-feral horses in this research would indicate the outcomes of positive reinforcement through beneficial “banking” of outcomes, and adaption to their environments. These “banked” stimuli might be a physical occurrence but also might be an associated internal biological outcome that has resulted from a previous experience. The implications of the reinforcement sensitivity theory have two avoidances from harmful stimuli concepts, one which corresponds with the semi-feral life style, that of fight-flight-freeze (FFFS), which is an action that can be seen in many semi-feral herds, resulting in escape behaviour and mediating the emotion of fear. The second behavioural outcome results in a behavioural inhibition reaction (BAS) through a process of goal resolving, leading to a feeling of anxiety through a process of risk assessments from previous experiences (Corr 2013). Both behavioural outcomes experienced by the semi-feral herds result in emotional change and welfare implications.

The increasingly complex process of timing and blinking has been studied primarily in humans and non-human primate, although limited research has shown that Avian American crows blink less in response to potential danger (Cross *et al* 2013).

Therefore, further investigation into the recording of partial blinks on semi-feral ponies as an indicator of dopamine tone within the brain is a necessity to enable it to be used as an indicator of stress levels within horses. This final point is of particular relevance when considered in the context of low sample sizes obtained for some breeds such as the Eriskay ponies. In this regard, future work should focus upon obtaining greater sample numbers.

Future research studying traditional methods for monitoring dopamine and cortisol levels through blood tests and heart rate variability should be correlated against the recording of partial blinks, this would enable the establishment of procedures for partial blink monitoring alongside full blinks in SBR research. To establish data surrounding the ramifications of partial blinks in respect of dopamine transmission, administration of an antagonist (i.e Sulpiride) and agonist (i.e Pergolide) whilst monitoring the changes in partial blink rate would provide enlightenment in this respect. This essential work would provide validation data to drive future studies which incorporate partial blinks as a proxy measure of dopamine tone.

7.2 BREED LEVEL DIFFERENCES FOR DOMESTIC VERSUS SEMI-FERAL.

For full blinks, domesticated Eriskay ponies demonstrated significantly lower blink rate values compared to Semi-feral equivalents. For partial blinks, Exmoor and Eriskay ponies demonstrated significant differences between domestic and semi-feral, with domestic partial blink rates being significantly higher compared to the semi-feral group. As reported by Speed (1951) the Exmoor pony is considered the most ancient of the UK breeds and may therefore show adaption and familiarity of the rural environment in which it lives and have evolved to survive within the rural setting. Also, the Exmoor ponies studied in this research was the largest group of the semi-feral breed, totalling approximately 40 in number, within a single herd, which may have had a significant effect on blink rate when compared to other herds studied. The effect on blink rate in Primates is significantly affected in large group sizes Tada *et al* (2013) potentially due to threat by other individuals within the group.

However, this trend was reversed when looking at the New Forest herd, with semi-feral groups showing significantly higher partial blink rates. Research carried out by Tada *et al* (2013) looking at environmental/ habitat effects on primates showed no significant correlations on blink rate when comparing habitat type, suggesting that other factors such as genetic differences between semi-feral and domestic were in play here. Again, further research focussing on genotypic comparison is warranted, which would again require expanded sample numbers and more accurate quantification of environmental factors relating to living conditions.

Observations were made that the New Forest semi-feral subjects carried out partial blinks every time they took a new bite from the grass, these observations correspond with the results obtained from research carried out by Doughty (2018) where mouth and jaw movements in humans can substantially affect the spontaneous blink rate.

Analysis revealed an overall difference at breed level with significant results being obtained for Eriskay ponies when comparing full blinks for the semi-feral to the domestic study group. The results showed that full blinks were significantly lower in the domestic cohort when compared to the semi-feral herd. However, these results were once again reversed when analysis of the partial blinks were performed, with significant results obtained for both Eriskay and Exmoor semi-feral herds being lower than the domestic counterpart.

Although, the results obtained for the New Forest horses showed that the semi-feral individuals had a higher blink rate than the domestic group. The Welsh section A showed no differences between both groups for partial blinks.

Blink rate ratio analysis showed that a significant trend for both Eriskay and Exmoor existed when comparing domestic to semi-feral groups. But no significant difference was observed for the New Forest breed at the blink ratio level.

This study explored Spontaneous Blink Rate as an indicator of dopamine levels in British Native breeds and to ascertain if differences exist between these breeds when comparing domesticated and semi-feral subjects. No studies to date have been carried out on semi-feral equids using spontaneous blink rate as an indication of stress and therefore as a non-invasive welfare assessment tool.

Limited research has been carried out investigating the differences of blinks rates between breeds of any species. Reported results for both brachycephalic and mesocephalic dog breeds show significant differences between breeds grouped with the above criteria, but the differences were concluded to be as a result of the physical conformation of the skull (Williams & Denny, 2020).

The significant increase in full blink rates seen in the semi-feral native ponies may be due to the requirement of the individuals to be aware of potential threats from within the herd and within their environment. These results would relate back to the observations of blink rates made by Hall (1945) where the fixation of the head on a stimulus resulted in a zero-blink rate in 90% of subjects, enabling the individual to gain total visual information. The reduction in blink rate followed by the increase in both full and partial correspond to the results obtained by Holmes (1938) and Blount (1927) that herbivores have a higher blink rate due to the involuntary survival mechanism of fight or flight. This may well correspond to the results obtained in this research where the semi-feral horses are reliant on flight from danger compared to the “danger” stimulants that domestic individuals were exposed to. Further investigation could be carried out by video recording of the Eriskay and semi-feral herds within the natural environment to monitor the effect that environmental stimuli exert on blink rate. SBR could be recorded and then related to known environmental stimuli, to ascertain the effect that the startle response had on SBR values.

Research in humans has shown that the blink rate decreases as the cognitive visual stimulus increases and may be controlled depending on the need to avoid missing visual input (Holland & Tarlow 1972, Goldstein *et al* 1992). Further research through the visual recording could highlight any environmental factors that may influence the blink rate in this manner.

7.3 BLINK RATE DIFFERENCES BETWEEN BREEDS IRRESPECTIVE OF DOMESTIC / SEMI-FERAL STATUS.

Significant differences were found between breeds irrespective of domestic or semi-feral management type, these differences were seen throughout the analysis of data. Full blink results showed that the Welsh section A ponies had a mean full blink of 1.060 per 15 seconds (4.24 per minute) compared to Eriskay mean 1.86 (7.44 per minute), New Forest mean 1.905 (7.8 per minute) and Shetland mean 1.75 (7.00 per minute) which were all significantly higher than the Welsh section A ponies. Comparisons between the Exmoor and

Welsh section A ponies for full blinks did not yield significant differences, but the Exmoor ponies showed a slight increased full blink rate, which may have been attributed to the herd size.

With reference to the Welsh section A Pony, this breed was revealed by Winton et al (2020) to exhibit the lowest levels of genetic diversity compared to other natives in the study, and so it is reasonable to postulate that the dopaminergic tone of this cohort has been affected as a result of sustained inbreeding in domestic conditions. This is evident by the low Full blink rate for the Welsh section A ponies at a mean of 1.060 (4.24 per minute) in this study compared to all the other breeds that had a mean of between 1.715 – 1.86 full blinks per 15 seconds (6.86 - 7.44 per minute).

The Partial blinks showed significant increase differences for Eriskays mean 7.286 per 15 seconds (29.14 per minute when compared to Exmoor ponies mean 6.876 per 15 seconds (27.50 per minute), but a significantly lower blink rate when compared to the New Forest ponies mean 9.395per 15 seconds (37.58 per minute). The semi-feral Eriskay ponies were located on the Holy Isle and had no direct contact with any large predators within their environment, in comparison with the New Forest ponies which were exposed to other predatory mammals and vehicles on the highways. New Forest ponies had significantly higher mean when compared to the Shetland 6.755 per 15 seconds (27.02 per minute) for partial blinks recorded. However, the Exmoor ponies mean 6.876 (27.504 per minute) showed significantly lower blink rates than both New Forest mean 9.395 per 15 seconds (37.58 per minute) and Welsh mean 8.909 per 15 seconds (35.64 per minute, which would support the current research into the ancient origins of the Exmoor breed (Speed *et al 1951)* that has been adapted to their environment over generations.

Blink rate ratio analysis showed that the significant trend for both Eriskay and Exmoor existed when comparing domestic to semi-feral groups. But no significant difference was observed for the New Forest breed at the blink ratio level, this may be due to the fact that the New Forest domesticated cohort resided within the New Forest boundaries but had a domesticated regime, with additional hay and hard feed during winter, anthelmintics and dentistry monitoring. This group were classified as domesticated, but their environment was not significantly different to the semi-feral group and therefore the results obtained may well have been skewed. Again, this group would benefit from additional numbers with a diverse, more traditional domesticated management regime.

As Spontaneous Blink rates have been clearly demonstrated as an indication of dopamine activity in the CNS (Merkies 2019) and therefore stress levels within the equine, breed differences have been identified in association with stereotypic behaviours Bachmann *et al* 2003, Albright *et al* 2009, Wickens and Heleski 2010. Thoroughbred were reported to be 3.1 times (Bachmann *et al* 2003) and warm bloods 1.8 times (Wicken & Heleski 2010) more likely to show crib-biting behaviour than other breeds included in the study. Whilst Thoroughbred horses are also more likely to exhibit weaving behaviours when stabled

(Ninomiya *et al* 2007) compared to other breeds. These results may be indicative of a genetic susceptibility to stereotypic behaviours. Although, it could be argued that these breeds of horses are more prevalent in high level competition dressage, racing and eventing, which would be deemed as a stress inducing sporting environment.

Although the lowest mean of partial blinks was recorded by the Shetland group at mean of 6.755 partial blinks per 15 seconds (27.02 per minute), the individuals in this group were all semi-feral and this may have influenced the results.

7.4 GENDER RELATED DIFFERENCES

Significant differences were recorded for full blinks irrespective of breed, with females having a higher recorded blink rate when compared to both stallions and geldings.

The results obtained in this research correspond with data recorded from the data-base of European Normal Control Database of DaTSCAN (ENC-DAT) which aimed to set up a large data base of normative data using Dopamine Transporter (DAT) imaging as a diagnostic tool for both Parkinsonism and Dementia (Varone *et al* 2013. The gender results obtained from this human study showed a significant effect on SBR in the caudate and putamen, where women had a higher DAT availability (Varrone *et al* 2013). The results from this study also showed an average related decline availability of 5.5% per decade in both genders. Similar results were obtained from research carried out by Yamamoto *et al* (2017) to establish both age related and gender-based differences in blink rate for healthy controls in Japan.

No Known research to date has investigated the effects of gender on SBR or the relationship between gender and breed for SBR in equids.

The increased blink rate of females within the semi-feral herds may be attributed in part to the fact that the females had foals at foot and were therefore in protection mode for their offspring during potential danger and flight situations. This may well account for the significant results obtained between genders.

Additional research would help to establish the effects of gender on blink rate and the effects of neutering on males with reference to SBR. No research to date has been carried out on the effects of neutering and sexual hormones on the SBR. This could be important with regard to the significant difference between the genders. Within this research males included stallions and geldings.

7.5 DIURNAL AND SEASONAL INFLUENCES ON DOPAMINE ACTIVITY.

Following analysis of the results obtained for the blink rate collection times there were no significant differences observed between data collection periods made preceding noon or after for either semi-feral or domestic subjects in this research study. Although research carried out by (Tada *et al* 2013) on eye blink rate behaviours in primates showed that diurnal primates exhibited more eye blinks when compared to nocturnal species. Research carried out by (Ugwu *et al* 2021) on cattle and Doughty (2006) on humans also showed no significant results of blink rate being affected by time of day. In comparison, the results reported by Giuseppe *et al*. (2000) in humans, showed a non-variable rate in SBR during morning, midday and afternoon but a significant increase at the 20.30h time point. This is supported by an increase in dopamine activity. This increase in dopamine levels at this time period corresponds to a rising sleep drive and is thought to counteract this sleep drive by a dopamine mediated activation period. Clinical research on patients with dopamine- related illnesses show an increased diurnal variation in dopamine levels (Wang *et al*. 1994).

Therefore, it can be assumed that collection time within a day would not have a significant effect on data obtained as the results indicated, although further data collection during an hour period either side of 20.30h would complement the studies carried out by Giuseppe *et al*. and investigate the existence of a crucial nocturnal time-point for dopamine transmission in the horse.

Seasonal collection data for the New Forest semi-feral subjects also showed no significant differences between spring (March-May), summer (June-August) and Autumn (September-November). Studies carried out by Depue *et al* (1989 & 1990) tested dopamine activity in patients with seasonal affective disorder (SAD) showed an increase in SBR which was normalised by intensive light therapy. These increases in SBR were not evident in the results obtained in this study but blink rate monitoring was not carried out during the December to February. Further investigation in the semi-feral herd during the winter month would enable this phenomenon to be compared to data observed in research by Depue *et al*.

As an observational point blink rates for semi-feral subjects appeared to be higher during summer months due to fly numbers and individuals with longer forelocks appeared to blink less than those with forelocks that did not cover the eyes. However, no direct data was collated from this observation, although future analysis would be possible from data records and photographic identity portraits.

Therefore, the data obtained in this research could be used to validate SBR as an indicator of welfare status, adding to the recent work of Harvey *et al.* (2023) who investigated the use of this measurement to extend the existing 5 domains model. Overall, the results from the current study can be used to inform research in free living equids, with a view to assessing negative and positive mental experiences in welfare assessment approaches.

From the results obtained during this research the Null hypothesis that there are no significant differences between British Native Breeds is rejected. The results also indicate that there are also significant differences between semi-feral and domesticated groups within breeds and irrespective of breeds.

7.6 BLINK RATE AVERAGES

The full blink rate averages obtained in this research for British Native pony breeds are Eriskay 7.467 per minute (224.01 per 30 mins), New Forest 7.62 per minute (228.6 per 30 mins), Exmoor 5.044 per minute (151.32 per 30 mins), Shetland 6.86 per minute (205.8 per 30 mins), Welsh 1.061 per minute (127.32 per 30 mins) irrespective of whether they are semi-feral or domestic. When compared as a group regardless of breed but considering whether they are domestic or semi-feral the following results were obtained. Domestic 3.897 per minute (116.91 per 30 minutes), Semi-feral 6.712 per minute (201.36 per 30 minutes). The results obtained from this research are considerably lower than those obtained by both Roberts *et al* 2016 of 547.72 (ponies, cobs and sports horses) and Dakin 370.86 (Thoroughbreds), all expressed as mean per 30mins.The discrepancy observed in this research may have arisen due to differences in the counting environment, compared to Roberts *et al* quantified the SBR in stabled subjects, which in itself may have applied a degree of stress leading to the elevated values recorded. In addition, the results obtained in this study when compared to results obtained in previous studies would indicate that there are factors between breeds that need to be considered when carrying out research featuring SBR, which may be attributed to factors such as genetic variation. On the other hand, the significantly higher blink rate for semi-feral equids regardless of breed may be attributed to environmental factors discussed previously within the report. Continuation of the research with an expansion of individual numbers, more sophisticated recording technology, which would enable both visual and sound monitoring of the individual's behaviour and blink rate would help to eliminate environmental stress stimulation. Results obtained may then potentially be attributed to differences in genetic inheritance.

The average blink rates obtained from this research are lower than those recorded for humans (26.13) and the domestic pig 30-36 per minute (Stevens and Livermore 1978) but higher than those obtained by Kaminer *et al* (2011) for rats 5.3 $^{+}_{-}$ 0.3 and 8-22 for primates per minute (Hall 1945).

8.0 FUTURE WORK AND PROTOCOL IMPROVEMENTS.

Replication of this study would enable a larger data set to be established, concentrating on improving data from domesticated Exmoor, Shetland, New Forest and Eriskay breeds. Increasing numbers of semi-feral Shetlands and Welsh Section A would also enhance these data. Although traditionally longer periods for monitoring of SBR are seen in other research reports on domestic Equids, the use of short repetitive monitoring enabled the natural herd grazing behaviour of the semi-feral groups to be maintained, whilst limiting the effects of observer on the group. However, this meant that the individuals were always moving and interacting with the herd, preventing the continuation of many observation periods and reducing the data set size. To reduce error in the experimentation period it would enhance accuracy if each subject during data collection periods were recorded using digital imaging recording facilities and analysed at a later data using an electronic counting facility.

Improvements made to the recording technology, would also enable the herd interaction and dominance to be considered during the data analysis and the effect on stress of individuals to be taken into consideration when drawing conclusions from the results. Periods of data collection within the semi-feral herds were also affected by "natural" stressor i.e. bird movements in the bushes and other spontaneous "startle" factors, these produced a period of non-blinking and cessation of movement as reported by Mott *et al.* (2020) following research carried out using a controlled startle response.

Research into the DA levels in the mesolimbic dopaminergic pathway can be used as an indicator of neuronal activity, and the ability of the individual to cope with stress leading to a modulation in behavioural responses. Using an immunohistochemistry procedure to identify the DA producing cell lines in the brain, they can be sub-divided into Groups A8-A16. The group of A9 cells located in the substantia nigra pars compacta projecting into the dorsal striatum, form the nigrostriatal pathway. This pathway which is primarily involved in the control of motor functions also controls goal directed behaviours, reward related behaviour and cognitive process (Baik 2020). Using this information it would be interesting to extend the research of the two Eriskay groups in particular to investigate potential differences in the activity and stress stimuli, looking at acute or short-term stressors and behavioural responses. This would of course involve the sacrifice of living animals for research purposes, which would require careful consideration from an ethical standpoint. However, this would provide the opportunity to compare SBR with the post-mortem indicators eluded to above. Alternatively, horses (of any breed) already designated for slaughter could be utilised to yield similar validatory information.

The significant increase in full blink rates seen in the semi-feral native ponies maybe due to the requirement of the individuals to be aware of potential threats from within the herd and within their environment. These results would relate back to the observations of blink rates made by Hall (1945) where the fixation of the head on a stimulus resulted in a zero blink rate in 90% of subjects enabling the individual to gain total visual information. The reduction in blink rate followed by the increase in both full and partial correspond to the results

obtained by Holmes (1938) and Blount (1927) that herbivores have a higher blink rate due to the involuntary survival mechanism of fight or flight. This may well correspond to the results obtained in this research where the semi-feral horses are reliant on flight from danger compared to the "danger" stimulants that domestic individuals were exposed to.

The increased blink rate of females within the semi-feral herds may be attributed in part to the fact that the females had foals at foot and were therefore in protection mode for their offspring during potential danger and flight situations. This may well account for the significant results obtained between genders. Study periods during the Stallion turn out in the New Forest would give a data set for the effects of stallion presence on mares and could be compared to periods when the stallions are not present in the forest. It would also give an opportunity to monitor additional stallions in semi-feral conditions. Data sets for the stallions at the New Forest and the Holy Isle could also be compared to establish if there are any differences for habitat type.

From the significant results obtained the procedure of Spontaneous Blink Rate recording could be used for welfare checks during the annual drifting process for administration and identification of the semi-feral herds of the New Forest, Dartmoor, Exmoor and the Welsh Mountains. During the group penning of the drift procedures welfare checks could be made by SBR as an indicator of stress levels. Eye flutters were recorded on the New Forest semi-feral herds during the group penning and branding during the autumn drifts, this being an indicator of high levels of dopamine levels.

The results obtained in this research for partial blinks recorded needs to be further investigate and verified using additional conventional methods, such as cortisol levels (Peeters *et al* 2011) or heart rate (Mohr *et al* 2000) for comparison of the results to verify that partial blinks can be used for dopamine tone and stress level indication. This future work would be necessary to clarify the results obtained from this research before data could be used as a welfare prediction monitoring system.

By continuation of the development of a base-line data of SBR for British native breeds and other domestic equids the establishment of a "normal" blink rate will validate the results obtained from research looking into Equine emotional state (Hall *et al* 2018), stress levels (Mott *et al* 2020) and the development of stereotypic behaviour patterns (Kirtsy *et al* 2016). The establishment of the "Happy Equine Athlete" by the Federation Equestre Internationale (FEI) to help protect the equine athletes during competitions and training through negative reinforcement could potentially utilise the SBR monitoring for welfare situations during competitions and to help establish welfare surveillance without direct intervention on the horse during competitions (Waran & Randle 2017).

The importance of inter blink time would also enhance this blink rate research to compare the interblink times of equids with other species. The differences between predators and herbivores were noticeable. Inter blink rates for elephants were relatively low at 4.1 seconds between blinks in comparison with dogs at 25-30 seconds and Lions at a period of 67 seconds. This confirms the research made from observation that predators have a lower blink rate compared to herbivore prey (Blount 1927). Additional data recording of interblink

intervals for the semi-feral and domestic groups would give additional valuable data for behavioural indicators of welfare and stress levels due to environmental stressors.

The opportunity to continue investigation into a comparison of brain and cranial size for both herds of Eriskay ponies would provide a valuable source of data for the 'domestication Syndrome' research proposed by Clutton-Brock (1999), Wright *et al* (2020). The two herds in this research would provide a good data set as the Eriskay population do not have any human intervention or management. Similar herds found on the Isle of Eriskay in Scotland could provide an additional data set for this comparison. It would be important to take into the account of the error factor for the measurement of body mass as the semi-feral individuals have no additional food supply over winter and are generally lean in comparison to those domesticated individuals (Clutton-Brock 1999, Healy 2021). Validation of the literature review carried out by Balcarcel *et al* (2021) which reported a reduction by -15.8% brain mass change with domestication and an endocranial volume change in domestic subjects of -13.9% could be compared to the results obtained from such a study. Comparisons of dissected brain structure between the two groups would provide evidence of effect of domestication on these two groups if feasible.

Further potential for research to continue with the Eriskay herd on the Holy Isle would enable the study of grazing habits, consumption of plant species throughout the season and the effects of this on gut health and microbiome. The changes seen in the microbiota within the gastro-intestinal tract (GIT) is in constant interplay with the internal cells, leading to effects on the host immune system, inflammation and general metabolism and energy. Due to the size of the horse's gastro intestinal tract and the microbial fermentation of the cellulose in the caecum and colon the various microbial populations are prone to fluctuations and are effected by both internal and external stressors, which may lead to digestive diseases which are associated to systemic diseases like laminitis, obesity and Equine metabolic syndrome. (Chaucheyras-Durand et al. 2022). The monitoring of both gut flora and a wide plant species consumption may have a benefical effect on the gut brain axis, leading to changes in both health and welfare through nutritional links with the GIT not seen in the domestic population kept on a much narrower concentrate diet.

9.0 FINAL CONCLUSIONS

From the significant results obtained the procedure of Spontaneous Blink Rate recording could be used for welfare checks during the annual drifting process for administration and identification of the semi-feral herds of the New Forest, Dartmoor, Exmoor and the Welsh Mountains. During the group penning of the drift procedures welfare checks could be made by SBR as an indicator of stress levels. Eye flutters were recorded on the New Forest semi-feral herds during the group penning and branding during the autumn drifts, this being an indicator of high levels of dopamine levels, indicating a stressful procedure for the individual and herd.

Blink rate data for individuals could be ascertained and recorded to establish a base level under normal circumstances. The individual could then be monitored on a regular basis to highlight changes to the blink rate, over time this could have the potential to indicate an eye issue or change to the health of the eye. An increase to the base rate may indicate “a dry eye” condition (Kaminar *et al* 2011) or the change in anxiety levels during a change in management procedures as recorded by Roberts *et al* (2016) and Dakin (2020). Changes in SBR could also indicate a general health issue with internal stressors increasing dopamine tone and therefore the process of SBR could be included by Agisters as a welfare check during their monitoring procedures.

The results obtained in this research for partial blinks recorded need to be further investigate and verified using additional conventional methods, cortisol levels (Peters *et al* 2011) or heart rate (Mohr *et al* 2000) for comparison of the results, to verify that partial blinks can be used for dopamine tone and stress level indication. This future work would be necessary to be able to clarify the partial blink rate results obtained from this research before data could be used as a welfare prediction monitoring system.

By continuation of the development of a base- line recordings of SBR for British native breeds and other domestic horses the establishment of a “normal” blink rate will validate the results obtained from research looking into Equine emotional state (Hall *et al* 2018), stress levels (Mott *et al* 2020) and the development of stereotypic behaviour patterns (Kirtsy *et al* 2016). The establishment of the “Happy Equine Athlete” by the Federation Equestre Internationale (FEI) to help protect the equine athletes during competitions and training through negative reinforcement could potentially utilise the SBR monitoring for welfare situations during competitions and to help establish welfare surveillance without direct intervention on the horse during competitions (Mcgreevy e*t al* 2011).

The opportunity to continue investigation into a comparison of brain and cranial size for both herds of Eriskay ponies would provide a valuable source of data for the ‘domestication Syndrome’ research proposed by Clutton-Brock (1999), Wright *et al* (2020). The two herds in this research would provide a good data set as the Eriskay population do not have any human intervention or management. Similar herds found on the Isle of Eriskay in Scotland could

provide an additional data set for this comparison. It would be important to take into the account of the error factor for the measurement of body mass as the semi-feral individuals have no additional food supply over winter and are generally lean in comparison to those domesticated individuals (Clutton-Brock 1999, Healy 2021). Validation of the literature review carried out by Balcarcel *et al* (2021) which reported a reduction by -15.8% brain mass change with domestication and an endocranial volume change in domestic subjects of -13.9% could be compared to the results obtained from such a study. Comparisons of dissected brain structure between the two groups would provide evidence of effect of domestication on these two groups if possible. It would indicate any differences to the dopamine receptor site between the domestic and semi-feral groups. Comparisons of the neural structures in the substantia nigra (SN) and ventral tegmental Area (VTA) in the midbrain, would give an indication of the significance of the dopamine tone of the individuals in the two groups and the links between dopamine levels and the flight or fight reactions for survival of the semi-feral nature of the individuals.

Further research into the changes in dopamine neurotransmission between the two study groups, would also give an indication into any possible behavioural responses that are linked to startle responses between the semi-feral and domestic groups within a particular breed, that maybe associated with reward anticipation as described by Baik (2020) and reward directed behaviour. With the application of an artificial startle stimuli with varying intensity and duration the release of altered dopamine levels could be monitored through blink rate recordings and used to imply the welfare and emotional state of the individuals under examination.

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11. APPENDIX

APPENDICES 1: GEOGRAPHICAL LOCATION OF STUDY POPULATIONS WITHIN THE BRITISH ISLES.

Table A1.

Location	Breed	Latitude	Longitude	Altitude (meters above sea level)
New Forest				
Janesmoor Plain	New Forest	50°55'19"N	1°39'20"W	119
Fritiam	New Forest	50°55'34"N	50°55'34"N	113
Lyndhurst Boltons Bench	New Forest	50°52'21"N	1°34'26"W	40
Sulfters Inclosure	New Forest	50°53'24"N	1°40'43"W	91
Parkhill Lawn Ponds farm	New Forest	50°51'41"N	1°32'58"W	25
Somerset				
Mendip Hill	Exmoor	51°16'57"N	2°40'48"W	255
Gloucestershire				
Barton End	Exmoor			
Daneway Cirencester	Section A Welsh	51°43'46"N	2°5'25"W	114
Lea	Section A	51°89'36"N	-2°49'30"W	100.5
Scotland				
Holy Isle	Eriskay	55° 31' 17.99" N	-5° 04' 12.00" W	314 highest point
Comrie	Eriskay			
Gifford	Eriskay			
North Berwick The Law	Exmoor	56° 2' 54.79" N	2° 42' 54.03" W	138
The Shetland Isle	Shetlands	60°17'36"N	1°14'59"W	51

Domestic Populations **Semi-Feral Populations**

11.2. BRITISH NATIVE PONIES IN THIS RESEARCH.

11.2.1 EXMOORS

The presence of free roaming ponies on Exmoor was first recorded in 1086 by tax inspectors of King William the I. (Morris et al. 1985). Farmers were reported to own pack horses and

unbroken wild ponies that lived and survived on the moors. Young stock were annually rounded up and sold to domestic service.

Current mitochondrial DNA research has indicated that the Exmoor pony is a direct descendant of a wild horse that were located in North European mainland in 7,000BC and a population of Nordic wild horses lived and survived in Britain (Sommer et al 2011). Mitochondrial DNA studied from seven Exmoor ponies showed that there was little DNA variation showing clusters with low variability, where there may have been a direct maternal link from the North European wild pony and the Exmoor pony. This has been confirmed following mtDNA work carried out by Cieslak et al (2010) where 11 pre-domestic haplotypes have been found in 17 Exmoor pony samples suggesting a maternal link to the North European pony. This has been shown to be true of studies of mtDNA of the Late Pleistocene Alaskan horse (Vila *et al* 2001, Jansen *et al* 2002).

Following DNA analysis made by Cieslak *et al* (2010) results showed that some mtDNA haplotypes were widely dispersed over Asia, Europe and Alaska before the horse was known to be domesticated, but other haplotypes were restricted to certain geographical regions and were present in the remains of wild horse populations. The presence of some of these unique haplotypes are in the Exmoor pony. There have been little influence on the Exmoor pony from ancient domesticated breeds.

Other biological evidence would also suggest that the Exmoor pony is a direct descendant of the North West European wild ponies that lived during the Late Pleistocene period >10,000 years. The anatomical similarities of the jaw and teeth morphology analysed by Speed J.G (1951), Speed J.G and Etherington M. G (1952) show characteristic deep jaw and teeth set in a radial pattern. (Hans J.P.M Hovens and Toon A J M Riijkers 2013).

DNA studies investigating coat colour of ancient wild breeds show that there was little variation, with the majority being Bay/ Dun, with coat colour variations being seen in Siberia and Eastern Europe around 3000BC coinciding with the domestication of horses. Unlike other British Native Breeds, the Exmoor ponies are uniformly bay with dun points, other breeds showing a variety of chestnut, white and bi and tri colours. Przwalshi's horses are the only other wild horse breed that shows the uniformity in colour(Pruvost *et al*. 2011).

Due to the limitation of trade routes across Exmoor and their geographical location, with no large sea ports nearby, the influence of external blood stock did not occur. This has meant that the blood lines of the Exmoor pony have remained very pure to the original stock. Before 1818 Exmoor was designated a Royal Forest and was a hunting ground, under the control of a Crown Warden. The Warden was responsible for the running of Stallions within the forest and these were true to type. In 1818 the Royal Forest was sold to Sir Thomas Acland, who took thirty of the Exmoor Ponies which had been running on the forest to his own estate following the dispersal sale and began his own breeding herd. Acland unlike others who had brought ponies from the sale kept his herd true to type in the knowledge that these ponies could survive on Exmoor.

Those herds that were crossed with blood from other breeds could not survive on Exmoor and soon died out.

Descendants of Acland herd survives to date and are known as the Anchor herd which can be found on Winsford Hill. In 1921 concern was raised for the survival of the Exmoor herds without introgression from outside blood stock, and the Exmoor stud book was started. All Exmoor ponies were inspected and all those true to type were registered, this continues today to ensure that the Exmoor pony continues to roam free and to exhibit the traits and display the natural instincts of it ancestors (Baker 2008).

Figure A1: Exmoor semi-feral population located on Blackdown Mendips.

11.2.2 SHETLANDS

Originated in the Shetland Isle North Scotland, the ponies are adapted to harsh weather and scant grazing (Hendry 1897). The origins of the Shetland ponies are unknown, but similarities exist with those from cob type ponies from the Tundra and the ponies that migrated from the southern Europe across the land bridges in existence during the ice age, and cross breeding with Celtic mountain ponies from the orient. Excavations have proved that they have inhabited the isle since the Bronze Age and are likely to have been domesticated since that time. Due to the isolation of the ponies on the islands the breed has remained true to origins, any cross breeding, by necessity, would have been made with other small individuals, which has led to the two type variations of the breed.

The first, a lighter type with higher tail carriage, smaller head and slender limbs. The second type is the heavier boned animal with longer larger head. The breed is known for it's ability to survive harsh winters with little feed, it's strength and hardiness and longevity. The Shetland ponies were traditionally used for cultivation, haulage of peat, seaweed and were ridden. They also used as draught ponies following the development of roads, after the mid nineteenth century.

In 1847 the law changed and children were banned from being sent to work down the coal mines, with the small stature of the Shetland it was the perfect occupation for these strong small hard working ponies. During this period several breeding studs were initiated for the proliferation of pony numbers for working in the coal mines one of these was the Marquis of Londonderry's Stud, these ponies were bred for their docile nature, bone and strength. During this period the Shetland Pony Stud book was formed in 1890, the first in Britain (Shetland Pony Breed Society).

Figure A2: Shetland Semi-feral ponies located on the Shetland Isles.

Results obtained from extensive research carried out by Orlando & Librado (2019) have shown that Shetland ponies studied have a high load value for deleterious mutational loads than would have been expected if natural selection had taken place. This would indicate that historically a breeding selection process would have occurred increasing the occurrence of deleterious mutations compared to samples analysed from fossilised remains. This confirms the stud book's history of the stallions used at stud.

11.2.3 ERISKAYS

The name of Eriskay pony will only be found post 1968, these native ponies were found on the Western Isles of Scotland and varied slightly between islands. The original Eriskay pony was used for all farming requirements, surviving on limited vegetation native to the islands. As farming requirements advanced during the 19th Century, the small Scottish breed was genetically altered to be more powerful, by the introduction of registered Highland stallions. Consequentially, these larger more powerful animals were an improvement for the work

required but were unsuitable for survival on the islands due to forage restrictions and weather conditions. Subsequently, there remained only a small herd of smaller original stock left on the isolated island of Eriskay south of Uist in Scotland. All Eriskay ponies are derived from this small founder's population in the 1960 resulting in a high coefficient of inbreeding, these founders have similarities to the Western Isle type of Highland pony. The Highland breeds on the mainland is an older breed, but has introgression from Spanish, Arab and Clydesdale. The Eriskay ponies have similar genetic ancestry to other pony breeds, with the Highland pony having genetic resemblance to the Clydesdale and no other pony breeds. Due to the small number of 'founders' within the breed and low level of genetic variability and allelic variation the RBST as a high priority on this distinctive separate breed. Due to the decline in numbers, a local Society Comann Each nan Eilean (CEnE) was set up in 1972 to preserve the breed and the Eriskay Pony Society in 1986.

To enable the survival of the breed, due to a lack of pure bred Eriskay stallion, six Highland pony stallions were chosen to increase the breed number. These records have been meticulously kept and the genetic lines can be traced through the breed. However, it was later discovered that there was a single pure breed stallion, Eric, who went on to have three colts, which were used to re-establish a pure line. Breeding records were not kept prior to the three colts being established as sires, therefore the Eriskay Society now have OES records stating that the offspring come from Original Eriskay Stock. Recent DNA studies have shown that there are genetic similarities with ponies from Faroes, Iceland, Connemara and Newfoundland (Word of mouth from Eriskay Society). The Holy Isle Eriskay ponies are the last surviving remnants of the original native ponies from the Hebridean Isle of Eriskay (south of South Uist), and have ancient Celtic and Norse origins.

Usually, the ponies spend their time on the plateaus of the hills or on the fields at the south end of the Holy Isle, but they regularly graze the fields surrounding the Centre. The situation of the ponies on Holy Isle seems to be unique in the world. They are living a wild existence, finding their own balance in the herd and between herds. The island habitat gives a rare opportunity to see them living naturally without human intervention as herds established through time.
Over the years, Lama Yeshe has observed how after a period of unrest and fighting, the ponies seem now to have settled into three groups: one with the main stallion surrounded by a group of females and young ones; another with a female pony as "boss" among older horses; and a small group of young males. In the last years, there has hardly been any fighting.

Figure A4: Eriskay Semi-feral ponies located on the Holy Isle.

11.2.4 WELSH SECTION A

The Welsh Mountain pony Section A is the smallest of the Welsh ponies, with a maximum height of 1.2m. All other Welsh ponies are thought to have been developed from the small Section A pony by breeding with larger ponies. They have a distinctive movement with forward and elevated front leg movement, used mainly as children's' riding ponies or harness ponies. They can be any colour not including Skewbald or piebald. The Welsh Pony and Cob Society, founded in 1901, believe the ponies to be descendants from the Celtic ponies, which have existed for over a thousand years in the Welsh mountains. The first stud book in 1902 contained the records of 38 stallions and 571 mares. The stallion Dyoll Starlight is the founding sire of the modern breed, introducing a combination of the Welsh and Arab breeding, giving rise to the characteristic dish face of section A.

Figure A5 Semi-Feral Section A Welsh ponies located at Daneway Gloucestershire.

11.2.5 NEW FOREST

The New Forest breed is indigenous to the New Forest in Hampshire in southern England, where horses have lived since before the last Ice Age, remains dating back to 500,000 BC have been found within 50 miles (80 km) of the heart of the modern New Forest. The introduction of selective blood lines after the WWI have altered the original lines of the breed. These changes have currently been restricted and only pure bred stallions are currently released during covering periods (New Forest Breed Society).

Figure A6 Semi-Feral New Forest Ponies located in Lyndhurst Hampshire

11.3 WEATHER DATA FOR SAMPLE PERIODS SEMI FERAL

Weather conditions were recorded during each sample period as the conditions may have had an effect on the blink rate during the observation period. Weather data was accessed from the meteorological web site and complied into a table for recording purposes, the nearest weather station for each location was used for the data collection.

11.3.1 NEW FOREST: SEMI-FERAL NEW FOREST (ASHURST BRIDGE)

Date	Location	Temperature ^{0}C	Dew Point ^{0} C	Wind Speed kn	Rainfall mm/h	Rain Amount mm	Pressure (MSLP) hPa	Wind Direction	Humidity %
11.04.19 (13.00)	Ashurst Bridge	11.1	4.5	1.7	0	0	1023.7	SSW	64.0

19.06.19 (16.00)	Ashurst Bridge	18.7	14.8	0.9	0	0.3	1007.1	WNW	78.0
20.06.19 (12.00)	Ashurst Bridge	17.7	11.7	1.7	0	0.3	1013.0	WSW	68.0
21.8.19 (16.00)	Ashurst Bridge	20.8	11.2	2.6	0	0	1024.5	S	54.0
22.08.19 (11.50)	Ashurst Bridge	21.8	14.7	2.6	0	0	1024.3	S	64.0
09.10.19 (12.55)	Ashurst Bridge	14.4	9.0	4.3	0	0	1005.2	WSW	70.0
12.03.20 (12.55)	Ashurst Bridge	10.2	2.3	11.3	0	1.3	1011.0	SW	58.0

11.3.2 SCOTLAND: SEMI-FERAL HOLY ISLE ERISKAYS (TARBERT AND KILWINNING)

Date	Location	Temperature ^{0}C	Dew Point ^{0} C	Wind Speed kn	Rainfall mm/h	Rain Amount mm	Pressure (MSLP) hPa	Wind Direction	Humidity %
05.07.19 (13.00)	Tarbert	15.0	13.9	6.1	0	0	1014.5	N	93.0
05.07.19 (09.45)	Kilwinning	12.9	8.0	0	0	0	1025.0	N	66.0
18.09.19 (12.45)	Tarbert	14.7	8.4	6.1	0	0	1027.0	WSW	66.0

11.3.3 SCOTLAND SHETLAND ISLANDS SEMI-FERAL SHETLANDS (GULBERWICK WEATHER STATION)

Date	Location	Temperature ^{0}C	Dew Point ^{0} C	Wind Speed kn	Rainfall mm/h	Rain Amount mm	Pressure (MSLP) hPa	Wind Direction	Humidity %
08.07.19 (12.00)	Gulberwick	13.2	10.2	7.0	0	0	1020.0	NW	82.0
09.07.19 (13.50)	Gulberwick	12.6	9.9	7.8	0	0	1019.2	ESE	84.0

11.3.5 MENDIP HILLS SOMERSET, SEMI-FERAL EXMOORS: (BISHOP SUTTON)

Date	Location	Temperature ^{0}C	Dew Point ^{0} C	Wind Speed kn	Rainfall mm/h	Rain Amount mm	Pressure (MSLP) hPa	Wind Direction	Humidity %
29.08.19 (13.45)	Bishop Sutton	21.5	13.9	6.6	0	0	1019.3	ENE	66.0

11.3.4.6 GLOUCESTERSHIRE DANEWAY : SEMI-FERAL SECTION A (CIRENCESTER)

Date	Location	Temperature ^{0}C	Dew Point ^{0} C	Wind Speed kn	Rainfall mm/h	Rain Amount mm	Pressure (MSLP) hPa	Wind Direction	Humidity %
16.03.22 (15.32)	Cirencester	7.6	7.3	0.9	0	0	1014.5	WSW	98.0

11.3.7 GLOUCESTERSHIRE FOREST OF DEAN, HARWICKE: SEMI-FERAL SECTION A

Date	Location	Temperature ^{0}C	Dew Point ^{0} C	Wind Speed kn	Rainfall mm/h	Rain Amount mm	Pressure (MSLP) hPa	Wind Direction	Humidity %
26.02.22 (10.32)	Cirencester	16.0	7.3	1.9	0	0	1013.5	S	76.0

11.3.8 NEW FOREST, FRITHAM: SEMI-FERAL SHETLANDS (ASHURST BRIDGE)

Date	Location	Temperature ^{0}C	Dew Point ^{0} C	Wind Speed kn	Rainfall mm/h	Rain Amount mm	Pressure (MSLP) hPa	Wind Direction	Humidity %
12.03.20 (14.25)	Ashurst Bridge (16.23)	10.2	2.3	11.3	0	0	1011.0	SW	58.0

11.4. DOMESTIC COLLECTION DATA:

11.4.1 SCOTLAND: DOMESTIC ERISKAY COMRIE AND GIFFORD (PENCAITLAND & COMRIE)

Date	Location	Temperature ^{0}C	Dew Point ^{0} C	Wind Speed kn	Rainfall mm/h	Rain Amount mm	Pressure (MSLP) hPa	Wind Direction	Humidity %
19.04.22 (12.52)	Pencaitland	19.1	3.7	0	0	0	1018.0	SSE	36.0
20.04.22	Ruby's Nest Comrie	12.5	6.3	1.9	0	0	1034.6	ENE	66.0

11.4.2 GLOUCESTERSHIRE BARTON END: DOMESTIC EXMOOR (COTSWOLD WEATHER STATION)

Date	Location	Temperature ^{0}C	Dew Point ^{0} C	Wind Speed kn	Rainfall mm/h	Rain Amount mm	Pressure (MSLP) hPa	Wind Direction	Humidity %
04.08.22 (13.55)	Barton End	21.0	8.6	6.1	0	0	1017.2	NW	45.0

11.4.3 GLOUCESTERSHIRE, CIRENCESTER: DOMESTIC SECTION A (COTSWOLD WEATHER STATION)

Date	Location	Temperature ^{0}C	Dew Point ^{0} C	Wind Speed kn	Rainfall mm/h	Rain Amount mm	Pressure (MSLP) hPa	Wind Direction	Humidity %
05.08.20 (10.58)	Coates	18.6	15.8	7.0	0	0	1008.9	SE	84.0
12.10.20 (10.55)	Coates	10.4	8.7	1.7	0	0	1017.1	SE	89.0
03.06.21 (09.55)	Coates	16.6	13.3	0.9	0	0	1015.7	SE	81.0

11.4.4 NEW FOREST, FRITHAM: DOMESIC NEW FOREST (ASHURST BRIDGE)

Date	Location	Temperature ^{0}C	Dew Point ^{0} C	Wind Speed kn	Rainfall mm/h	Rain Amount mm	Pressure (MSLP) hPa	Wind Direction	Humidity %
12.03.20 (12.10)	Ashurst Bridge (16.23)	10.2	2.3	11.3	0	0	1011.0	SW	58.0

APPENDIX 2 RAW DATA FOR SBR

Raw data for trial. Spontaneous eye blink rate data