THE INFLUENCE OF FLAVOUR ON VOLUNTARY WATER INTAKE IN HORSES

Miranda Filmer

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Abstract

The equine athlete is subject to increasing demands on its physiology; more efficient transport means we are able to travel horses for further and longer, as well as competing them in more challenging conditions. As with humans, the industry surrounding maintenance and recovery of athletes is ever growing, with more and more products being developed in order to gain a competitive edge. Prolonged transport can result in dramatic losses in bodyweight and therefore dehydration, caused by excessive sweating and increased faecal output; this can have a dramatic effect on performance, as electrolyte derangements cause a range of issues such as muscle degeneration and impaction colic. In this study, two synthetic variations of flavours, banana and cherry, were selected to be added to water to determine if they encouraged a greater voluntary water intake compared with plain water and might therefore be a mechanism for mitigation of dehydration in horses. Two trials were carried out using Latin square and Randomised Block designs. Trials 1a and 1b used 6 horses and three different concentrations of each flavour with the aim to ascertain the preferred concentration for both flavours. Trial 2 consisted of 12 horses and tested the palatability of the flavours when offered alongside plain water. Each trial consisted of 3 repetitions in order to eliminate bucket location bias. All horses were without water for 1 hour before each trial period and underwent a standardised exercise test before being offered the waters. Trials 1a and 1b showed no preferred concentration for either flavour. There was a tendency, albeit weak for a preference for the medium concentration of banana and weak concentration of cherry thus those concentrations were chosen for Trial 2. The results from Trial 2 showed that plain water was preferred when offered alongside the 2 flavoured waters, average voluntary fluid intake for plain water was 5.33 litres, which was more than double that of the flavoured waters with average intake for banana of 2.07 litres and cherry of 2.19 litres. In summary, adding banana and cherry flavour to water did not encourage an increase in voluntary fluid intake, and as such cannot be used as a method for mitigating dehydration in horses. Further research is required to ascertain if other flavours, are preferred in water, as this could be added to electrolyte solution in order to make them more palatable and so encourage drinking.

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Authors 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 presented to any other education institution in the United Kingdom or overseas.

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

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Abbreviations

ADH - Antidiuretic hormone

ATP - Adenosine triphosphate

ECF - Extracellular fluid

ESGD – Equine squamous gastric disease

GFR - Glomerular filtration rate

ICF - Intracellular fluid

NRC – National Research Council

ViF – Voluntary fluid intake

Chapter 1: Introduction

‘You can lead a horse to water, but you cannot make it drink’
Old English Proverb

1.1 Rehydration of the equine athlete

Water makes up 65% of the total equid body mass (Geor, 2000), and is essential for maintaining functions such as thermoregulation, digestion, and electrolyte distribution. The increasing demands placed on modern-day performance horses during both competition and travel means that they are particularly susceptible to dehydration (Marlin *et al*., 1996). Even mild dehydration can have a major impact on performance, so it is vital that every effort is made to maintain sufficient water intake during times of stress and exertion (Hyyppä *et al*., 1996; Nyman *et al*., 1996). Human athletes are now keenly aware of the debilitating effects of dehydration and horse owners are now equally aware that to achieve the best performance from their horses they need to ensure they are fully hydrated going into competition. However, a key difference is that humans can be educated and made aware of the importance of maintaining a hydrated state, and thus understand the importance of drinking before thirst develops. The same cannot be done with horses who simply respond to normal physiological responses that trigger drinking. Furthermore, horses are more susceptible to dehydration as the hypertonic nature of their sweat, increases their capacity for dehydration compared to humans (Geor and McCutcheon, 1998). It does not take extreme exercise to dehydrate a horse, factors such as prolonged travelling or hot weather can have a major impact on hydration status; Van den Berg *et al.* (1998) found that the sodium output of horses that had been transported for 8 hours was 71mmol higher than the control group, and Stull and Rodiek (2000)

reported body weight losses of 6% after a transport period of 24 hours and partially attribute this to sweat losses. As humans we can foresee situations that could cause dehydration and drink accordingly, whereas the horse relies on the biological stimuli of thirst to make it re-hydrate. The thirst response in horses is primarily triggered by changes in plasma osmolality and sodium concentrations (Schott and Hinchcliff, 1998; Sufit *et al.* 1985), and utilisation of this response can be used to encourage voluntary water intake. The decision to swallow a potential food source is based on olfactory and gustatory cues (Scott, 2001; Van den Berg et al., 2016a), which combine to determine the palatability of the food source, so a positive stimulus results in the consumption of a substance. Previous studies have found that horses have a preference for a sweet taste, with several flavours; namely cherry, banana, and fenugreek, being the most popular when added to feed (Danel and Merkies, 2009; Randall *et al.,* 1978). The current study drew on this knowledge in order to determine how the addition of some of the above-mentioned flavours might influence voluntary fluid intake (hereafter ViF). There have been limited studies conducted into the taste preferences of horses, and these studies took place over fifteen years ago Kennedy *et al.* (2001) and Goodwin *et al.* (2005a). The majority of palatability studies have been done using feed, and only a handful, such as Mars *et al.* (1992) and Randall *et al.* (1978) exist that asses flavour preference using water. The current consensus (Butudom *et al.,* 2002; Nyman *et al.,* 1996; Schott and Hinchcliff, 1998) is that adding electrolytes to water is the most effective way of voluntary rehydrating horses as it stimulates the physiological response to drink. Whether an appealing taste which stimulates further consumption of the substance may also act as a stimulant to ViF was the main objective of the current study.

1.2 Study

There is a distinct lack of information regarding the flavour preferences of the horse when it comes to fluid intake; preference testing in feed trials is predominantly done using the Latin square design, and this trial followed that procedure. The flavours investigated in the present study; banana and cherry, were selected based on the previous palatability trials of Kennedy *et al.* (2001) and Goodwin *et al.* (2005). The flavours for this study were synthetically produced, as they were more practical to work with and were much more concentrated, so only a small amount was needed to achieve the desired concentration of flavour.

Whilst there is a general consensus about the flavour of banana and cherry enhancing the palatability of feed, no study has investigated what flavours are preferred when added to water, and if these are more palatable than water alone. The overall aim of this study was to determine whether flavouring water would encourage horses to drink more than when offered plain water. Two trials were conducted, each with their own objectives.

The objectives were to

1. Determine the preferred concentration of cherry and banana flavours when mixed in water and offered to horses.
    - Hypothesis – There will be no difference in the intake of fluid for either banana or cherry flavoured water according to the concentration of the mixture.
2. To determine horses' preferred flavour when offered water, cherry or banana flavoured solution.
    - Hypothesis – There is no difference in the intake of fluid when horses are offered plain water, cherry flavoured water or banana flavoured water

Chapter 2: Literature Review

2.1 Fluid homeostasis in the Horse

Similar to humans, water makes up 65% of the horse's total body mass, (Geor, 2000). Water is a component of all cells and is essential for the movement of dissolved substances, including minerals such as sodium, potassium and calcium, and hormones such as aldosterone, in and out of cells via osmosis through the phospholipid bilayer (Johnson, 1998). Body fluid can be divided into a number of different categories; extracellular fluid (hereafter ECF), which includes plasma, lymph, interstitial and transcellular fluid; and intracellular fluid (hereafter ICF). All of these have different roles within the body.

2.1.1 Physiology of body fluid

Alterations in the makeup of the body fluid can significantly increase the risk of medical problems such as Azoturia, colic, and an inability to thermoregulate. As Johnson (1998) informs us, the composition of the fluid must be strictly regulated. Changes in tonicity of these body fluids have a great impact upon intracellular fluid (ICF) and any significant changes could impair cellular function (Carlson, 1987). A primary function of body fluid is to regulate a horse's temperature, when its temperature begins to rise, the horse must move heat from its core to the periphery and this is partially done by the evaporation of sweat. This process is facilitated by an increase in arterial pressure, which causes water and electrolytes to be expelled from the vascular system and into the interstitial space, allowing it to be taken up by the muscles to create sweat (McKeever, 1998). Within the sweat gland, two processes are responsible for sweat production. A small amount of sweat is produced by the necrosis of a small number of secretory cells, the contents of which are part of what is expelled as sweat. Secondly, there is the secretory process itself,

which involves the transport of water and ions and is facilitated by a thermal stimulus to the sweat gland; when this occurs, the intercellular spaces between the cells in both the duct and the fundus of the gland dilate, allowing transport of fluid and electrolytes to occur (McCutcheon and Geor, 1998). If necessary, this fluid can be returned to the vascular system using the lymphatic system. Fluid can also be lost passively via skin diffusion and the lungs (National Research Council hereafter NRC, 2015); though fluid loss from respiration is difficult to quantify, losses via vaporization can be between 0.8-2.1l per day (Hodgson *et al.,* 1993), though this can increase in warm or humid conditions. Furthermore, intercompartmental fluid shifts are vital to maintaining muscle function during exercise. McKeever (1998) explains that during exercise, an influx of protein and water into the vascular compartment causes an increase in plasma volume, which plays a key role in maintaining cardiac filling pressure during increased vasodilation of the working muscles.

Similarly, fluid shifts containing electrolytes are also critical to maintaining healthy cell function. Electrolytes are electrically charged ions, and are comprised of minerals such as sodium, potassium and chloride; these have different roles when dissolved in body fluid. Sodium and chloride are primarily found in the ECF and blood plasma. Chloride is involved in changes in plasma tonicity and maintaining the acid base balance of body fluid (McCutcheon, 2001), it also is a key component in bile and therefore crucial for digestion. Sodium and Potassium play key regulatory role in the energy of cellular systems, specifically the transport of ATP (Ellis and Hill, 2005). Potassium is predominantly found in the ICF and is essential for facilitating biochemical reactions between cells (McCutcheon, 2001). The ratio between the ICF to ECF is a determinant of action potentials in excitable tissues (Johnson, 1998). During exercise potassium is released from the muscles into the blood plasma and

this subsequent hyperkalemia of the plasma triggers an increase in blood flow to the muscle from which it has been released, allowing the muscles to continue functioning; without potassium, the horse would experience muscle fatigue and longer term weight loss would occur (McCutcheon, 2001), similar to the effects of a sodium deficiency.

Other vital macro minerals are magnesium and calcium. Magnesium is a crucial ion in the blood; involved in over 300 enzyme reactions, such as generation of cellular energy and the decoding of genetic information (Crandell, 2011). When absorbed by the muscle, magnesium works as a muscle relaxant. A deficiency in magnesium caused from a lack of delivery to the muscles from the blood can cause spasm. Calcium has an antagonistic effect to magnesium, being key to muscle contraction; an increased concentration of calcium in the cell allow the actin-myosin filament interaction, producing a contraction (Foreman, 1996).

2.1.2 Gastrointestinal fluid and the Renal system

Fluid is also used in the renal system to balance the concentration of nutrients and minerals within the body and is key to removing any unwanted components such as lactate and ammonia. The gastrointestinal regions provide a substantial fluid and electrolyte reservoir which can be called upon when a horse undergoes prolonged exercise. This is demonstrated by the fact that sodium chloride and potassium contents of ingesta were lower in ponies that had undergone exercise, meaning they had been absorbed during exercise than when at rest (Schott and Hinchcliff, 1998). Fluid is regulated via the kidneys, the glomerular filtration rate (GFR) is key to urine flow, as explained by McKeever (1998), changes in the GFR depending on the horse's hydration status cause changes in renal blood flow and determine how many essential electrolytes are excreted or reabsorbed. Endocrine factors such as the

release of aldosterone can also control kidney function; the release of aldosterone, which is reported to be primarily a result of an increase in plasma potassium, causes an increase in the retention of fluid and electrolytes (McKeever, 1998).

Decreases in the GFR also result in more electrolytes and water being reabsorbed into the blood, if increased then more are excreted. Excess sodium can be excreted with increased urine production as a homeostatic response to hypernatremia (Schott and Hinchcliff, 1998). The GFR can remain unchanged if the horse is hyperhydrated, unless undergoing high intensity exercise; Hinchcliff *et al.* (1990) reported that renal blood flow averages approximately 15mL/kg/min; though McKeever (1998) explains that high intensity exercise can cause a 73% decrease in the GFR. Water and electrolytes are taken back into blood plasma from the kidneys via tubular reabsorption which is ultimately dependent on renal blood flow (McKeever, 1998), if the horse is exerted, the blood flow is directed to the cardiovascular system to facilitate sweating; as a result urine output, and therefore less fluid and electrolytes are lost via excretion.

2.1.3 Role of saliva in bodily function

Another important body fluid is saliva, comprising of approximately 99% water, with the rest being minerals such as calcium and chloride; a horse's daily saliva production can be between 35 and 40 litres a day (Kentucky Equine Research Staff, 2014a). Saliva's role is to aid the passage of food through the oesophagus and into the stomach. When the gastric tissues are well bathed in saliva, there is less chance of ulceration in the a-glandular region of the stomach, referred to as Equine Squamous Gastric Disease (ESGD) (Sykes *et al.,* 2015). In order to prevent ESGD, saliva must be produced constantly, by round the clock access to forage, horses only produce saliva when chewing (Kentucky Equine Research Staff, 2011a). Secretions

of saliva help ease the passage of food into the stomach, where more fluid in the form of gastric juices, secreted from the mucous membrane of the stomach, begin the breakdown of the consumed food (Janssens, 2002); protein is broken down into proteoses and peptones, and triacylglycerols into fatty acids (Davies, 2018). The well-hydrated food then flows into the small intestine where the enzymatic digestion occurs and absorption of proteins, carbohydrates and fat take place. Fluid is also vital for the prevention of colic; a lack of water consumed could lead to impaction colic, where a blockage forms in the gut as there is not enough fluid to assist the flow of chyme along the digestive tract. Fluid bulks-up the digesta which then allows the peristaltic waves that move the food to be more effective. According to Waite (2015), a mature horse must consume at least 10 gallons of water a day to help keep the feed that is consumed well hydrated throughout digestion.

2.2 Causes and impacts of dehydration

The consumption of water by the horse is essential for the homeostasis of fluid balance and the prevention of dehydration, a lack of water is often more rapidly fatal to the horse than a lack of food. Depending on the type of horse, its work load, and weather conditions, a horse can require anything between 18.9 to 75.7 litres of water a day (Mars *et al.,* 1992); a maintenance water intake of an adult non-working horse fed alfalfa hay has been found to be around 5 litres per 100kg of body weight (Groenendyk et al., 1988). Dehydration occurs because of water loss, which can happen via four main routes; faecal, urinary, respiratory and cutaneous losses (NRC, 2015), these losses can be brought on by a variety of different conditions and factors. Horses are capable of losing vast quantities of water, this can be between 10-15 litres per hour during exercise (Carlson, 1987), as the horse's sweat is hypertonic relative to the blood plasma, it can lose around 10g of electrolytes per litre

of sweat (Marlin and Nankervis, 2002). The reason for this high loss is due the horse's high sweating capacity which is attributed to a high mass specific oxygen uptake and poor surface area to bodyweight ratio (Hodgson *et al.,* 1994). Marlin and Nankervis specify that horses have evolved to sweat at three times the rate of humans in order dissipate heat; dehydration would reduce the horse's ability to sweat effectively and would struggle to bring down its temperature as a result, leading to heat exhaustion. Cooling down a horse post exercise is imperative in order to minimise further electrolyte losses via sweat. Even a light degree of dehydration can reduce performance, Marlin and Nankervis (2002) state that a loss of water amounting to just 2% of a horse's body mass would affect performance, so maintaining hydration of the equine athlete is key (Geor, 2000). Usually, fluid losses occur from the ECF, however as dehydration increases, losses will occur from the ICF as well, which can have a detrimental effect on performance (Marlin and Nankervis, 2002).

2.2.1 Indicators of dehydration

Before investigating the causes and consequences of dehydration, it is necessary to identify the markers which alert us to the problem. Dehydration is predominantly characterised by poor skin turgor, dry mucous membranes, minimal and concentrated urine production, and poor gastrointestinal motility which is highlighted by dry and minimal faeces (Foreman, 1996). Clinically, dehydration can be assessed by haematology; the measurement of blood gases and serum electrolytes are also useful (Foreman, 1996). A common way to assess dehydration is the skin tent test, this is done by assessing the skins propensity to return as quickly as possible to its normal contour after being raised in a pinch; if the skin does not return rapidly, then this is a key marker of dehydration (Pritchard *et al.,* 2006). A good indicator for

electrolyte loss and dehydration is thumps, a diaphragmatic flutter. Though the condition is not harmful itself, it indicates a body wide electrolyte imbalance and alkalosis leading to low ionized calcium; the ‘thumps’ manifests as diaphragmatic contractions synchronised with the heartbeat as the phrenic nerve becomes hypersensitised running over the right atrium of the heart; this is predominantly caused by excessive sweat production resulting in losses of minerals such as calcium and magnesium (Al-Qudah and Al-Majali, 2008).

2.2.2 Water and electrolyte losses

The vaporization of water is an effective way of losing heat; it is the active process of sweating, a cutaneous loss that contributes most to dehydration. Sweating is induced by hot or humid weather, travelling, and exercise, and sometimes all of these occur simultaneously. Water losses via sweat can be responsible for 70-92% of total evaporative loss during exercise (Hodgson *et al.,* 1993). Due to the isotonic to hypertonic nature of equine sweat, losses result in a hypoosmotic hypohydration (Geor and McCutcheon, 1998), meaning that the body has lost many of its important electrolytes and nutrients, such as proteins, as well as water. Most important, are the losses of sodium, potassium and chloride; the concentration of sodium in a horse’s sweat is around 150 mmol/Litre which is similar to the sodium plasma concentration. The potassium concentration in sweat is can be 15 times greater than plasma potassium concentration and chloride sweat concentration is twice as high as plasma concentration and is similar to the sum of sodium and potassium concentrations combined (Flaminio and Rush, 1998). This contributes to hypovolemia, a decrease in the volume of the blood plasma, which limits perfusion to the muscles and vital organs caused by inefficient oxygen and substrate utilisation (Muñoz *et al.,* 2010). This is characterised by the inefficient circulating volume and it

is the abnormalities of perfusion pressure at the baroreceptors of the muscles and vital organs responding to changes in vessel wall stretch (Johnson, 1998). Electrolyte losses provide a basis for muscle cramps, acid base imbalances and heart arrhythmias, eventually leading to total exhaustion (Muñoz *et al.,* 2010). Furthermore, dehydration can be detrimental to total body potassium depletion as equine kidneys are not able to conserve potassium when deprived of water (Schott and Hinchcliff, 1998). This can cause muscle weakness as potassium is vital in mediating the flow of blood to the working muscle (Johnson, 1998). In a study on successful and unsuccessful endurance horses, Muñoz *et al.* (2010) found that the losses of water and electrolytes were highest in the horses that did not complete the endurance event, highlighting the negative impact dehydration can have on performance. The unsuccessful horses showed higher packed cell volumes and total plasma protein concentrations, confirming their dehydration. The slower speed of the unsuccessful horses was also noted, supporting the decreased performance due to dehydration hypothesis because of haemodynamic comprise.

2.2.3 Water loss and temperature

It is commonly acknowledged by authors such as McCutcheon and Geor (2000), and Morgan *et al.* (1997), that exercise during increasing environmental temperatures can cause evaporative fluid loss to significantly increase. Exercising alone increases perspiration rate, however when combined with high temperatures this loss is intensified. Dehydration rates dramatically increase with sustained high environmental temperatures, as the skin to air thermal gradient is reduced which prevents the body from dissipating heat. Similarly, the horse is also at a disadvantage as it has a high metabolic capacity but a small surface area for the dissipation of heat; as only two thirds of metabolic heat can be dissipated via

sweating, the rest is stored and contributes to an increase in core temperature (Hodgson *et al.,* 1994). Increased heat retention induces muscle fatigue because of poor blood perfusion of the working muscles caused by an increased heart rate and decreased stroke volume and cardiac output (Kohn *et al.,* 1999). Geor and McCutcheon (1998) state that a high plasma osmolality has been found to impair thermoregulation, therefore fluid loss via increased sweating in high temperatures could bring heat related health issues such as myopathy, and diarrhoea, leading to further dehydration (Foreman, 1998).

2.2.4 Effect of transportation

Dehydration is a common consequence of prolonged transportation. Marlin and Nankervis (2002) explain that long journeys by air or road can result in body weight losses of 2kg/h in a 500kg horse, and this figure is representative of horses that are acclimatised to long travel in 'ideal' conditions, an anxious horse could be capable of much greater losses. These bodyweight losses occur as a result of decreased water intake, increased sweating and increased water content of faeces.

Friend (2000) used a prolonged period of up to 33 hours of transportation in high temperatures of up to 37 degrees Celsius, to examine the effects that dehydration can have. Horses were categorized into four groups; two penned groups, one with access to water and the other not, and two transported groups, one of which were offered water during the journey. Friend (2000) noted that after 30 hours of travelling, the non-watered horses were deemed unfit to continue, which highlighted the importance of water intake in such conditions; though this experiment appears highly unethical and highlights a number of welfare issues. It was also explained that dehydration can increase weight loss, as total body water makes up a 65% of the horse's body mass. Both the groups that did not have access to water showed

greater drops compared with the groups that had access to water. The weight loss is most likely a combination of sweat and urinary and faecal losses. Faeces is the main route of water loss for a horse at maintenance; adult horses fed alfalfa hay lose between 3-3.8 litres of water per 100kg of body weight under non stressful conditions (Freeman *et al.,* 1999). However, this can increase when horses are transported, in the case of Friend's (2000) study, the transported horses lost more water via faeces than the control horses kept in pens, as the novelty of the trailer increased defecation; though because they did not sweat as much, their overall weight loss was less than the penned horses as it was thought that the convective cooling via transportation caused less sweating and the aluminium open topped trailer that the horses were transported in would possibly reflect the sun. In both the penned and transported groups, weight loss was greater when the horses did not have access to water during the trial. Losses via urine are unlikely during dehydration, as the kidneys regulate fluid homeostasis and when water intake is low, urine production is reduced; the normal urine loss is determined to be around 0.5 litres per 100kg of body weight (NRC, 2015). Dehydration induced weight loss can have an affect days after the strenuous exercise that caused the initial weight loss. Despite an additional sodium supplement, Jansson *et al.* (1995) found that sodium continued to be lost via urine in the 2 days following exercise which resulted in an approximate 2% loss of body weight. In addition, water could be lost via depletion of the reservoir in the gut, which can give the 'tucked up' appearance often seen after a prolonged period of activity (Schott and Hinchcliff, 1998)

2.2.5 Physiological effects of dehydration

Dehydration can have a wide range of effects across many different systems. A reduction of fluid in the large intestine may interfere with the bacteria in the gut,

affecting digestion which could in turn increase the risk of colic (Marlin and Nankervis, 2002). A lack of mucus in the respiratory tract leading to the drying of the airway could limit the removal of bacteria and allergens, and therefore cause inflammation of the airway and reduced lung function (Marlin and Nankervis, 2002). Effects on the muscular system are more fully documented; Muñoz *et al.* (2010) aimed to investigate the differences in hydration marker hormones and electrolytes in endurance horses that were successful or eliminated in competition. It was found that the eliminated horses presented higher levels of dehydration, with a greater electrolyte loss and increased activation of antidiuretic hormones compared with successful ones. As Muñoz *et al.* (2010) explain, the higher lactacidemia in the dehydrated horses led to reduced blood flow to the muscles. The defective muscle metabolism, specifically the reduction in oxidative phosphorylation and electrolyte derangements such as shifts in fluid and electrolytes down a concentration gradient into the damaged muscle, can precipitate unwanted metabolic conditions such as Azoturia, or Exertional Rhabdomyolsis Syndrome (Arighi *et al.,* 1984; Foreman, 1996; Valberg, 2006). Azoturia is predominantly characterized by the alterations to the horse's gait due to muscle spasm or necrosis, and if severe the horse may be entirely unable to move. The muscle degeneration results in the release of myoglobin into the blood plasma which is then filtered into the kidneys causing myoglobinuria. This can starve the muscles of oxygen (Jackson, 1970), a common symptom of this is dark brown urine. Severe myoglobinuria can result in acute renal failure as the renal tubules can become blocked by myoglobin casts (Arighi *et al.,* 1984). In a well hydrated horse working sub maximally, the conversion of glycogen to glucose to pyruvate normally occurs aerobically, although a small amount can be metabolised anaerobically into lactic acid without causing any harm (Jackson, 1970).

Dehydration may cause packed cell volume to increase and reduced uptake of oxygen by haemoglobin, this will cause insufficient intracellular oxidative muscle enzymes to produce adenosine triphosphate (ATP) aerobically (Foreman, 1996). Therefore ATP must be produced via anaerobic glycolysis, the conversion of glycogen to pyruvate produces higher concentrations of lactic acid, because of an increasingly acidic environment the calcium flux is affected, resulting in the impediment the actin-myosin contractile process (Foreman, 1996), weakening the muscles and forcing the muscle cells to rupture.

The losses of vital electrolytes can trigger a wide range of issues, as described by Flaminio and Rush (1998). Absolute loss of sodium can cause tachycardia, hypotension, increased capillary fill time, muscle spasms and fatigue. It can also further dehydration as because as much fluid is lost and sodium is depleted, causing a loss of the drink response, furthering dehydration. In addition, excessive chloride loss causes bicarbonate to be reabsorbed by the kidneys allowing the development of metabolic alkalosis (Flaminio and Rush, 1998). Potassium losses can be one of the most dangerous. Dehydration increases sodium reabsorption at the expense of potassium, lack of potassium can form the basis for skeletal muscle paralysis, gastrointestinal hypomobility, and the hyperirritability of long nerves leading to cellular irritability and rhabdomyolysis. Calcium losses affect the sodium channels resulting in nerve irritability and involuntary muscle contractions as the depolarization threshold of nerves to electrical stimulation is lowered, as in the detailed ‘thumps’ above.

Dehydration can in some cases cause stomach ulcers; Andrews *et al*. (2005) explain that the glandular lower section of the stomach is normally protected by prostaglandins, an active lipid compound responsible for maintaining the mucus

needed to protect the lining of this section from gastric acid. Ulcers are more common in the upper part of the stomach as it lacks the mucus needed to protect it from ulceration. However, dehydration can result in the mucus in the lower part of the stomach becoming reduced, leading to ulcers. An increased intake of sodium causes the horse to use its water reserves to flush the excess sodium from the body, and if it cannot replace its water content, then the fluid balance will be affected, leading to ulcers.

2.3 Thirst controls and response

The old English proverb ‘You can lead a horse to water, but you can’t make it drink’, has sparked research into exactly what triggers the thirst response in horses and whether we can use this to maintain a healthy hydration status. As humans, we are aware of what makes us dehydrated and can drink more water accordingly. We are able to recognise the importance of drinking and we can make the decision to drink before thirst develops to attenuate dehydration (Düsterdieck *et al.,* 1999). A horse simply relies on its biological stimuli to trigger the thirst response; though an unwillingness to drink despite signs of dehydration is a common problem, especially in horses that have undergone exercise (Nyman *et al.,* 1996) and those that have had Lasix® administered . The primary stimulus for the thirst response is increased plasma tonicity and consequently osmolality, a loss of electrolytes reduces plasma hypertonicity; as equine sweat is hypertonic relative to the plasma, greater amounts of electrolytes are lost compared with humans whose sweat is hypotonic, therefore equines have the potential to develop a greater magnitude of dehydration and subsequently thirst response (Butudom *et al.,* 2003).

2.3.1 Influence of electrolytes on thirst

Marlin *et al.* (1998) stress that ingesting water alone will fail to stimulate the thirst response due to decreases in plasma sodium and osmolality, as urine output increases this decreases the stimulus to drink. This has also been observed in humans, Maughan *et al*. (1996) has shown that drinking plain water is less effective than drinking water with electrolytes as a rehydration strategy. However, Marlin *et al.* (1998) do mention that if sufficient electrolytes are given in feed following situations that result in high fluid loss, such as competition or travel, then water may be an adequate method of rehydration; providing a reluctance to eat does not occur post exertion, which can sometimes occur.

The predominant control of the horse's thirst response is an increase in plasma osmolality, specifically an increase in plasma sodium concentration (Butudom *et al.*, 2002). Osmoreceptors in the hypothalamus monitor the levels of sodium in the blood, when dehydration occurs, sodium levels in the blood begin to increase, which triggers the release of the antidiuretic hormone (ADH); ADH increases the permeability of the kidneys, allowing them to reabsorb pure water to help dilute the blood-sodium back to the optimal level (Marlin and Nankervis, 2002). If this is not enough to reverse the dehydration, the thirst response is activated. Previous studies (Jansson *et al.,* 1995; Sosa-Leon *et al.,* 1995) have successfully used saline to rehydrate horses but this has been via a nasogastric tube, it is deemed unethical to use this method when prior to and during competition, so a less invasive way has been found. Marlin and Nankervis (2002) explain that the most effective way to rehydrate a horse is by offering the horse its normal feed supplemented with electrolytes, as large volumes of electrolytes are not easily consumed in water as the amount a horse will readily consume is only around 0.9g per litre, a horse would need to drink approximately 22 litres of isotonic fluid to replenish the lost electrolytes,

whereas it can consume 22g of electrolytes in one feed; this will stimulate the thirst response so the horse can rehydrate voluntarily. However, if it is not possible to feed, electrolytes in water and oral electrolyte pastes can be effective, such as between phases of a one-day event or in between polo chukkas, the idea of this is to ensure the horse is fully hydrated before exercise begins. The supplementation of electrolytes after prolonged exercise is vital for maintaining fluid balance, and the provision of electrolytes in water remains a popular way to trigger the thirst response. Butudom *et al.* (2002) found that offering salt water was sufficient enough to increase water intake in horses dehydrated by frusemide and endurance exercise. Although full replacement of body fluid losses was not achieved, saline solution of 0.45% and 0.9% sodium chloride decreased the magnitude of dehydration to approximately 2% (Butudom *et al*., 2002), this figure has been found to be a similar value that has been observed in human athletes (Hubbard *et al.,* 1984). Furthermore, Butudom *et al.* (2002) noted that body weight losses the day after the dehydration decreased as a result of the rehydration that occurred as a result of drinking the saline solutions, suggesting that many of the electrolytes consumed in the initial recovery period were retained in the body fluids of the horses. This advocates the use of salt water as a rehydration technique in horses that need to continue to perform the days following dehydration, such as three-day eventing or endurance competitions, as the retention of electrolytes from the saline solution would maximise recovery in addition to stimulating voluntary drinking as a result of changes in plasma tonicity. It is proposed by Butudom *et al.* (2002) that rehydration with water not supplemented with salt, such as in this study, is not recommended as the hypotonic nature of the fluid would lead to the inhibition of the thirst response and therefore delay recovery by an

additional day; however, Butudom *et al.* (2002) recommend that horses be trained to drink salt water during and after exercise, prior to using it as a rehydration strategy. The use of salt as a means to stimulate the thirst response is further supported by Nyman *et al*. (1996); their study aimed to test three different voluntary rehydration strategies by offering just plain water, plain water after administering a 30g salt paste, and a saline solution of 0.9% sodium chloride, during a 62km endurance ride. The horses that were given the saline solution had the highest total voluntary water intake and made up the most of their bodyweight losses compared to the other two treatments; water intake remained high in this group even when the saline was replaced with water one hour after the ride had finished. Nyman *et al.* (1996) believe that drinking saline can enhance fluid balance maintenance during prolonged exercise and competitions lasting several days; the uptake of sodium chloride together with water corresponds to increases in plasma osmolality, plasma sodium, and a drop in total plasma protein concentration, which are all known to stimulate the thirst response and so will ensure the horse keeps drinking even after the treatment has ceased. This further proves the benefit of adding salt to water to ensure rehydration. Though the salt paste group did not have as high a water intake as the saline group, water intake post ride was still higher than the group given plain water; as water intake only began to increase in the salt paste horses after the ride had finished. This shows a delay in the thirst response with this strategy; this is attributed to the way in which the paste was given which could have caused net water shifts between the extracellular volume and the gastrointestinal tract and it was recommended that should salt paste be used it should be given either several hours before the activity to give horses time to increase their water intake so they are euhydrated, or after the activity so fluid losses can be replaced the following day.

The use of salt pastes during exercise is cautioned, as it causes a disturbance in the distribution of fluid between the body fluid compartments. The purpose of the plain water group was to ascertain to what extent voluntary water intake could replenish electrolytes from the body's own stores. The fluid intake of water alone was significantly lower than the other two treatments and so body weight loss was greater; this is due to the fact there were no significant changes in plasma osmolality, plasma sodium, or total plasma protein concentration. As drinking plain water would dilute plasma sodium concentrations the thirst response would not be activated as this would explain why water intake was lowest in this group. The findings of Nyman *et al.* (1996) could explain why we did not see high water intakes in this study, as drinking water without salt prevents the necessary changes in the blood plasma needed to trigger the thirst response. Furthermore, Nyman *et al.* (1996) stress the palatability of solution used to enhance voluntary rehydration is crucial, like Butudom *et al*. (2002), horses in their study were offered the saline solution at home prior the start of the trial in order to become used to it and were only used if they accepted it; not all horses find saline palatable, as found by Randall *et al.* (1978).

Electrolyte pastes are another way to encourage a horse to drink (Düsterdieck et al., 1999). Once the electrolyte paste reaches the intestine, the electrolytes draw water from the blood into the gut, this causes the concentration of sodium in the blood to increase and stimulates the thirst mechanism (Gray, 2012). Many riders use these electrolyte pastes to combat dehydration, however whether this method's aim is to replace lost electrolytes or enhance voluntary water intake is debated. Schott and Hinchcliff (1998) state that 10-15 grams per day is a sufficient maintenance dose for a 450kg horse; when dehydrated, sodium in the blood becomes concentrated and

the horse must rectify this by consuming water to return to the correct fluid – electrolyte homeostasis.

This corresponds to plasma osmolality stimulating thirst. In an experiment by Sufit *et al.* (1985) they found that ponies usually drink when there is a decrease in osmotic pressure or decrease in the volume of body fluids. The overnight water deprivation was shown to increase osmotic pressure and plasma protein, and this stimulated the ponies to drink as much, if not more water than the control pony that was offered water *ad libitum*. When water was consumed by the water deprived ponies, osmotic pressure and blood volume were restored within an hour. Sufit *et al.* (1985) discovered that the increase for osmotically induced thirst is an 8osm/L increase in osmotic plasma pressure. This has been likened to the thirst response of wild horses who have to travel long distances for water in arid conditions, resulting in increased osmotic pressure and hypovolaemic changes (Sufit *et al.,* 1985). However, it has been found that increased plasma osmolality is a stronger thirst stimulus than hypovolaemia, but that is not triggered until a significant fluid loss has occurred. Osmolality increases after sodium bicarbonate administration as it increases serum sodium concentration (Schott and Hinchcliff, 1998) which highlights its effectiveness as a means to stimulate thirst and therefore rehydrate. However it is noted by Nyman *et al.* (1996), that if high amounts of sodium are lost during sweat the plasma sodium concentration may be unchanged, and the thirst response will not be triggered as it relies on an increase in plasma sodium concentration, which will not occur because an equivalent amount of plasma sodium is lost in urine as well as sweat, which is particularly common in endurance horses (Flaminio and Rush, 1998). This is also a common problem in racehorses. Many racehorses are given furosemide, or Lasix®, which causes water loss, before racing as it prevents exercise-induced pulmonary

haemorrhage (Hinchcliff *et al.* 2005), as well as reducing bodyweight, resulting in faster runs. However, this also affects the electrolyte balance, Furosemide causes a massive increase in urinary sodium and chloride excretion, this loss can be up to 50 times that of an untreated horse, calcium loss also increased (Pagan, 2015). Due to the high loss of sodium, the thirst response in horses treated with Furosemide is unlikely to be triggered, so they must be given supplementation to aid their recovery. Kentucky Equine Research have developed a two-stage program called Race Recovery™, consisting of a paste, high in sodium and chloride, given immediately after racing to stimulate thirst, in a study by Pagan (2015) it has been shown that it increased water intake by 17% in 24 hours coupled with a 30% increase in body weight. Following this the horses are given a powder fortified with electrolytes and minerals such as sodium, calcium and magnesium, to replace what has been lost via sweat and urine.

Another interesting method for stimulating the thirst response is the supplementation of electrolytes with glycerol; in human athletes, glycerol has been labelled as a 'hyperhydrating' substance that can enhance endurance performance (Montner *et al.,* 1996) and help with thermoregulation by increasing sweating rate (Lyons *et al.,* 1990). Düsterdieck *et al.* (1999) sought to find out if glycerol was as effective in hydrating horses as it is in humans, they hypothesized that glycerol and electrolyte supplementation would produce a higher water intake than the administration of electrolytes alone. It was found that both a large dose of electrolytes and a combination of electrolytes and glycerol in the form of oral pastes enhanced voluntary water intake and therefore attenuated weight loss in the horses performing a 60km endurance ride, a strong negative correlation was found between weight loss and total water intake. However, the administration of electrolytes in glycerol did not

produce a higher water intake than when electrolytes were given in water, despite plasma osmolality being higher in the horses given glycerol. Düsterdieck *et al.* (1999) postulate that this could be because glycerol is well distributed around the body fluid compartments and so failed to produce an effective osmotic stimulus for thirst. They also explain that plasma sodium concentration could be a more important stimulus for thirst than plasma osmolality.

2.3.2 Drinking behaviour of horses

It has been observed that horses drink peri prandially, meaning they often drink at the time they eat, but although this can be modified by water source and availability. Water intake is episodic, the number of drinking bouts for adult horses watered using pressure valve bowls, float valve bowls, and buckets ranged from 16-21 episodes per day with duration of 10-52 seconds (Nyman and Dahlborn, 2001). These drinking episodes are brief; in fact, geldings in Nyman and Dalhborn's trial only had a daily drinking time between 3-15 minutes, though Sufit *et al.* (1985) recorded daily drinking times of 21-27 minutes in ponies, much of this was peri prandial, the relationship between feeding and drinking is further discussed in sections 3.3.4 and 4.3.5.

Drinking commonly increases in the first few hours post exercise, before returning to normal. Drinking patterns can be influenced by the source of the water, horses tend to prefer deeper bowls, as shallow one may restrict the ability to drink quickly (McDonell *et al.,* 1999). An experiment by Nyman and Dahlborn (2001), found that horses favoured drinking from buckets as opposed to the shallower pressure or float valve bowl; horses drank 98% of their daily intake from buckets, but spent more time drinking from the valve bowls even though they consumed less water. The phenomenon of involuntary dehydration caused by an upset plasma sodium

concentration is also observed in human athletes and normally contributes to around 2% loss in body weight due to lack of fluid. This loss is said to be greater in horses as the magnitude of involuntary dehydration is greater (Butudom *et al.,* 2002). Interestingly, it is not just the composition of the fluid that can mute thirst response; gastric filling is said to be a key factor in satiating thirst. Butudom *et al.* (2004) have found that volumes consumed immediately after exercise are similar to the capacity of a horse's stomach, these volumes were influenced by the temperature of the fluid, and so this must be taken into account when devising rehydration strategies; the relationship between temperature and water intake is discussed in section 2.3.4.

2.3.3 Influence of diet on thirst

Ingestion of a meal can significantly alter fluid balance in horses (Schott and Hinchcliff, 1998); diet can impact water intake depending on the composition of the consumed feed and the amount that is eaten. The moisture content in what is consumed influences how much water the horse needs to drink to stay hydrated. Pasture has a high moisture content of over 80% and so the horse can use metabolic water to help hydrate itself, Marlin (2019) explains that a horse kept at grass 24/7 can get up to 50 litres a day from the pasture, whereas a horse kept in and fed *ad libitum* hay may only receive 5 litres a day from metabolic water. Therefore, a horse fed on pasture will not need to drink as much water as a horse fed on hay and grain. However, forage is an important component in the diet, as it allows the digestive tract to function correctly during exercise, as keeping blood flowing to an active healthy digestive tract stimulates thirst (Pagan and Huntington, 2010), which in turn maintains hydration status. The dietary composition, particularly mineral composition of the diet can also affect water intake. For example, a forage diet which is high in fibre triggers a high water intake as it increased the water

holding capacity of the digesta within the gut and so decreases faecal dry matter excretion compared with concentrate diets. Diets high in sodium and potassium also have the same effect by increasing urinary excretion (NRC, 2015). In addition, it has been found that ponies fed a pelleted meal had increased plasma protein and sodium concentrations one-hour post prandial, which increase the thirst response (Schott and Hinchcliff, 1998). It was also noted that a secondary decrease in plasma volume and increase in osmolality occurred in the ponies 6-8 hours after eating, triggering a second thirst response.

2.3.4 Influence of temperature on thirst

Ambient temperatures can fluctuate significantly which influences water intake. Cold temperatures below that of 3^{o}C have been found to decrease water intake by 6-14%; high temperatures of 30^{o}C or over can increase water intake by as much as 79% if being exercised simultaneously (Geor *et al.,* 1996). This links back to the role of electrolytes in thirst response, as hot temperatures will trigger the mechanisms for electrolyte loss, i.e. sweating. The temperature of the water itself can impact water intake; it has been noted by Butudom *et al.* (2004) that horses prefer luke-warm water to cold when given a saline solution after exercise. However, the ambient temperature can have an impact on the preferred temperature of the water; in cold conditions, horses were found to prefer luke-warm water, but when temperatures were milder, i.e. between 15^{o}C and 29^{o}C, horses did not show a preference for either icy or warm water (McDonnell and Kristula, 1996). This is supported by Kristula and McDonnell (1994), who examined the effect of water temperature on water consumption in ponies during cold weather conditions (-7^{o}C to 5^{o}C). When provided with buckets of hot water at 46-49^{o}C twice daily, ponies drank 38% more water than when provided with near freezing water; it must also be noted that the majority of

drinking episodes occurred within three hours of being given the water, highlighting the ponies desire to drink before the water cooled too much. It was also found that providing the ponies with hot water twice daily, rather than having continuous access using bucket heaters, was a simpler, more effective way of offering water; it is worth noting this, as horses travelling in cold conditions will only have intermittent access to water, and on arrival should be offered warm water. The provision of warmer water in cold temperatures not only ensures water consumption but can also prevent medical problems such as impaction colic, as water is vital for healthy gut function. Sufficient water can also help to prevent Azoturia, where high lactic acid content in the blood can have a harmful effect on muscle metabolism. No preference for either hot or cold water was found when offered in ambient temperatures between 15-29^{o}C; ponies drank similar volumes of icy and warm water (McDonnell and Kristula, 1996). Interestingly, the total daily water consumption per pony was similar in both the cold weather and hot weather studies, again stressing the importance of offering warm water in cold conditions to maintain a healthy water intake. However, in both studies exercise was not included, and it was not conducted as a preference test as warm and icy water were offered separately. Further studies are also required to examine the effect of water temperatures on water consumption during a wider variety of environmental factors such as humidity and type of forage available.

2.4 The physiology and controlling mechanisms of palatability and preference

2.4.1 Anatomy and physiology of the taste and smell senses

For horses, taste and smell are two senses that are very closely linked, often thought to be inseparable and are referred to gustation (taste) and olfaction (smell). The senses of taste and smell play an important role in food preferences and selection. Orosensory characteristics together with post ingestive mechanisms allow the horse

to make either pleasant or unpleasant associations with what it has consumed. Food can be rejected as a result of sensory input and its link to post-ingestive consequences (Van den Berg *et al.,* 2016a), these learned aversions have also been recorded in other animal species such as rats (Garcia *et al.,* 1972), and ruminants (Burritt and Provenza, 1991).

There has been limited research into the anatomy and physiology of gustation and olfaction in the horse. A common issue is to liken these with other herbivorous animals, and much discussion is reliant on comparative studies in other animals and humans. It is the assessment of the external environment that precedes the roles of gustation and olfaction. This assessment is two-fold; physical, involving the utilisation of senses such as vision and touch that assess the external environment, and gustation and olfaction that evaluate the chemical environment. It is these chemical senses, and the evaluation of the external environment that allow the horse to make an informed decision (Murphy *et al.,* 1999); the decision to swallow a potential food source is ultimately based on gustatory cues (Rawson, 1990). Therefore, gustation and olfaction play a crucial role in nutrient intake and the avoidance of consuming toxins (Scott, 2001), as a result, diet and feeding behaviours are controlled by gustatory and olfactory responses, as well as the levels of glucose and fat in the blood, which physically control the hunger response . Ultimately these two systems play a vital role in the proliferation of a species, and any compromise in their function would have implications in diet selection and therefore the survival of the animal. However, the response to perceived stimuli may be subject to individual variation and prior experience, and it is thought that age can influence these functions, though there is little research to confirm this. The use of intake, when presentation is replicated during experiments, as an indicator of palatability may be inaccurate and

affected by inconsistencies such as the levels of blood glucose which control the hunger response (Rook *et al.,* 1997). Rogers (1990) stated that hunger and palatability may act independently of each other and may account for variation in preference within individuals, this could also be the case for water intake.

Gustation is the physical action of tasting; substances dissolved by the saliva coming into contact with specialized receptor cells in the tongue and throat region. The sensation of taste is produced from raised areas on the tongue called papillae, upon which the taste buds sit (Davies, 2018); these are comprised of clusters of taste cells. Taste at the front of the tongue is served by sensory nerve fibres which are distributed by a branch of the facial nerve, taste at the back of the tongue is controlled by the glossopharyngeal nerve, which also conveys temperature and texture senses, taste buds on the soft palate are served by the vagus nerve (Fails and Magee, 2018). Traditionally there are four taste sensations that horses can detect; sweet, salt, bitter and sour. The individual taste cells have a membrane which is responsible for detecting the chemical substances that are associated with these tastes (Fails and Magee, 2018). However, it has been found that a fifth taste sense known as umami, its responsible for imparting a savoury quality to food stuffs when glutamate is present with sodium. Like humans, horses are thought to have a sweet tooth, and favour flavours like apples, carrots and honey; although it is also known that horses can tolerate more bitter flavours (Davies, 2018).

However, to experience more complex sensory experiences, the basic sense of taste must work in conjunction with the olfactory system; if something smells bad, then the horse might refuse what it is being offered or pick around the strange substance (Kentucky Equine Research Staff, 2014b). The horses' olfactory system is highly complex. Anatomically, the nasal passages contain two tightly rolled turbinate bones

designed to increase the surface area of the nasal passage and divide each of the nasal passage into three channels; the dorsal, medial and ventral meatus (Davies, 2018). The dorsal meatus is the channel responsible for conducting air breathed into the olfactory system, and air breathed into this region is picked up by olfactory nerve cells throughout the mucous membranes (Davies, 2018). The anatomy of these neurons is complex; each apex has a single dendrite with a tuft of hair like projections bearing the chemical receptors, an axon from each neuron passes through the mass of fibres of the cribriform plate of the ethmoid bone into the cranial vault, this constitutes the olfactory nerve (Fails and Magee, 2018). These fibres synapse with the large olfactory bulbs on neurons that connect with the limbic system of the brain; because these large bulbs have an extensive epithelium, and because densities of receptor cells remain constant per unit surface area volatile odours play a much larger sensory role than in humans (Saslow, 2002). Interestingly, olfaction is known to have connections to the hypothalamus, a part of the brain which generates emotional responses; therefore, smells are capable of eliciting emotions and behaviours (Fails and Magee, 2018), highlighting the importance of smell in the palatability of a food stuff.

2.4.2 The process of selection using gustation and olfaction

It has traditionally been assumed that the role of taste and smell in horses is similar to other herbivores, however this is not the case. Horses have a well-developed sense of taste and can easily differentiate between differences in flavour and texture in relation to nutrient density (Ellis and Hill, 2005). As horses are hind gut fermenters, they are unable to vomit so they must be especially wary about what they chose to ingest (Van den Berg, 2014). As a result, horses can be very sensitive to the inclusion of new tastes into their diet, this could either be a taste aversion or

neophobic response. The development of aversions and preferences also takes in to account the nutritional value of the food, stemming from the need to select the best quality food in order to survive in the wild. However, diet selection cannot solely be explained by nutritional needs and sensory cues may outweigh nutritional value (Van den Berg *et al.,* 2016b). This is especially the case in stabled horses, where sensory cues may be more important due to the monotonous nature of their diet (Cannas *et al.,* 2009). In the wild, horses are socially motivated, apparent by herd grazing; and an increased is intake is noted when others in the herd are observed eating (Houpt, 1990); it can be assumed this is the same for drinking.

The process of food selection and ingestion for horses is threefold; food recognition by sight and smell, orientation with vision and proprioception, and finally grasping, chewing and swallowing (Lawrence, 1990). The role of pre-ingestive feedback has been extensively studied in ruminants, but little is known about horses.

Interestingly, many animals are more sensitive to the inclusion of chemicals in water than feed (Goatcher and Church, 1970a), though the taste response in feed will have a bigger influence on selection and palatability than water would. The comparison of feed palatability and water trials is difficult as the data cannot easily be compared.

2.4.3 Factors affecting palatability

Palatability can be defined as the overall sensory perception of a feed by an animal and can be measured as the characteristics, such as investigation and intake, that trigger a sensory response (Ellis and Hill, 2005). These characteristics combined, with previous experience are the first controlling factors of food intake. Rook *et al.* (1997) state that palatability is the short-term response to a feed, before the influence of post-ingestive stimuli; if post-ingestive stimuli are to be included, then palatability would become acceptance, according to Rook *et al.* (1997). If palatability

is considered to be just the initial response to a feed, then it makes it incredibly difficult to measure, and would be subject to conditioning and learned behaviour. It may be that a preference for sweet substances and aversion to bitter substances is based on learning of post-ingestive consequences (Rook *et al*., 1997), however, if selection of food was based on short term palatability alone, there would be a limited survival benefit throughout evolution.

It has been shown that pre-ingestive stimuli can override post-ingestive stimuli and sensory feedback can influence preferences if post ingestive signals are lacking (Van den Berg *et al.,* 2016b). The ability of the horse to avoid a substance that has a negative post-ingestive consequences has been studied by Houpt *et al.* (1990). For aversion to be learned the negative consequences must occur in the thirty minutes following ingestion, and learning would not occur if consequences occurred after this time. As a result, horses are at risk of chronic poisoning if the symptoms take time to materialize. Furthermore, when negative consequences are associated with a more palatable feed, learning is less effective (Houpt *et al.* 1990). Prior experiences influence taste response in two ways; through a conditioned aversion or preference to a previously encountered feed, and from learned associations between post-ingestive consequences and taste cues.

It is known that horses can display what we would describe as ‘fussy eating’, however this neophobic response is an essential innate behaviour as it is the mechanism for avoiding toxic plants in the wild (Van den Berg and Hinch, 2016). Over the last few decades, the variety of commercial feed products has rapidly expanded, and flavours are now commonly used in hard feed in an attempt to try and limit the cautiousness that horses display while feeding. Although there are some studies on flavour acceptance of feed, such as Goodwin *et al.* (2005a&b) and

Kennedy *et al.* (2001), the use of flavours in water to encourage intake has received little attention. Feed is developed with the intention of it being consumed consistently with the influence of taste affecting whether or not the horse wishes to continue to consume the substance. Van den Berg and Hinch (2016) reported that the characteristics of useful flavours are often not taken into account, with flavours classified as non-nutritive, providing only an aromatic non-caloric taste, or nutritive, providing calorie content as well as taste. It must also be noted that as a foal, horses develop preferences through cultural transmission and social facilitation (Ellis and Hill, 2005), so a sense of preference is developed from a very young age.

2.4.4 Preference and influence of flavours

The four basic tastes; sweet, salty, bitter and sour (Goatcher and Church, 1970a) are primary in classification and any tastes that cannot be described in terms of these groups would be a mixture of two or more and not a separate taste altogether. It is debated by Goatcher and Church (1970a) that there is another taste group, alkaline; however, it is concluded alkaline simply produced a complex sensation via stimulation of specific receptors rather than a taste itself. In each of the main taste groups, there is not one single chemical which would completely explain the stimulating properties of the chemicals within the taste groups, so many concepts are responsible for characterizing substances to a particular group (Goatcher and Church, 1970a). The sweet taste is attributed to a mixture of sugar derivatives, alcohols and glycols; salty is characterized predominantly by sodium chloride, with the cation playing a slightly larger role in stimulation than the anion; sour taste is produced mainly by mineral and organic acids; whilst the bitter taste is evoked by quinine, tannins and caffeine (Goatcher and Church, 1970a).

The environmental factors that influence taste are predominantly affected by the nature and temperature of the taste medium, and the visual and positional cues; though, the effects of temperature have been little studied (Goatcher and Church, 1970a). In laboratory kept animals, visual and positional influences played a crucial role in both food and liquid choices (Young, 1948). Bias such as this also occurs in larger domesticated animals but has not been as extensively studied. Intra-organic factors such as age, disease, and genetics also can influence the sense of taste. The effect of age is somewhat unclear, but it is thought that taste sensitivity is reduced in young and old animals and at its maximum when in the prime of life (Bottom, 2008).

Many flavours are added to feed in an attempt to improve palatability by masking unpleasant flavours. A limited range of fruit flavours are particularly used in the commercial equine market, although there has been little research to prove their effectiveness (Goodwin *et al.,* 2005a). The ultimate aim of flavouring feed substances is to increase intake. Many studies have been conducted in attempts to determine flavour preference as the value of this knowledge would have a huge impact on the commercial feed market. In humans it is accepted that food acceptance and therefore, nutrition is essential for optimum health (Ennis, 1998), and this is also being realised in the equine athlete.

Van den Berg *et al.* (2016b) aimed to determine the influence of odour, taste, and nutrients on the preference of horses, and hypothesised that nutritional content would be ranked first followed by taste then odour. The flavours used were selected from different classes for contrast; banana (fruit), coconut (nut), spearmint (herb) and cinnamon (spice). Pellets with varying crude protein levels were used to test nutritional preference. Initially, most horses showed a neophobic response but after

and adaptation period it appeared that pellets high in crude protein and therefore nutritional value were the main driver for selection. Taste proved to be the secondary influence of selection when the nutritional value was low. Odour was also found to affect intake, banana and coconut recorded the highest intakes, this supports other studies such as Kennedy *et al.* (2001) and Goodwin *et al.* (2005a), that have proved fruit flavours to be preferable, as they provide a sweet aromatic sensation.

A study by Kennedy *et al.* (2001) sought to evaluate whether the fruit flavours of apple, cherry, citrus and teaberry, could increase the intake of oats in thoroughbreds. It was found that more of the flavoured oats were consumed than plain oats. Interestingly, cherry was the most popular flavour by some margin. Building on this, Goodwin *et al.* (2005a) sought to determine flavour acceptance and preference using 15 flavours in standard base diets. An initial acceptance trial found echinacea, coriander and nutmeg to be totally or partially rejected and therefore were excluded from further investigation. Garlic has been thought in the past to improve palatability, though accepted in Goodwin *et al*'s (2005a) trial, it was not as palatable as other flavours. Garlic flavoured meals were eaten unless a more palatable option was given, this explains the strong role of garlic as a commercial flavour as most diets for the kept horse are fairly bland. It was found that banana and fenugreek were the flavours with the shortest consumption times, and that banana was the most accepted flavour.

Mars *et al.* (1992) stress that a horse's response to flavour is important, as it can help us to medicate the sick horse and to maintain electrolyte levels when horses are placed under stress. Mars *et al.* (1992) aimed to investigate water intake in horses after transportation and whether flavoured water could increase intake of unfamiliar water in the new location. Horses were transported for 4 hours from their usual

dwelling to a research centre where the experiments were undertaken, half were offered familiar water from the home and half unfamiliar water, which undoubtedly differed in its dissolved solids levels and therefore flavour. Although there was little variation between water intake of the two water sources at the unfamiliar location, horses that remained in their familiar location drank more than in the new location, this highlights that horses under stress will drink less and need to be encouraged to drink on arrival in the unfamiliar location. Mars *et al.* (1992) found that adding apple flavouring to the unfamiliar water source increased water intake regardless of whether it was given in their home environment or the unfamiliar one. This study proved that the stress of a new location can reduce water intake and the addition of a flavour could rectify this problem.

Although work has been done that investigates the palatability of specific flavours, little has been done on the basic taste preference of horses (Merkies and Carson, 2011). A common practice is to use a sweetener to enhance intake, Danel and Merkies (2009) found that a moderate concentration of 10g/100ml solution of sucrose was most accepted, which is consistent with Randall *et al.* (1978). On studying the taste response of the horse with the basic tastes of sweet, salty, bitter, and sour presented in water against an unflavoured control, preference was indicated if the horse's intake was higher than 60%, anything less than 60% was considered indifference. Preference for the sucrose solution was evident at 2.5g, 5g, and 10g/100ml, but no preference was shown at 20g/100ml, Randall *et al.* (1978) concluded that horses showed a similar taste sensitivity to sheep and pygmy goats. The sodium chloride solution used for the salty taste began with aversion recorded at 0.63g/100ml, and aversion increased as the concentration of sodium chloride increased. This finding of Randall *et al.* (1978) is consistent with that of Goatcher

and Church (1970b), who found similar taste aversion in cattle and goats. The response to a sour solution of acetic acid was found to be similar in horses to goats and sheep as stated by Goatcher and Church (1970c); Randall *et al.* (1978) found an aversion at 0.16ml/100ml with a pH of 3.1. Finally, the aversion to the bitter solution of quinine hydrochloride exhibited at 20mg/100ml was found to be less sensitive than pygmy goats as studied by Goatcher and Church (1970c). Only one horse in Randall *et al.*'s (1978) study showed an aversion to sucrose. This rejection by an individual could be as a result of increased taste sensitivity, although sensitivity was not found towards other solutions, or an aversion to sucrose. Inter species variation has been also been found in calves, with indifference and pronounced preference being exhibited (Kare *et al.,* 1965).

Similarly, Merkies and Bogart (2013) describe how unpublished results showed that horses had an aversion to bitter tasting solutions, but when sucrose was added the bitter taste was masked, highlighting that a sweet flavour will probably be more successful in improving water intake. This aversion to bitterness is explained further by Merkies and Carson (2011); who found that the more acidic the water, the more it was rejected by the horses. However, it was noted that none of the acidic solutions used were completely rejected, implying that although one option is preferred, the others will not be completely discounted.

2.5 Chemical composition of cherry and banana

Bananas are one of the most important food crops in the world, coming from a class of plants known as Musaceae they are a source of fibre, carbohydrate, potassium, vitamins and phytonutrients. Specifically, they are rich in vitamins B6 and C, and minerals such as manganese and potassium. The biological composition of bananas varies depending on the cultivar, the abiotic and environmental factors, and the

nutrient status of the soil (Elayabalan *et al.,* 2017). The flavour of the banana can vary depending on the stage of ripening, though it is predominantly volatile chemical compounds that give the banana its unique taste and odour. Generally, the scent of the banana is described as being sweet, floral and fruity, 30-40 aroma compounds have been found to give banana its aromatic profile (Phung, 2014). Isoamyl acetate is described as the flavour molecule, though is it not the most abundant compared to other aromatic compounds, it has a strong flavour which can be tasted in concentrations as low as 2 parts per million (Phung, 2014). Isoamyl acetate is often sold as pure banana oil and is commonly used to add the banana flavour to food products.

Cherries are a stone fruit belonging to the Rosaceae family of small tree fruit; there are two types of cherry, *Prunus cerasus* (tart), and *Prunus avium* (sweet). Like Bananas, they are a rich source of phytonutrients, vitamins and minerals; furthermore, they have high antioxidizing properties due to high concentrations of Anthocyanins, which are said to help reduce inflammation and oxidative stress, therefore aiding recovery after strenuous exercise (Kelley *et al.*, 2018). Unusually, they contain more glucose than fructose, making them extremely sweet and palatable (Kennedy, 2014). The aroma of cherries is reported to stem from glycosydically bound volatile aromatic compounds; notably, (E)-hexenal and benzaldehyde which are the dominant flavour constituents in both sweet and sour cherries (Serradilla *et al.,* 2017). Sour cherries possess higher concentrations of carbonyl benzaldehyde, whereas sweet cherries possess more benzyl alcohol and 1-hexenol; other compounds such as acids are also present (Serradilla *et al.,* 2017). However, to recreate all of the many compounds that constitute the flavour of cherries in a lab would be highly expensive and time consuming so to make the

artificial flavour, only the compounds (Z)-3-hexenol and 2-heptanone are used (Kennedy, 2014).

Both the flavours of banana and cherry are described as sweet and fruity, stemming from volatile and aromatic chemical compounds. This supports the use of these flavours in this study. Though the flavours for this study will be artificially produced, the necessary flavour will be still achieved using chemical compounds. From a review of relevant literature, the flavour of banana appears to be the most common in terms of increase food intake, the reason for this must be due to its composition and how that affects its odour and flavour, this explains why is has been chosen for this study. However, though Mars *et al.* (1992) proved the apple flavour to be palatable when added to water, cherry was chosen as the second flavour for this study. This is because market research conducted by Natural Animal Feeds (NAF) has shown the cherry flavoured treats to be more commercially viable and was preferred over the apple flavour in Kennedy *et al.*'s (2001) study.

Chapter 3: Trial 1 - The effect of three different concentrations of either banana (Trial 1a) or cherry (Trial 1b) flavoured water when offered to 6 polo ponies

3.1. Introduction

The objective of this trial was to investigate which of three dose rates (concentrations) for the flavours of banana and cherry was preferred by the horses by measuring the greatest fluid intake in 6 horses. The null hypothesis of 'There will be no difference in the intake of fluid according to the concentration of flavour'. The Animal Ethics Review group of the Royal Agricultural University approved the experimental design and procedures of this work.

3.2.1 Methodology

The dose rates were determined by the manufacturers of the flavours, Natural Animal Feeds (NAF), and were classed as weak, medium, and strong and are detailed as in Table 1:

TABLE 1. DOSE RATES FOR EACH CONCENTRATION FOR BOTH FLAVOURS IN TRIAL 1.

Concentration	Powder to water ratio
1. Weak	2.6g per litre of water
2. Medium	5.3g per litre of water
3. Strong	10.6g per litre of water

Both the banana and cherry flavours were synthetically produced, as is common in animal feeds; the banana flavour used Propylene Glycol as a carrier and the cherry flavour used Glycerine, Ethanol and water as carriers. Though banana and cherry flavoured products are currently commercially available in the animal feed market,

the flavoured powders used here were developed by NAF specifically for this study and have not been released commercially.

3.2.2 Experimental Design

An initial pre-trial was carried out before the trial began, in which the buckets were set out in the stable in a similar pattern to that proposed for the trial. This aimed to reduce exploratory behaviour following the presentation of a bucket configuration that may have been unlike what the horse was used to. The buckets in this training period did not contain any of the flavours.

Two Latin square design experiments were carried out, one for each of the flavours. Each trial had six horses, three repetitions, each one with a different bucket order, and three concentrations for each flavour, 6 horses x 3 concentrations x 3 repetitions, therefore n = 54. A diagram detailing the different bucket orders for each repetition can be seen in Appendix 1. As the two flavours were being trialled separately the trial was split into Trial 1a (banana flavour) and 1b (cherry flavour). To ensure preference was not down to bucket location the order of the buckets along the stable wall containing each concentration were changed for each repetition. This ensured that intake was due to concentration and reduce any possible influences of bucket location. This was done for both Trial 1a and 1b.

3.2.3 Animal Management

The 6 horses were selected at random from polo ponies from the Royal Agricultural University yard at Fossehill, Cirencester, Glos GL7 6JS, consisting of three mares and three geldings with an average age of 8.1 (±2.3) and a range of 5 to 11 years old. All ponies were of a similar height of approximately 15.2hh, were scored as 4 on the Henneke body condition scoring system (Appendix 2) and their training regime categorised as light to moderate work, in keeping with the stage of training for the

polo season. Each horse was maintained on a healthcare programme, monitored by their usual carer, and were deemed to be in good health prior to the beginning of the trial. All horses were fed a similar diet consisting of chaff and a concentrate mix, received unsoaked meadow hay as forage, hay was cut and baled on the RAU farm at Fossehill, and were stabled in similar loose –boxes (dimensions 3.6 x 3.6 m squared) in an American barn style stable block, and bedded on soaked wood pellet bedding, manufactured by Verdo based in Andover.

3.2.4 Trial Procedure

The horses began their one hour of exercise at 8am, with each repetition of offering the flavoured waters starting at 9am. Daily air temperatures ranged from 3.7^{o}c to 6.8^{o}c. As a result of exercise horses did not have access to water for approximately one hour prior to the beginning of the preference test. Three clearly labelled 12 litre buckets each containing 10 litres of water with a different dose of the flavour were placed 5 inches apart on the floor along one wall at the front of the stable; each horse kept the same three buckets throughout Trial 1a and 1b and each bucket was thoroughly cleaned between each repetition and between Trial 1a and 1b to avoid cross contamination between odours and flavours. The order of the concentrations changed each time and each repetition of the experiment is as follows:

- Repetition 1 = Weak – Medium - Strong
- Repetition 2 = Strong – Weak - Medium
- Repetition 3 = Medium – Strong – Weak

The buckets were spaced far enough apart so the horse had to make an effort to move from one to another, but not so far that it would discourage drinking from the bucket furthest to the horse. Each test period took four hours, and each horse underwent one repetition daily. No disruption was caused to the horse's normal routine. The trial was then repeated with the cherry flavour.

All horses underwent light exercise via a horse walker for one hour prior to the beginning of the trial and then were returned to their usual stable. The first ten minutes on returning to the stable was forage free. Ethogram 1 (table 2) was used to record the horses' initial reactions to each of the concentrations and whether or not they displayed a preference during this first ten minutes, this was named Observation period A. After 10 minutes a 4-6 kg hay net was given to each horse in order to determine if eating influenced fluid intake. Horses were then left for a further 10 minutes without observation. During the next 20-30 minutes, a second period, Observation B, began to determine if foraging influenced preference for flavour concentration. Checks were carried out to ensure that no water had been spilt or a horse had finished all of one bucket, bucket refills were unnecessary in trial one as no horse emptied any of the buckets. Ethogram 2 (table 3) was used in observation period B and was modified to include the consumption of forage.

TABLE 2. ETHOGRAM 1 – OBSERVING PREFERENCE AND DRINKING BEHAVIOUR (OBSERVATION A - MINUTE 1-10)

Behaviour	Description
Investigating weak concentration	Sticking muzzle into water bucket but not actually drinking
Investigating medium concentration	Sticking muzzle into water bucket but not actually drinking
Investigating strong concentration	Sticking muzzle into water bucket but not actually drinking
Drinking weak concentration	Consuming water from the bucket containing the weak concentration
Drinking medium concentration	Consuming water from the bucket containing the medium concentration
Drinking strong concentration	Consuming water from the bucket containing the strong concentration

TABLE 3. ETHOGRAM 2 – OBSERVING PREFERENCE AND DRINKING BEHAVIOUR IN RELATION TO FORAGING (OBSERVATION B - MINUTE 20-30)

Behaviour	Description
Investigating weak concentration	Sticking muzzle into water bucket but not actually drinking
Investigating medium concentration	Sticking muzzle into water bucket but not actually drinking
Investigating strong concentration	Sticking muzzle into water bucket but not actually drinking
Drinking weak concentration	Consuming water from the bucket containing the weak concentration
Drinking medium concentration	Consuming water from the bucket containing the medium concentration
Drinking strong concentration	Consuming water from the bucket containing the strong concentration
Foraging	Doing at least one of the following: • Investigating/manipulating material on the ground with muzzle • Pulling hay from hay net • Hay in mouth • Jaws moving in a lateral and medial direction

3.2.5 Measurements taken during each repetition

Forage intake was determined by weighing the hay net using a hanging weigh scales at the beginning and end of each trial repetition. Water intake in litres was measured at three points during each repetition, the buckets used displayed litre markers on the inside to ensure ease and efficiency of measuring. The amount of each concentration drunk was recorded after each of the observation periods, and at the end of the trial.

3.2.6 Data Analysis

As no previous information existed on water preference testing, experimental power (number of replicates necessary to detect significant differences) was chosen on previous experience. However, to test the ideal replicate number a post-trial power-based calculation was carried out to ensure the sample size used was sufficient to pick up any significant differences, should there be any. The formula used was as follows (Cornish, 2006):

$$n = f(a\beta)\frac{2s^2}{\delta^2}$$

f(α,β) is a value calculated from α and β, where α is the significance level, set at 0.05. β is the power, which was set at 90%, allowing the use of f=10.5 from table 4 below.

TABLE 4.

	β			
α	**0.05**	**0.1**	**0.2**	**0.5**
0.05	13.0	10.5	7.9	3.8
0.01	17.8	14.9	11.7	6.6

δ is the smallest difference in means which was chosen to be 5 litres. Finally, s, is the standard deviation of 2.4, this has been calculated post trial 1 using voluntary fluid intake data across both flavours and concentrations. Therefore:

$$4.83 = 10.5\frac{11.52}{25}$$

The fluid intake data was tested for normality using a Q-Q (Quantile-Quantile) plot. To determine if bucket location influenced intake a repeated measures ANOVA (Genstat 18) was used. As this test showed no significant influence of location, a

one-way ANOVA was done with horse and concentration as factors thus n=54. Spearman's rank correlation was used to assess the relationship between hay intake and fluid intake per repetition for both the banana and cherry flavour separately.

3.3 Results - Trial 1a and 1b - Flavoured Water Concentration Preference

3.3.1 Analysis of the flavour concentrations

The power calculation indicated that the minimum sample size for this trial was 5, thus the 6 horses used in this trial were sufficient to detect if any preferences were demonstrated between flavour concentrations.

Data for both trials were normally distributed as shown by Q-Q plots (Appendices 3&4). The QQ plots revealed that both flavours had high r^2 values; 0.83 for banana and 0.90 for cherry, and neither have any significant outliers. All horses sampled all 3 concentrations offered; 1= weak (2.6g per litre), 2= medium (5.3g per litre), and 3= strong (10.6g per litre), for both flavours.

The results for the volumes of banana flavoured water drunk are shown in Figure 1 below. No significant preference was noted for a particular concentration. Although the individual variation for the amount of each concentration drunk was high, this was the case for each concentration so amount drunk was due to animal variation rather than concentration of the flavour.

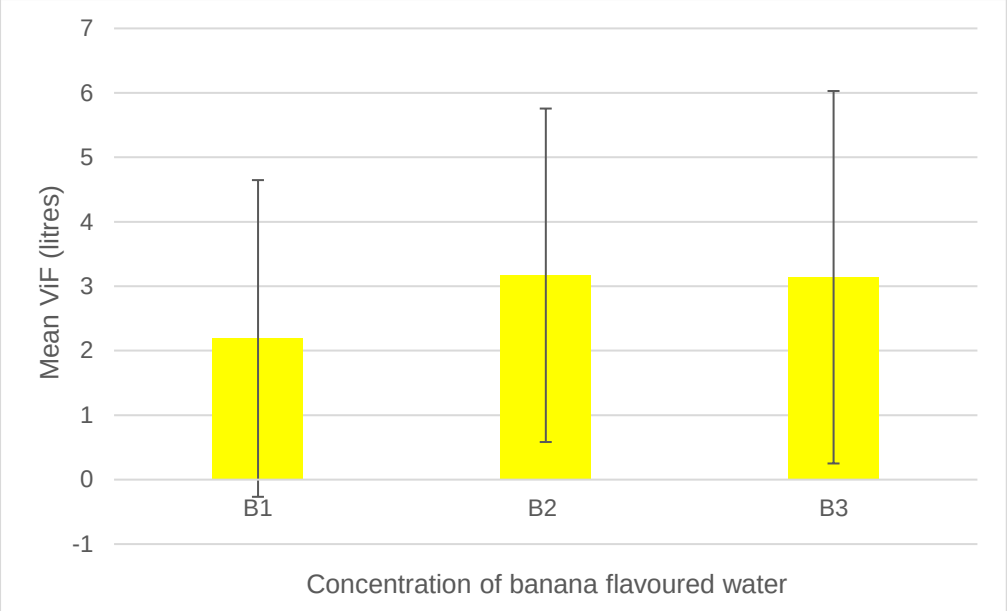

FIGURE 1. MEAN VOLUNTARY FLUID INTAKES (± SD) FOR 6 HORSES WHEN OFFERED 3 CONCENTRATIONS B1 = WEAK, B2 = MEDIUM AND B3 = STRONG FOR BANANA FLAVOURED WATER ON 3 SEPARATE OCCASIONS.

The results for the amount of cherry flavoured water drunk are shown in Figure 2 below. Again, no significant preference was noted for a particular concentration. There was a high individual variation for the amount of each concentration drunk, particularly for C3 but again due to animal variation rather than concentration of the flavour.

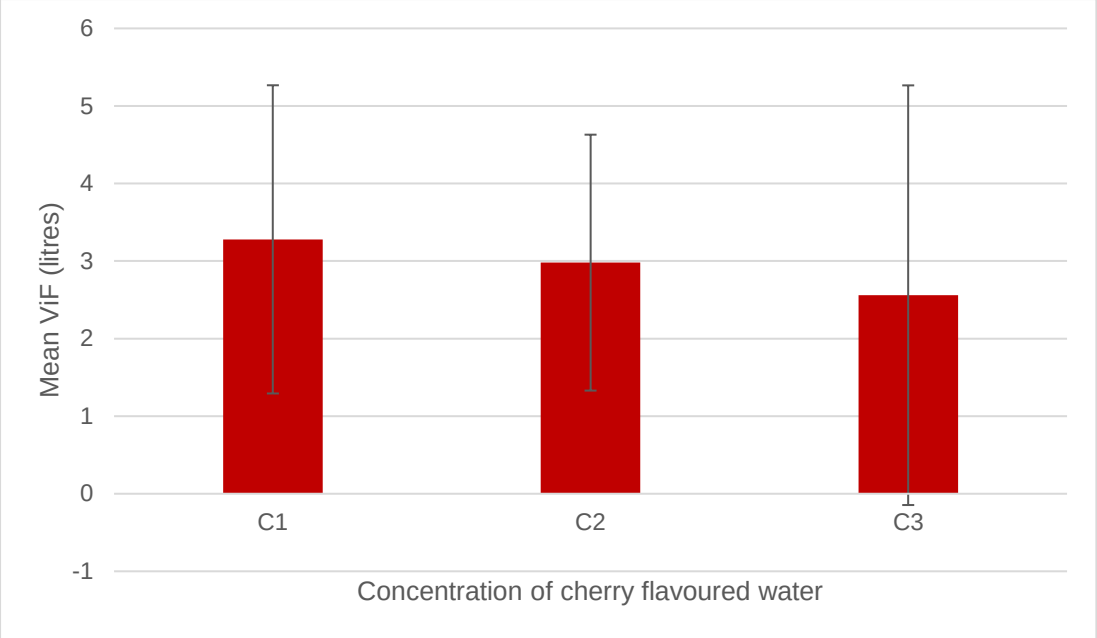

FIGURE 2. MEAN VOLUNTARY FLUID INTAKES (± SD) FOR 6 HORSES WHEN OFFERED 3 CONCENTRATIONS C1 = WEAK, C2 = MEDIUM AND C3 = STRONG FOR CHERRY FLAVOURED WATER ON 3 SEPARATE OCCASIONS.

3.3.2 Voluntary fluid intake in relation to the repetitions for the banana flavour (Trial 1a)

As detailed in Table 5, horses did not show any positional preference for drinking that might have confounded the results on preferred concentration of the banana flavour.

TABLE 5. MEAN VOLUNTARY FLUID INTAKE (LITRES) (+/-SD) FOR EACH CONCENTRATION, WEAK, MEDIUM AND STRONG ACROSS 6 HORSES FOR EACH REPETITION IN TRIAL 1A

	1. Weak	**2. Medium**	**3. Strong**	**S.E.D**
Repetition 1	0.83(+/-1.60)	2.67(+/-2.58)	2.92(+/-3.29)	0.80
Repetition 2	3.25(+/-3.02)	3.33(+/-2.63)	2.08(+/-2.24)	0.58
Repetition 3	2.75(+/-2.20)	4.58(+/-2.61)	4.42(+/-3.02)	1.27

3.3.3 Voluntary fluid intake in relation to the repetitions for the cherry flavour (Trial 1b)

For the cherry flavour, none of the 6 horses showed any positional preference for drinking that biased the results on preferred concentration. Table 6 below shows that no horse appeared to prefer a particular bucket location and voluntary fluid intake of each concentration was not affected by a favoured bucket location.

TABLE 6. MEAN VOLUNTARY FLUID INTAKE (LITRES) FOR EACH CONCENTRATION (+/-SD) ACROSS 6 HORSES FOR EACH REPETITION FOR TRIAL 1B

	1. Weak	**2. Medium**	**3. Strong**	**S.E.D**
Repetition 1	3.00(+/-2.52)	3.17(+/-2.06)	2.83(+/-2.33)	0.559
Repetition 2	3.33(+/-1.75)	3.33(+/-1.88)	2.17(+/-2.62)	0.642
Repetition 3	3.50(+/-1.94)	2.45(+/-0.94)	2.67(+/-3.50)	1.019

3.3.4 Voluntary fluid intake for the three concentrations of banana flavoured water (Trial 1a)

The mean ViF values across the 6 horses for the 3 concentrations of banana were not different from each other, P=0.376, as shown in Table 7 below. Therefore, the null hypothesis of ‘There will be no difference in the intake of fluid for the banana flavoured water according to the concentration of the mixture’ was accepted. The medium concentration proved to be the most consumed, so this was the concentration that was chosen for the flavour preference trial (Trial 2).

TABLE 7. MEAN VOLUNTARY FLUID INTAKE (LITRES) (+/-SD) FOR 6 HORSES WHEN OFFERED 3 DIFFERENT CONCENTRATIONS OF BANANA FLAVOURED WATER.

Banana concentration	**1. Weak**	**2. Medium**	**3. Strong**	**S.E.D**	**P value**
Mean ViF (litres)	2.19(+/- 2.45)	3.17(+/-2.58)	3.14(+/-2.88)	0.783	0.376
Median ViF (litres)	1.5	3	2.25	-	-

3.3.5 Voluntary fluid intake for the three concentrations of cherry flavoured water (Trial 1b)

From Table 8 below, it can be seen that like banana, the mean ViF values for the 3 concentrations of the cherry flavour are not statistically significant (P= 0.428). Therefore, the null hypothesis of 'There will be no preference in concentration for cherry' was accepted. The weak concentration was the most preferred so was chosen for the flavour preference trial (Trial 2).

TABLE 8. MEAN VOLUNTARY FLUID INTAKE (LITRES) (+/-SD) FOR 6 HORSES WHEN OFFERED 3 DIFFERENT CONCENTRATIONS OF CHERRY FLAVOURED WATER.

Cherry concentration	**1. Weak**	**2. Medium**	**3. Strong**	**S.E.D**	**P value**
Mean ViF (litres)	3.28(+/-1.98)	2.98(+/-1.65)	2.56(+/-2.7)	0.553	0.428
Median ViF (litres)	3.5	3	1.5	-	-

3.3.6 Voluntary fluid intake and hay intake data for both flavours across all 3 repetitions

Table 9 below shows that for the banana flavoured water repetitions, there is a significant relationship between the amount of forage consumed and voluntary fluid intake, this positive correlation is shown in Figure 3, as hay intake increases so does total fluid intake. There is no such relationship for the cherry flavoured water repetitions (Figure 4).

TABLE 9. SPEARMAN'S RANK OUTPUT FOR THE RELATIONSHIP BETWEEN HAY INTAKE AND TOTAL FLUID INTAKE FOR BOTH FLAVOURS ACROSS ALL 3 REPETITIONS

	P Value	**Correlation value**
Banana flavoured water	0.002*	0.6
Cherry flavoured water	0.182	0.089

*denotes significant relationship

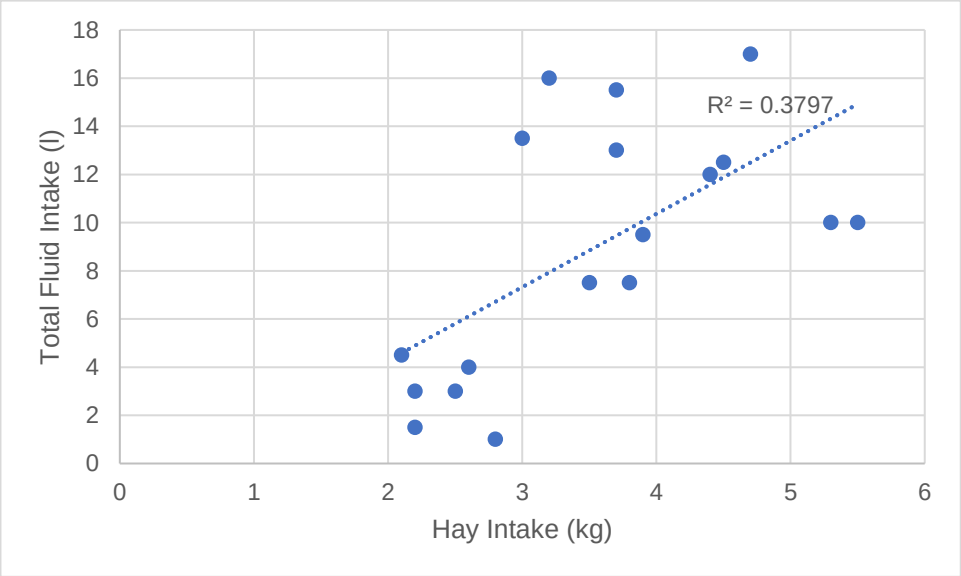

FIGURE 3 SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN HAY INTAKE AND TOTAL FLUID INTAKE FOR ALL 6 HORSES ACROSS ALL 3 REPETITIONS FOR THE BANANA FLAVOUR

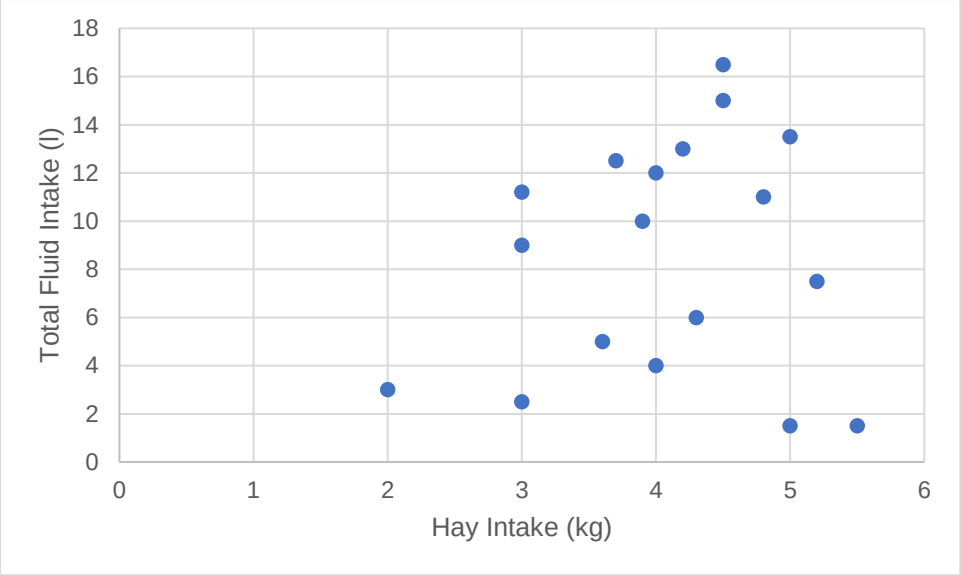

FIGURE 4. SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN HAY INTAKE AND TOTAL FLUID INTAKE FOR ALL 6 HORSES ACROSS ALL 3 REPETITIONS FOR THE CHERRY FLAVOUR

3.3.7 Observational data on drinking behaviour across the 3 replicates for before forage and during forage periods for Trial 1a

As can be seen from Figures 5 and 6 below, there were very few investigations of the buckets or drinking episodes made in either of the observation periods. The medium concentration in observation period A was investigated the most and subsequently had the equal highest number of drinking episodes.

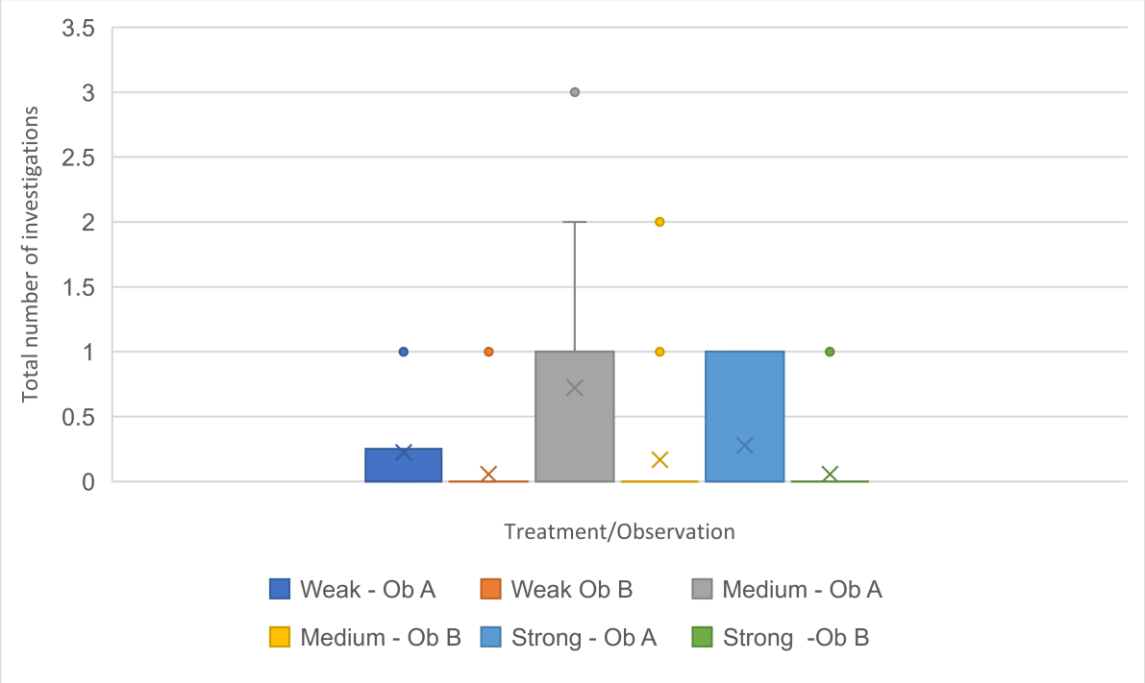

FIGURE 5. BOX AND WHISKER PLOT SHOWING THE TOTAL NUMBER OF INVESTIGATIONS MADE BY THE 6 HORSES TO EACH OF THE FLAVOUR CONCENTRATIONS DURING OBSERVATION PERIODS A (BEFORE FORAGE) AND B (DURING FORAGE) ACROSS ALL 3 REPETITIONS FOR THE BANANA FLAVOUR

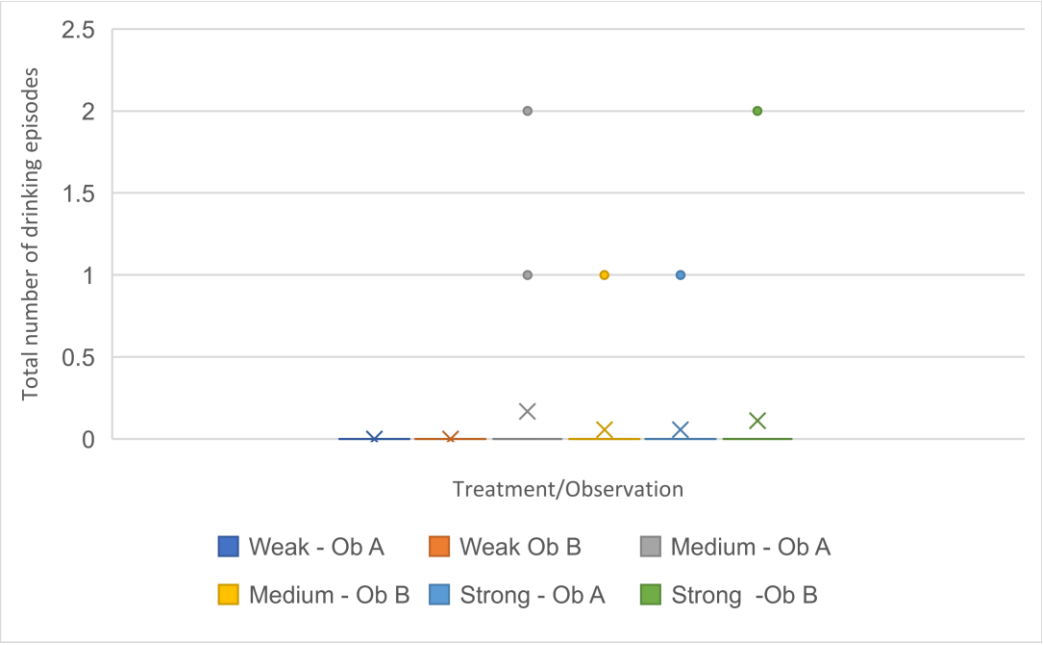

FIGURE 6. BOX AND WHISKER PLOT SHOWING THE TOTAL NUMBER OF DRINKING EPISODES THAT OCCURRED DURING OBSERVATION PERIODS A (BEFORE FORAGE) AND B (DURING FORAGE) ACROSS ALL 3 REPETITIONS FOR THE BANANA FLAVOUR

Table 10 below shows that no fluid was consumed by any of the 6 horses during the first observation period and very little was consumed during observation period B across all 3 repetitions.

TABLE 10. TOTAL AMOUNT OF FLUID CONSUMED BY ALL 6 HORSES AFTER EACH OBSERVATION PERIOD ACROSS ALL REPETITIONS FOR THE BANANA FLAVOUR

Banana	**Total amount drunk after observation A (litres)**	**Total amount drunk after observation B (litres)**
Weak	0	1
Medium	0	1
Strong	0	0.5

3.3.8 Observational data on drinking behaviour across the 3 replicates for before forage and during forage periods for Trial 1b

Like the banana flavour, the number of investigations of the buckets made by the six horses during both observation periods was low (Figure 7). No drinking episodes from any of the cherry flavour concentrations occurred during either of the observation periods. However, some fluid was drunk in between the two observation periods, between minutes 10 to 20, as Table 11 shows.

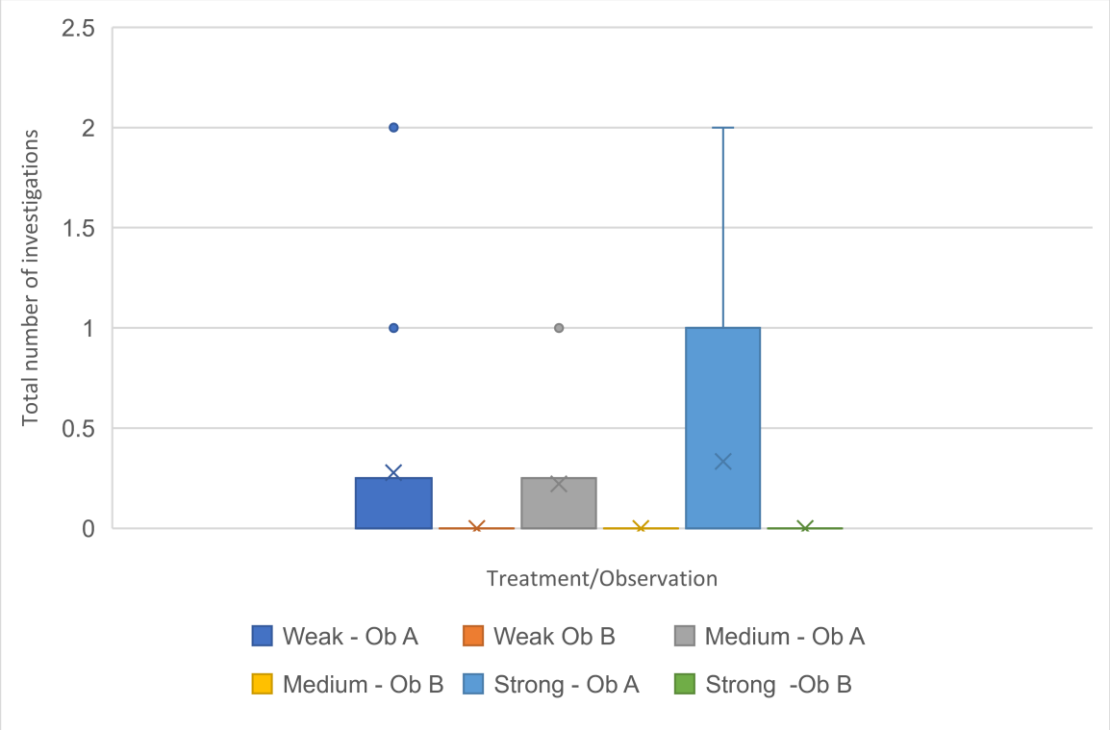

FIGURE 7. BOX AND WHISKER PLOT SHOWING THE TOTAL NUMBER OF INVESTIGATIONS MADE BY THE 6 HORSES TO EACH OF THE FLAVOUR CONCENTRATIONS DURING OBSERVATION PERIODS A (BEFORE FORAGE) AND B (DURING FORAGE) ACROSS ALL 3 REPETITIONS FOR THE CHERRY FLAVOUR

TABLE 11. TOTAL AMOUNT OF FLUID CONSUMED BY ALL 6 HORSES AFTER EACH OBSERVATION PERIOD ACROSS ALL REPETITIONS FOR THE CHERRY FLAVOUR

CHERRY	Total amount drunk after observation A (litres)	Total amount drunk after observation B (litres)
Weak	0	0
Medium	0	0.5
Strong	0	2

3.3.9 Summary of results from Trials 1a and 1b

It can be seen in Figure 8 that overall horses consumed more of the banana flavour than cherry; the total ViF for the banana flavour was 2.3 litres higher than the cherry flavour Apart from the weakest concentration, in which the mean ViF was higher for cherry, the banana flavour had higher mean ViF for the medium and strong concentrations.

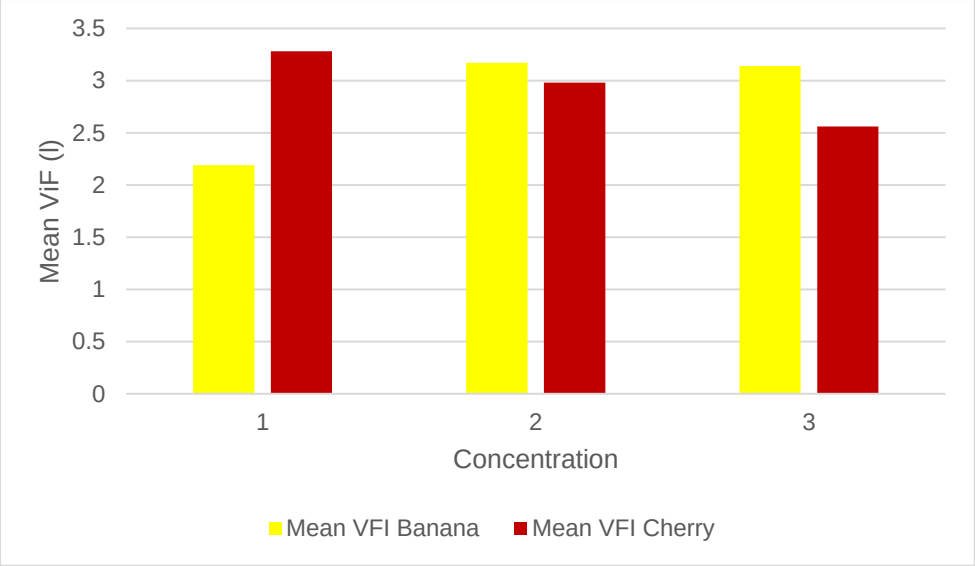

FIGURE 8. GRAPH COMPARING THE MEAN VOLUNTARY FLUID INTAKES FOR EACH CONCENTRATION OF EACH FLAVOUR; 1= WEAK, 2= MEDIUM, AND 3= STRONG

3.4 Discussion

3.4.1 Water intake levels

The horses used in this trial were approximately 500kg in bodyweight, so their water requirements, if calculated on the NRC (2015) recommendations would have been 25 litres /day. In this trial the average water intake across the six horses was 8.9 litres (banana) and 8.8 litres (cherry) over a 4-hour period. If the horses continued to drink at the same rate over the entire 24-hour period, they would have consumed 54 litres, however, it is highly likely that as with feed intake water intake is spasmodic and drops during the night-time, therefore the fluid intakes noted in these trials are likely to be in line with normal consumption. It is well documented that offering water from buckets will promote fluid intake compared with automatic drinkers (Nyman and Dahlborn, 2001; McDonnell *et al.* 1999); the choice in this trial of using 12-litre capacity buckets seemed suitable to encourage drinking.

The aim of offering *ad libitum* forage was to trigger thirst so that horses would want to drink. As the horses had minimal access to pasture, which has been found to

contribute highly to daily water intake as pasture has a high moisture content which allows the horse to use metabolic water to hydrate itself; it could be thought that the horses in this trial would need to drink more water as a forage diet which is high in dry matter and fibre triggers a higher water intake as it increases excretion; high water intakes have been attributed to forage diets high in fibre (Warren *et al.,* 1999). Danielsen *et al.* (1995) also reported high water intakes overnight in horses given forage the previous evening. It is suggested that the high fibre content in forage such as hay increases water in the large intestine (Cuddeford *et al.*, 1992), this water must be drawn from the blood into the gut, and results in a decrease in plasma volume and triggers a greater thirst response (Pagan, 2008). Sweeting and Houpt (1987) also found that hay intake increased water intake in a weeklong study. It is worth noting that there is little work done on hay and water intakes in the short term. Whilst the data for the banana flavour supports the theory that forage increases water intake, the data for the cherry flavour does not; however despite the apparent relationship in the banana flavour repetitions, hay and fluid intake values were not noticeably different from the cherry flavour repetitions, so the lack of relationship in the cherry repetitions is difficult to explain and intake appears random.

3.4.2 Influence of positional preference and other controls on intake

Horses had no preference in the location or order of the buckets for either the banana or cherry flavours. Though the preferred concentration for the banana flavour varied slightly across all 3 repetitions, on average the most preferred concentration was the medium strength so was chosen for Trial 2. In all the repetitions for the cherry flavour, ViF for the weakest concentration remained higher than the other two and so was chosen for Trial 2. For the cherry flavour, some of the difference between repetition 1 and 3, could have been caused by, a lower than average intake

for one horse 2 who drank very little from all concentration in repetition 3. Unrecorded factors on these days such as the weather or the horse's hydration status could have affected intake. Cymbaluk (1990) found that voluntary water intake is directly related to ambient temperatures; and showed that cold temperatures can reduce water intake by 6-14%. As the trial took place from November to February, with average monthly temperatures of 3.7 to 6.8 ^{o}c this may have influence some of the horses to drink less on certain days. Temperature of the water can also control drinking. As the water used in was in keeping with the ambient air temperature, approximately 5^{o}c, which may have influenced the amount of water consumed by some horses. Kristula and McDonnell (1994) found that ponies during cold weather conditions of -7^{o}c to 5^{o}c when provided with buckets of hot water at 46-49^{o}c twice daily, drank 38% more water than when provided with near freezing water. However, overall horses water intake in this trial was not below the recommendation set by NRC (2015) so while it might have been desirable in terms of stimulating choice for them to drink more, clearly neither the air or water temperature significantly affected intake. In future preference testing, it might be wise to take into account that horses prefer warm water in cold weather. It might also be wise to induce a strong thirst response by working the animal in order to induce sweating as this would also stimulate the thirst response. However, the fact that horses drank normally suggests that they did not indeed have a strong preference for any concentration.

3.4.3 Factors affecting fluid intake

The results presented here indicate that there was no pattern in drinking behaviour across the repetitions for the banana or cherry flavour, and ViF was not influenced by bucket positioning. In humans, mood has been shown to affect decision making (Yuen and Lee, 2003) with a positive correlation reported between mood and the risk

taking. The ‘mood’ of each horse could have impacted their choices in this trial with a more nervous, agitated horse less likely to consume a new substance. This ‘neophobia’ (Van den Berg and Hinch, 2016), could have the same effect for fluid intake as in this trial. With the introduction of a new odour, a large variation was initially recorded, and some of horses displayed a neophobic response whereas others did not; this variation in the horses’ initial response could be due to their individual dispositions and whether they are tolerant of changes being introduced into their environment. Very little exploratory behaviour was recorded during the current study; the lack of drinking episodes in the observation periods could indicate that many of the horses were intolerant of the unfamiliar solutions after having investigated them.

Although no significant difference in intake was noted between the three concentrations there was a slight increase in ViF for the medium concentration of the banana flavour. Standard deviations across repetitions were high and this could account for no clear difference seen. For example, horses 3 and 4 consumed much less fluid during each repetition than others; this could be due to a dislike of the banana flavour overall or their hydration status going into each repetition, if they were more hydrated to begin with, they might drink less, as hydration status was not measured for trial 1 we cannot be sure exactly what caused these two to drink less. On occasion, others drank much higher volumes in total; during repetition 3, horse 6 drank high volumes of the weak and strong concentrations, and horse 2 drank a high volume of the weak concentration, horse 5 showed a high intake for the medium concentration in repetition 2. Individual results such as these have the power to slightly skew the overall results and affect the mean ViF for each concentration. The mean voluntary fluid intake for the weak concentration for the cherry flavour was the

highest and the variation between the means for each concentration was more uniform. As with the banana flavour, the standard deviation from the mean of each concentration of cherry was more variable. Again, these variations could be due to individual taste preferences and water requirements. As the study had sufficient power to detect differences, these results are likely to be repeatable with any group of horses and highlight the difficulty of a ‘one size fits all’ flavour for animals with inherent individuality.

The restriction of water for one hour prior to the beginning of each repetition did not appear to alter drinking behaviour in any way; and the purpose of withholding forage for the first ten minutes of the trial was to see whether the horses had an immediate desire to drink on returning to the stable after exercise without the distraction of forage. Little was observed during the two observation periods at the beginning of the trial. No horse went straight to investigate or drink from the buckets and very few appeared to pay much attention to them during the observational periods. This could be because the horses did not have enough time to develop thirst before the beginning each repetition, withholding water for longer or more intense exercise in the hour before the trial might have resulted in the horses developing a greater thirst. The data shows that with both flavours, horses only began to drink after a longer period of time, as the data collected after the two observation periods in the first half hour of the trial showed that almost no water was consumed in this time. As forage was added after 10 minutes, and the next recording of water intake took place 20 minutes after this, it could be said that the horses needed time for the thirst response to be stimulated after beginning to eat forage. As discussed in section 3.4.1, many studies on hay and water intake are conducted over a longer time frame; and this should be taken into account in future trials.

In conclusion, there were no significant preferences for any of the concentrations for either flavour, this suggests that the horses may in fact not find these flavours palatable; if they were to find them palatable, a preference for either the strong or medium concentrations might be displayed. Although not significant, the preferred concentration for the banana flavour was the medium concentration, and for cherry the weak, and these will be used in trial 2.

Chapter 4: Trial 2 – Voluntary fluid intake by 12 horses when offered banana, cherry and plain water

4.1 Introduction

The purpose of this trial was to investigate if the two flavoured waters resulted in a greater fluid intake than plain water when offered to 12 horses. The optimum concentration for the two flavours determined in Trial 1 were used. The null hypothesis was 'There is no difference in the intake of fluid when horses are offered plain water, cherry flavoured water or banana flavoured water'. The Animal Ethics Review group of the Royal Agricultural University approved the experimental design and procedures.

4.2 Methodology

4.2.1 Experimental Design

The trial was run as a randomised block design experiment, consisting of 12 horses, three treatments (plain water, banana flavour, and cherry flavour), with 3 repetitions per horse, each one with a different bucket order, therefore n=36. The three repetitions had all three treatments offered simultaneously but the positioning of the buckets was different for each repetition to eliminate any positional preferences (Appendix 5).

4.2.2 Animal Management

The 12 horses, consisting of five mares and seven geldings with an age range of 5 to 19 years; the average age being 10.5(±4.8), were a combination of polo ponies from the Royal Agricultural University yard at Fossehill, Cirencester, Glos GL76JS, and event horses from a private family-run yard, Minety, Wilts SN169RJ. The horses' height ranged from 15hh to 17hh. All were in moderate to heavy work, with

bodyweight ranging from 441 to 632 kg. The trial took place during the summer months of June and July, so animals were at the height of their competition seasons, so all of them had a regular daily routine. Each horse was maintained on a healthcare programme and their daily health monitored by their usual carer. Nine out of the 12 horses were stabled in an American barn style stable block stabled in loose boxes (dimensions 3.6 x 3.6 m squared) in, and the remaining three in a row of stables facing outwards (dimensions 4 x 4m squared), all were bedded on dust free wood shavings, made by Equisupplies based in Brinkworth. All horses were fed their usual concentrate feeds and received dry hay as forage, hay was cut and baled on the RAU farm at Fossehill. The trials were conducted in the horse's usual stable and familiar surroundings.

4.2.3 Trial Procedure

Daily air temperatures ranged from 9.8^{o}c to 21^{o}c. The horses began their one hour of exercise at 8am, with each fluid choice repetition starting at 9am. The horses did not have access to water for two hours prior to the beginning of each measurement period. On data collection days a standardised exercise test (S.E.T) was carried out at 8am, this aimed to ensure all the horses were given the same exercise The S.E.T was 30 minutes in duration, consisting of ten minutes of walk, then five minutes of trot, then five of walk, then five of trot, then a final five of walk. Once completed, the horses were returned to their stable which were free from water and forage for a further 30 minutes. At 9am three clearly labelled 12 litre buckets each containing 10 litres of water with either banana flavour (5.3g per litre of water), cherry flavour (2.6g per litre of water), or plain water were placed on the floor 10 inches apart along the front wall of the stable; each horse kept the same three buckets throughout the trial; the buckets were cleaned thoroughly between each repetition to avoid cross

contamination of odours and flavours. As this bucket formation would be new to the horses, the day before beginning the first repetition, three buckets of water were placed in the layout used for the experiment so that horses would be familiar with the layout and not put off by a new bucket arrangement during the trial. Like trial 1, each test period took four hours, each horse undergoing one repetition daily, to reduce any potential disruption to the horse's normal routine. The horses were tested in groups of 3, 3, 2, then 4, and followed the same order of repetitions, which is as follows:

- Repetition 1 = Banana – Cherry – Plain Water
- Repetition 2 = Plain Water – Banana – Cherry
- Repetition 3 = Cherry – Plain Water – Banana

As with trial 1, the observation periods were kept at the duration of 10 minutes, however the timing of the second observation period was changed to later in the repetition, as almost all the horses in trial 1 only foraged in the second observation period as the forage had just been introduced, so moving the second period to later for trial 2 would allow the horses more time to forage and potentially develop thirst. At the beginning of each repetition, the first ten minutes were forage free, to see if the horses had an immediate desire to drink having been without water for the previous two hours. During this 10 minutes Ethogram 1 was used to record any initial reactions to the buckets and if an initial preference was apparent, Observation period A. Following observation period A, a 4-6 kg hay net was given to each horse in order to determine if eating influenced fluid intake. At the beginning of the second hour, a second 10-minute observation period was conducted, Observation period B, and aimed to spot any preferences now that the horses had been eating forage for some time. The ethogram used in this second observation period was the same as used in

the second observation period in Trial 1(Ethogram 2). Consistent with the Randomised Block Design, the repetition was changed another twice, so each flavour had been offered from a different location to ensure horses were not choosing a bucket based on location preference.

4.2.4 Measurements taken during each repetition

As in trial 1, Ethograms 1 and 2 provided a tally of behaviours, allowing any traits that would indicate a preference to be recorded. Forage intake was determined by weighing the hay net using a hanging weigh scales at the beginning and end of each trial repetition. Fluid intake in litres was measured at three points during each repetition. The amount of each fluid drunk was recorded after each of the observation periods, and at the end of the trial; the buckets used displayed litre markers on the inside to ensure ease and efficiency of measuring. In addition, the pH and temperature of each bucket was recorded at the beginning of each repetition to maintain consistency, as both pH and temperature can affect water intake. pH was measured using a TESTWEST Multi-Purpose Digital pH Meter and temperature with a THERMOMETERS DIRECT Digital Pocket Style Thermometer. Weather measurements such as air temperature, measured using a dry bulb temperature in ^{o}C, and humidity was calculated by temperature measured as dry and wet bulb and then humidity was calculated as a percentage using Table 12 below.

TABLE 12. RELATIVE HUMIDITY CHART

Dry Bulb (°C)	Number of degrees difference between the wet- and dry-bulb readings (°C)									
	1	2	3	4	5	6	7	8	9	10
10	88%	77	66	56	45	35	26	16	7	--
11	89	78	67	57	47	38	28	19	11	2
12	89	79	68	59	49	40	31	22	14	5
13	89	79	69	60	51	42	33	25	16	9
14	90	80	70	61	52	43	35	27	19	11
15	90	80	71	62	54	45	37	29	22	14
16	90	81	72	63	55	47	39	31	24	17
17	91	82	73	64	56	48	41	33	26	19
18	91	82	73	65	57	50	42	35	28	21
19	91	82	74	66	58	51	44	37	30	24
20	91	83	75	67	59	52	45	38	32	26
21	91	83	75	68	60	53	47	40	34	27
22	92	84	76	69	61	54	48	41	35	29
23	92	84	77	69	62	56	49	43	37	31
24	92	84	77	70	63	57	50	44	38	32
25	92	85	77	71	64	57	51	45	40	34
26	92	85	78	71	65	58	52	46	41	35
27	93	85	78	72	65	59	53	47	42	37
28	93	86	79	72	66	60	54	49	43	38
29	93	86	79	73	67	61	55	50	44	39
30	93	86	80	73	67	61	56	50	45	40
31	93	86	80	74	68	62	57	51	46	41
32	93	87	80	74	68	63	57	52	47	42
33	93	87	81	75	69	63	58	53	48	43
34	93	87	81	75	69	64	59	54	49	44

https://www.test-and-measurement-world.com/Terminology/Relative-Humidity-Table-or-Chart.html

Weather measurements were recorded on each day the trial took place as this too can influence how much a horse will drink; local data from the RAU weather station, located in Cirencester, just a few miles from the site where the experiment took place, was used. Finally, each horse was weighed at the beginning of the trial using a portable weigh bridge.

4.2.5 Data Analysis

The sample size was increased from 6 horses in trial 1 to 12 horses in trial 2 so as to ensure if there was a significant result it would not be missed due to a too small sample size, a post-trial calculation was performed to check the sample size used in trial 2 was accurate.

The same formula was used (Cornish, 2006):

$$n = f(a\beta)\frac{2s^2}{\delta^2}$$

TABLE 13.

	β			
α	**0.05**	**0.1**	**0.2**	**0.5**
0.05	13.0	10.5	7.9	3.8
0.01	17.8	14.9	11.7	6.6

f(α,β) is a value calculated from α and β, where α is the significance level, as with Trial 1 this was set at 0.05, β is the power, again set at 90%, allowing the use the value of f=10.5 from Table 13 above. δ is the smallest difference in means, as with trial 1, this has been chosen to be 5 litres. Finally, s, is the standard deviation of 3.1, this has been calculated post trial 2 using voluntary fluid intake data from trial 2 across all treatments. Therefore:

$$8.07 = 10.5\frac{19.22}{25}$$

This indicates that the ideal sample size for trial 2 would be minimum of eight horses to detect any significant differences; as 12 horses were used in trial 2, the sample size used here gave sufficient replicates to detect a significant difference in fluid intakes.

As with Trial 1, the data was tested for normality using a Q-Q plot. Data for this trial was not normally distributed and showed a positive skew with a r^2 value of 0.54 as shown by a Q-Q plot (Appendix 6). There are several outliers that stray higher than the trend line, most noticeably one voluntary fluid intake of 20 litres. Therefore, data was analysed using the General Linear Mixed Model (Genstat, 18) with $P<0.05$ taken as the level of significance; factors are 12 horses, 3 treatments x 3 replicates. As a significant difference was found between treatments, and the null hypothesis for trial 2 rejected, a post hoc 95% confidence interval test was carried out to identify differences in flavour preference. The GLME model also allowed for identification of any positional preferences in the data. Spearman's rank correlation was used to assess the relationship between hay intake and fluid intake per repetition.

4.3 Results Trial 2 – Flavour Preference

4.3.1 Sample Size and data analyses

The power calculation indicated that the sample size for this trial was 9; thus the 12 horses used in this trial were sufficient to detect if any preferences were demonstrated between the flavoured waters and plain water. Eleven out of the 12 horses drank from all three of the available treatments of banana favoured water (B), cherry flavoured water (C), and plain water (W), only one horse completely rejected the banana flavoured water on all three repetitions.

4.3.2 Voluntary fluid intake for the three solutions; banana flavoured water, cherry flavoured water and plain water

The ViF values differed between the water and the other two treatments $P = < 0.001$, with horses drinking an average of 5.33 litres of plain water which was more than double that of the other two flavours, (Table 14). A post hoc 95% confidence interval

test was calculated from the model predicted means and standard error. Table 14 below shows that the significant difference between the treatments is between plain water and the two flavoured water treatments. The null hypothesis of 'There will be no preference between banana flavoured water, cherry flavoured water, and plain water' must be rejected as the fluid intake difference was significant. This trial showed that horses prefer plain water, and fluid intake was considerably higher than for the two flavoured waters. Though the horses did not drink large amounts of the flavoured waters, intake for these two treatments ranged from 0 to 8 litres over the four-hour trial period, they did seem to be drinking enough to fulfil their hydration needs.

TABLE 14. MODEL PREDICTED MEAN AND ACTUAL MEAN VOLUNTARY FLUID INTAKE (LITRES) (+/-SD) AND CONFIDENCE INTERVALS FOR 12 HORSES WHEN OFFERED BANANA FLAVOURED WATER, CHERRY FLAVOURED WATER, AND PLAIN WATER ON 3 SEPARATE OCCASIONS.

	1. Banana	2. Cherry	3. Plain Water	S.E.D	P value
Model predicted mean ViF	0.73_a (0.28 - 1.17)	0.79_a (0.34 - 1.23)	1.67_b (1.23-2.12)	0.524	<0.001
Actual mean ViF (litres)	2.07(+/-2.34)	2.19(+/-2.17)	5.33(+/-4.49)	-	-

[ab] Values in the same row not sharing common letters differ significantly (P<0.0%)

The original scale means are the back-transformed means calculated by the GLMM

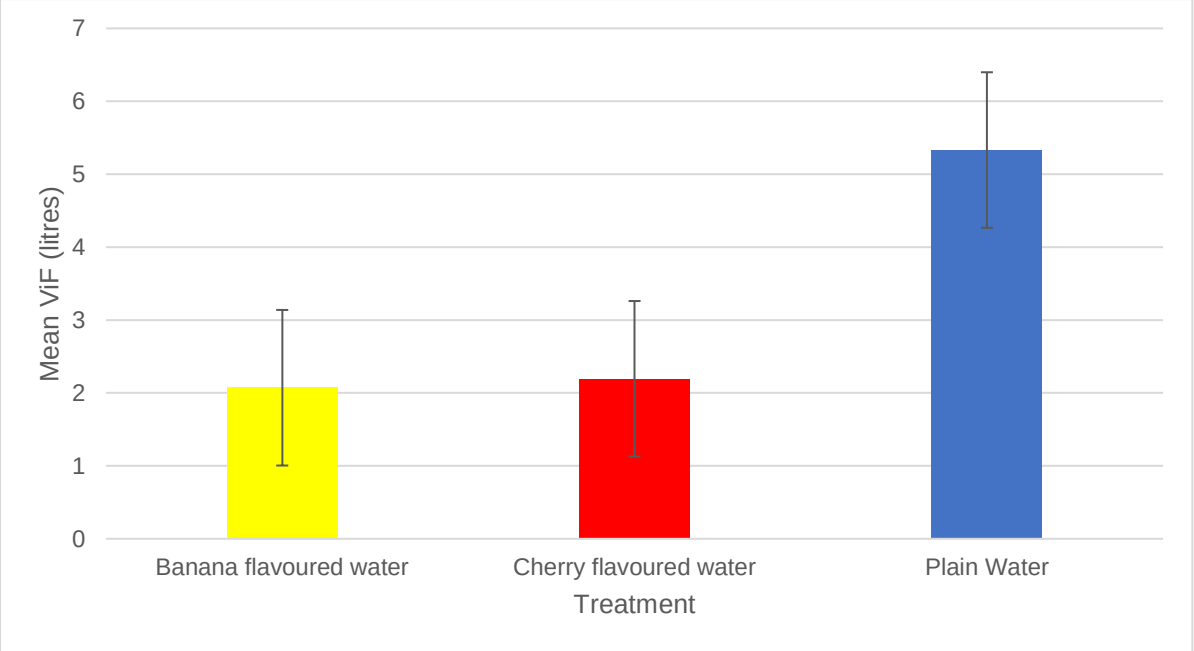

FIGURE 9. MEAN VOLUNTARY FLUID INTAKES (LITRES) (± SD) FOR 12 HORSES WHEN OFFERED 3 TREATMENTS; BANANA FLAVOURED WATER, CHERRY FLAVOURED WATER, AND PLAIN WATER ON 3 SEPARATE OCCASIONS.

4.3.3 Different intakes across Repetitions and Treatment

No significant differences in ViF were found between each repetition, or between treatment and repetition; with p values of 0.88 for treatment, and 0.3 for repetition. All repetitions had a similar mean voluntary fluid intake, as shown in Table 15.

TABLE 15. MEAN VOLUNTARY FLUID INTAKE (LITRES) FOR 12 HORSES ACROSS ALL 3 TREATMENTS WHEN LIQUID WAS OFFERED FROM 3 DIFFERENT POSITIONS IN THE STABLE (+/-SD)

	Model predicted mean ViF	**Actual mean total ViF (litres)**
Repetition 1	1.07	2.91(+/-3.36)
Repetition 2	1.08	2.96(+/-2.99)
Repetition 3	1.04	2.82(+/-3.98)
S.E.D	0.23	

The actual means are the back-transformed means calculated by the GLMM

In addition, no treatment was significantly preferred in one repetition, as can be seen in Table 16, the model predicted means for treatment per repetition do not differ largely from one another; it can also be said that no horses displayed a preference for a particular bucket location. Although it is clear that across all 3 repetitions, plain water had the highest ViF.

TABLE 16. MEAN VOLUNTARY FLUID INTAKE (LITRES) FOR EACH TREATMENT PER REPETITION (+/-SD) ACROSS 12 HORSES

	Banana-Model predicted mean ViF	**Banana-Actual mean ViF (litres)**	**Cherry-Model predicted mean ViF**	**Cherry-Actual mean ViF (litres)**	**Plain Water-Model predicted mean ViF**	**Plain Water-Actual mean ViF (litres)**
Repetition 1	0.88	2.41(+/-2.54)	0.62	1.89(+/-1.85)	1.70	5.48(+/-4.20)
Repetition 2	1.02	2.78(+/-2.62)	0.71	2.03(+/-1.71)	1.52	4.57(+/-3.87)
Repetition 3	0.28	1.33(+/-1.70)	1.02	2.78(+/-2.12)	1.80	6.06(+/-5.50)
S.E.D	0.39					

The actual means are the back-transformed means calculated by the GLMM

4.3.4 Voluntary fluid intake and other variables

As can be seen in Figure 10 below, there is no relationship between the temperature of the water, which ranged from 10^{o}c to 12^{o}c, and the total amount drunk by each horse during each repetition.

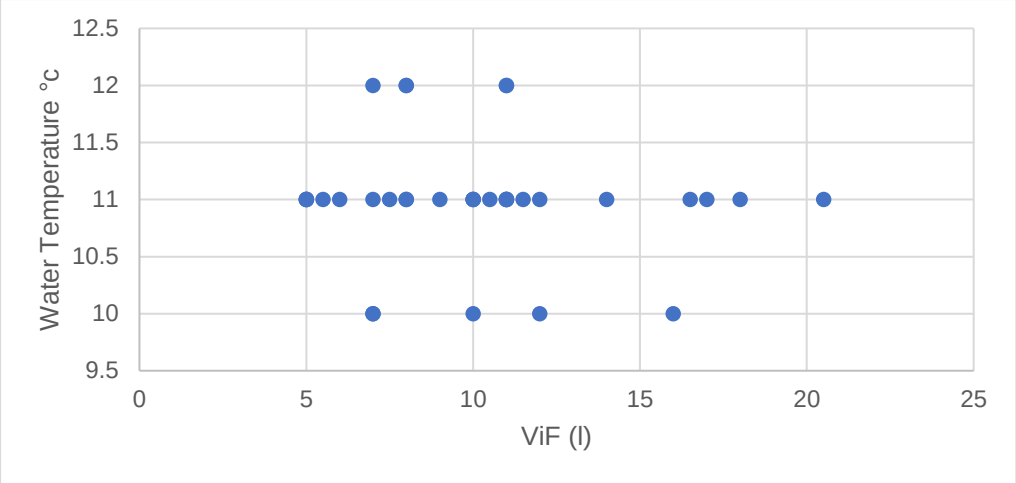

FIGURE 10. SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN THE TOTAL VOLUNTARY FLUID INTAKE (LITRES) OF EACH HORSE PER REPETITION FOR ALL THREE TREATMENTS AND FLUID TEMPERATURE

As can be seen in figures 11 and 12 below, neither air temperature of humidity affected the amounts drunk by each horse. Despite fluctuations in temperature ranging from 21ºc to 9.8ºc, and humidity ranging from 99% to 65.8%, the total volumes consumed during each repetition appear random and did not differ.

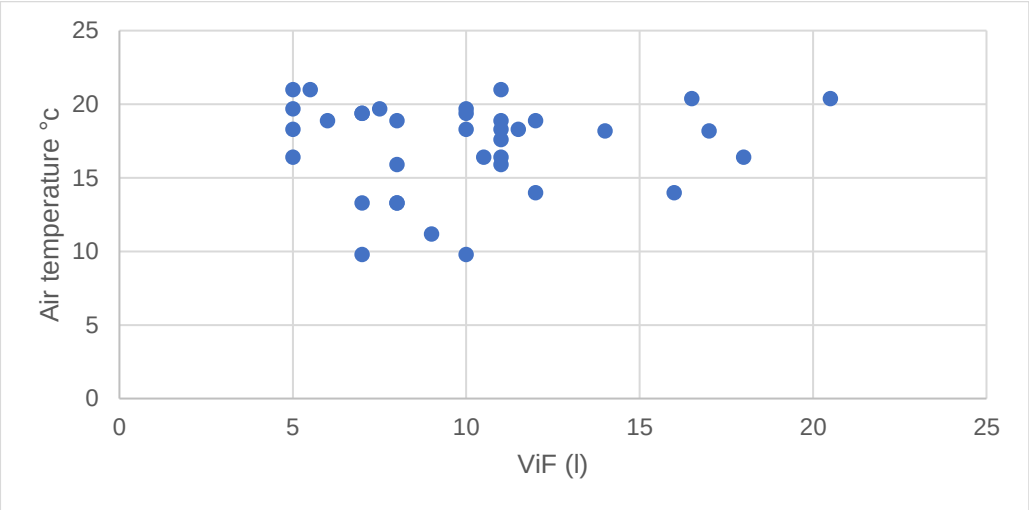

FIGURE 11. SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN THE TOTAL VOLUNTARY FLUID INTAKE (LITRES) OF EACH HORSE PER REPETITION FOR ALL THREE TREATMENTS AND AIR TEMPERATURE

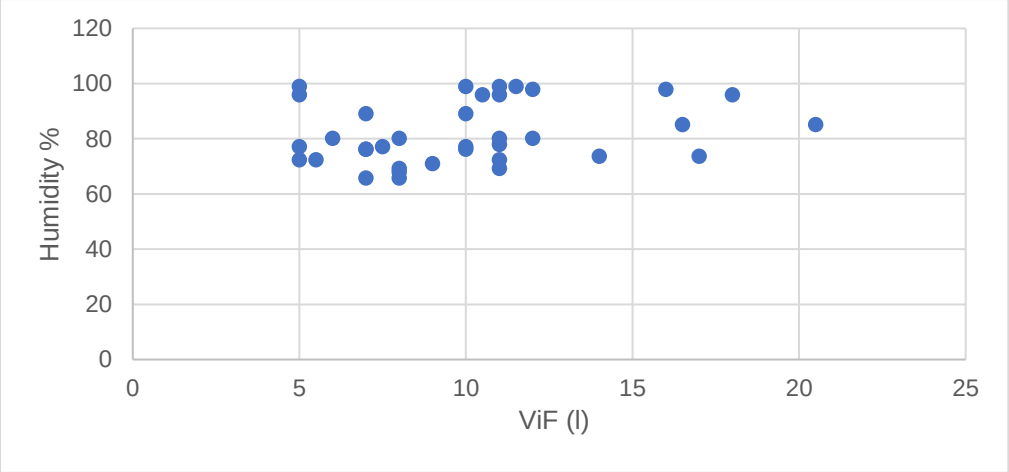

FIGURE 12. SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN THE TOTAL VOLUNTARY FLUID INTAKE (LITRES) OF EACH HORSE PER REPETITION FOR ALL THREE TREATMENTS AND HUMIDITY

As shown in figure 13 below, the average total amount of fluid consumed by each horse is not influenced by body weight.

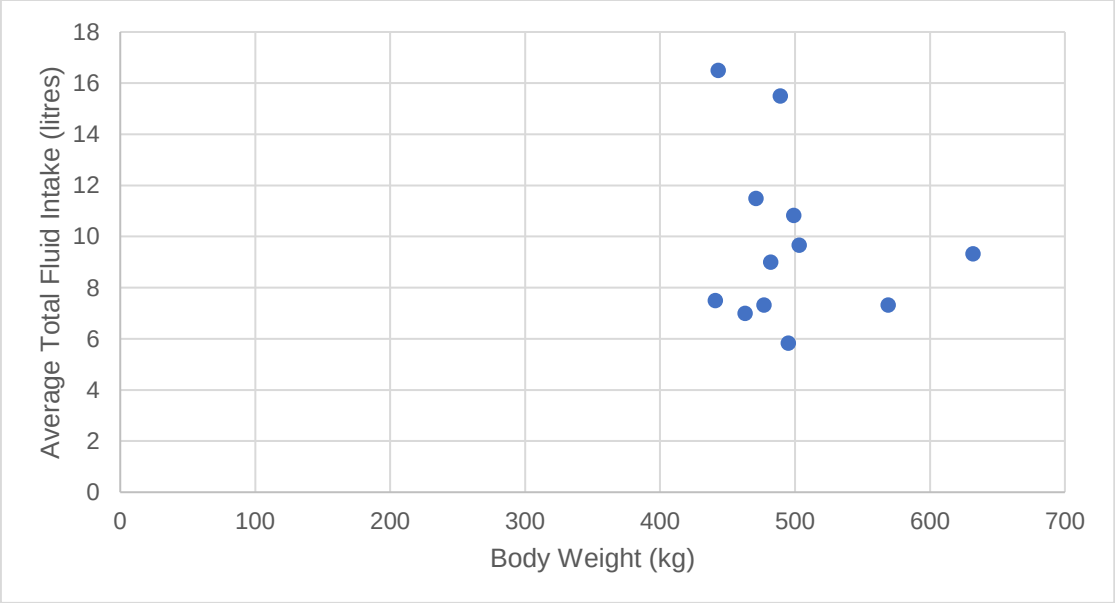

FIGURE 13. SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN THE AVERAGE TOTAL VOLUNTARY FLUID INTAKE (LITRES) FOR EACH HORSE AND HORSE BODY WEIGHT

Figure 14 below shows a weak positive relationship between the amount of hay consumed and the total ViF, Spearman's rank proved this relationship to be significant (Table 17).

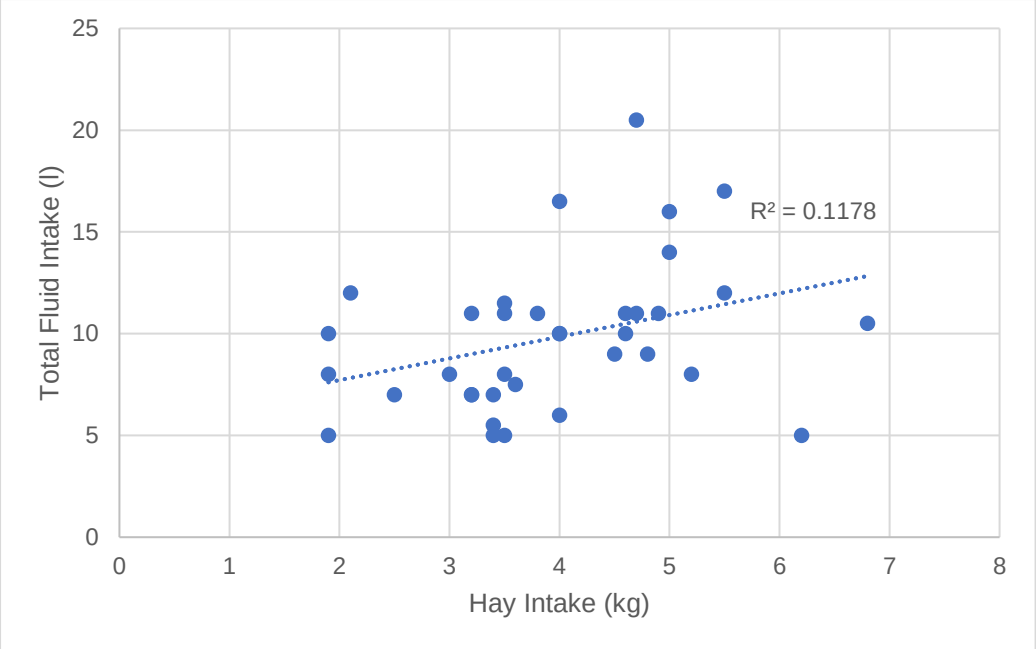

FIGURE 14. SCATTER PLOT SHOWING THE RELATIONSHIP BETWEEN HAY INTAKE AND TOTAL FLUID INTAKE (LITRES) FOR ALL 12 HORSES ACROSS ALL 3 REPETITIONS

TABLE 17. MEAN HAY INTAKE AND TOTAL VIF (LITRES) ACROSS 12 HORSES FOR EACH REPETITION (+/- SD), ALONG WITH THE SPEARMAN'S RANK OUTPUT

	Mean Hay Intake(kg)	**Original Scale mean total ViF (litres)**	**P value**	**Correlation value**
Repetition 1	4.45(+/-1.43)	2.91(+/-3.36)	0.003	0.41
Repetition 2	3.83(+/-0.99)	2.96(+/-2.99)		
Repetition 3	3.59(+/-0.97)	2.82(+/-3.98)		

*denotes significant relationship

As seen in table 18 below, there is little variation in the mean pH of each treatment. Plain water was the most alkaline, but only by 0.15; incidentally, plain water also had the highest mean voluntary fluid intake by an extensive margin.

TABLE 18. MEAN PH AND ORIGINAL SCALE MEAN VOLUNTARY FLUID INTAKE (LITRES) (CALCULATED FROM THE MODEL) FOR EACH OF THE 3 TREATMENTS ACROSS THE 3 REPETITIONS

	1. Banana	2. Cherry	3. Plain Water	S.E.D	P Value
Mean pH	6.75_a	6.74_a	6.90_b	0.016	<0.001
Original Scale mean ViF (litres)	2.07	2.19	5.33	-	-

[ab] Values in the same row not sharing common letters differ significantly (P<0.0%)

There is a significant difference between the two flavoured treatments and the plain water treatment, plain water is more alkaline in pH than the banana and cherry flavoured waters.

4.3.5 Observational data on drinking behaviour across the 3 replicates for before hay and with hay periods.

As seen in figures 15 and 16 below, the number of investigations made to each treatment do not differ greatly, the most investigations were made to the plain water treatment during observation B. Though there were not many drinking episodes in either observation period, the numbers for each treatment were again similar, and plain water in observation period B had the highest frequency of drinking episodes.

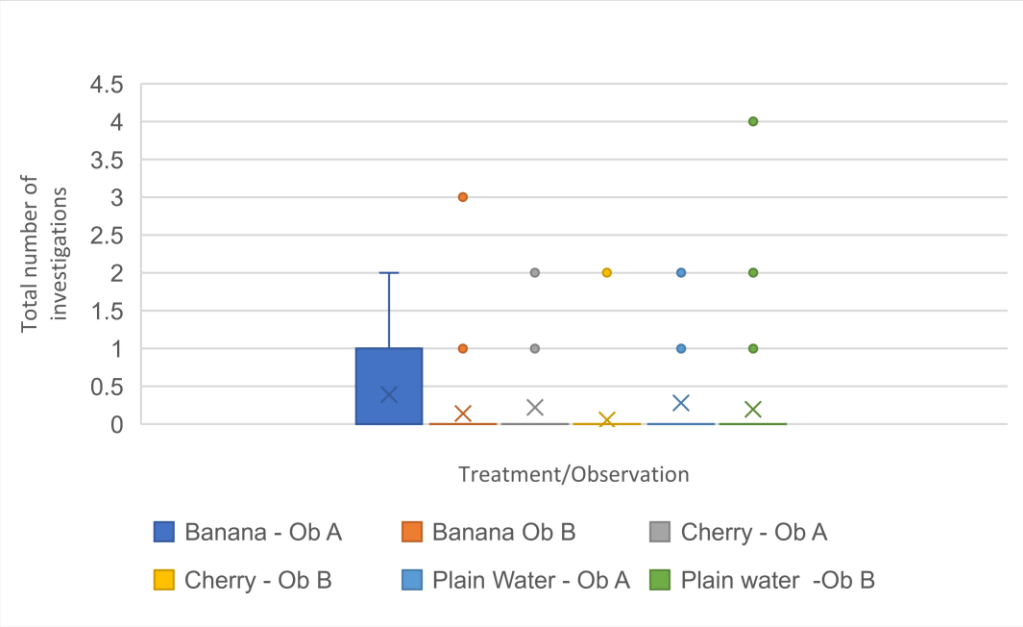

FIGURE 15. BOX AND WHISKER PLOT SHOWING THE TOTAL NUMBER OF INVESTIGATIONS MADE BY THE 12 HORSES TO EACH OF THE TREATMENTS DURING OBSERVATION PERIODS A AND B ACROSS ALL 3 REPETITIONS

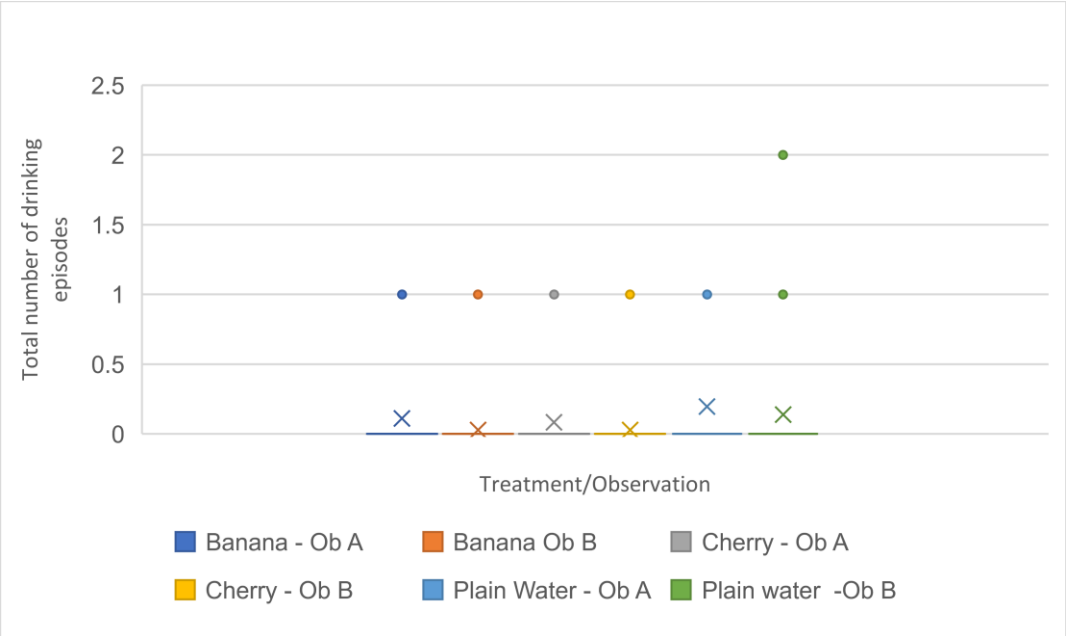

FIGURE 16. BOX AND WHISKER PLOT SHOWING THE TOTAL NUMBER OF DRINKING EPISODES THAT OCCURRED DURING OBSERVATION PERIODS A AND B ACROSS ALL 3 REPETITIONS

From table 19 below, it is clear that plain water was the most preferred treatment, with more than double the amount of the two flavoured treatments having been drunk after both observation periods.

TABLE 19. TOTAL AMOUNT OF FLUID CONSUMED BY ALL 12 HORSES AFTER EACH OBSERVATION PERIOD ACROSS ALL REPETITIONS

	Total amount drunk after observation A (litres)	**Total amount drunk after observation B (litres)**
Banana	4	32
Cherry	3.5	35
Plain Water	13.5	72

4.4 Discussion

4.4.1 Total fluid intake levels

The results for total fluid intake from this trial are similar to Trial 1 in that the average intake was 9.7 litres, over the four-hour trial period. Applying the same assumptions

regarding bodyweight and fluid intake this average was in line with normal consumption as explained by the NRC (2015).

Horses with a larger body mass require more water, therefore it would be expected that the larger horses in this trial drink the most, however body weight had no impact on fluid intake. Horse 1 was the largest but had an average total fluid intake of only 9.33 litres, and horse 7 at 443kg had an average fluid intake of 16.5 litres. Moreover, fluid intakes were not influenced by hay intake, as both horses consumed an average of 4.8kg during each repetition; thus although Sweeting and Houpt (1987), Danielsen *et al.* (1995) and Pagan *et al*. (1998) showed that forage intake influences voluntary water intake, there was clearly another reason for the variance in intake in this trial. Repetition did not influence ViF in any of the horses, nor did any other external factor such as water temperature, air temperature or humidity. The results presented here are therefore indicative of the influence of olfactory and gustatory assessment i.e., smell and taste of the horses, as evident in the studies of Bottom (2008) and Van den Berg *et al.* (2016a,b), who found that when horses were presented with new flavours, acceptance was based on the feedback gained from the senses of smell and taste.

4.4.2 Factors influencing the acceptance of flavours

The horses had no preference when it came to the flavoured treatments, but greatly preferred plain water to either cherry or banana flavour, the latter being the least favourite. These findings contrast with Kennedy *et al.* (2001) study, that found cherry to be the most palatable when added to oats, and Van den Berg (2016b) found banana to be the most popular flavour out of the 15 that were tested when added to a pelleted meal. Interestingly, Van den Berg (2016b) used organic, human grade flavour emulsions to achieve the required flavours, whereas synthetic flavours were

used in this study. No past studies have examined the effect of these flavours when added to water, and it is plausible that horses taste preferences in water are different to that in feed.

Overall, banana was rejected the most on the third repetition, as by this time the horses had begun to learn what was palatable and what was not. This again relates to the 'win stay' strategy as described by Hosoi *et al.* (1995), or rather in this case 'lose move'. It is mentioned by Rose and Kyriazakis (1991) that horses can take time to exhibit the learned behaviour necessary to accept or reject a food source, as they have a longer gut transit time, compared with poultry and pigs and as a result, post ingestive consequences take time to manifest. However, as the flavoured treatments had no nutritional value, the learned behaviour needed was solely taste. As explained by Van den Berg *et al.* (2016a), taste and odour are paramount in determining whether or not a horse will continue to consume a substance; ultimately, if something tastes bad, they will not continue to consume it.

Like with trial 1, very little exploratory behaviour was recorded from the ethograms, which suggests that none of the buckets possessed a particularly strong olfactory cue. In this study it seems that rejection occurred as a result tasting the flavoured treatments; therefore, it is clear that the majority of the horses found the flavoured treatments unpalatable. Even after observation period A across all repetitions, plain water proved to be noticeably more popular than the two flavoured treatments, and this was apparent after observation B also; changing observation B to later in the trial period after trial 1 did not result in more activity during observation period B during trial 2 however it did allow for more fluid to be consumed between the two periods which could indicate that the forage provided after observation period A was beginning to trigger the thirst response.

4.4.3 Possible reasons for the rejection of flavours

Intakes for plain water were high across all three buckets orders; however, some horses drank considerably more than others, which could have attributed to the average intake for plain water being so high, with the total intake for all 3 repetitions ranging from 42 litres for horse 8 down to 1 litre for horse 6. Horse 7 was another to prefer plain water, drinking 40 litres of plain water in total, with only 1 litre of the banana treatment and 8.5 litres of cherry overall. Horse 7's almost complete rejection of the banana treatment could have resulted in it being the least preferred treatment overall, despite cherry being the least preferred in two out of the three repetitions. Also noteworthy is the differences in the total amount of fluid consumed by each horse. Horses 7 and 8 drank distinctly more during each repetition than the rest; the majority of horses consumed between 7 and 11 litres across all three repetitions; however, these two horses consumed between 12 and 20.5 litres during each repetition. Hay intake for horses 7 and 8 were some of the lowest recorded throughout the trial so water intake could not have been related to foraging in this case, this contrasts with Sweeting and Houpt (1987), who noted a clear relationship between forage intake and water intake.

Interestingly, horse 10 preferred the banana treatment, drinking 13 litres overall, compared to 4 litres of both cherry and plain water, and horse 11 preferred the cherry treatment over the banana and plain water treatments, this highlights that horses can exhibit individual taste preferences. Although preference of the flavoured treatment was uncommon, the fact that some horses did show certain preferences between banana and cherry show a high degree of individuality, though the fact remains that plain water was still the most preferred. Individual taste preferences are evident in a study by Bottom (2008), who used sucralose and aspartame to assess the taste response of horses. When presented with the aspartame feed, two horses

showed a significant preference for the control feed and two showed a weak preference for the aspartame feed. Though preferences in Bottom's (2008) study were mainly insignificant, it does highlight the individual preferences of horses towards flavoured feeds.

4.4.4 Use of flavours in other studies

It is somewhat surprising that the two fruit flavoured treatments were so strongly rejected by the horses in this trial; it is widely accepted that fruit flavours have sweet flavour, due to the natural sugars within them. Evidence suggests that horses find the sweet taste more palatable; preference for sucrose solutions has been recorded at concentrations of up to 10g/100ml (Danel and Merkies 2009; Randall *et al*., 1978). It is known that the flavour of both banana and cherry are described as sweet, owing to the chemical compounds; in banana, the flavour is attributed to isoamyl acetate a strong flavour molecule that can be tasted at very low concentrations (Phung, 2014). The buckets in this trial each contained 10 litres of water then in theory they could contain up to 100g of sugar in order to be considered palatable; as the banana treatment contained 53g of the flavouring and the cherry 26g, as determined by trial 1, then it could be that they did not contain enough sweetener in order to seem palatable. One recommendation for further study would be to increase the concentrations of banana and cherry when added to water to see if this altered the results.

As explained by Randall *et al.* (1978), horses are fairly sensitive in terms of taste and it may be that the treatments were not sweet enough, however this does not explain why the plain water treatment, with no sweet flavour whatsoever, was preferred over a mildly sweet solution. Cherry can give two tastes; depending on the type of tree, cherry can either have a sweet flavour, *Prunus avium*, or bitter, *Prunus cerasus*; the

cherry flavour in this trial was artificially made, and was intended to mimic the sweet cherry flavour, it may be that the artificial powder was not a good representation of the flavour. Kennedy (2014) explains that cherries contain a high concentration of glucose, giving the cherry a sweet palatable taste, the cherry flavour was selected for this trial from its success in previous taste trials owing to its sweet taste.

Almost all trials testing the effect of flavours have focussed on increasing the intake of feed. Though Mars *et al.* (1992) aimed to ascertain if they could increase water intake by adding flavouring; it was noted that adding apple flavouring, increased water intake when horses were given unfamiliar water, the tendency of horses to display a neophobic response to a new source is widely accepted; though the apple flavoured water was also unfamiliar, its sweet taste would mean that horses found it more palatable. It is possible that in water, horses flavour preferences are different to that of feed, and more research is required to find those preferences, had apple flavouring been used in this trial, the outcome may have been different.

4.4.5 Rejection of flavours in other studies

It may be that the rejection of the flavoured treatments in this trial could be a result of the neophobic tendencies that horses can exhibit, it is acknowledged that they only sample small quantities when presented with an unfamiliar food source (Van den Berg and Hinch 2016). This is supported by Bottom (2008) who found that horses showed a strong aversion when presented with a novel feed flavoured with orange juice, and so displayed a strong preference for the control feed.

Bottom (2008) also mentions the preference for the control feed could be a conditioned preference as a result of the exposure to it during the 13-day training period, this is also supported by Kennedy *et al.* (2001). Had the horses in our study had a training period in which to become accustomed to banana and cherry

flavoured water, they might have displayed more of a preference, instead of opting for the familiar source of plain water. It is worth noting that although the horses would have experienced the banana and cherry treatments in trial 1, this was for a short time of 4 hours on 3 different days and would probably not have been enough for them to become familiar with it.

Contrary to this, Goodwin *et al.* (2005b) explain that the diets of stabled horses are often monotonous, and that providing sensory variety in the form of flavour could increase dietary variety and as a result, intake, whilst minimising any other consequences associated with changing feed. This was the aim of our trial, attempting to see if adding a flavour to the water would cause the horses to drink more by adding variety in the form of flavour; however, it had the opposite effect. Evidently, as apparent in this current study, horses do not experience long-term monotony when it comes to water, as overall, they did not appreciate new flavours being added. It is worth noting that according to Goatcher and Church (1970a), that many animals are more sensitive to any changes in their water source, and so it may be that the horses found the flavours too overpowering when in water but would explain why they are deemed palatable in feed. However, it must be noted that as with Goodwin *et al.* (2005b) study, the majority of the horses in this study also sampled all options; this can be attributed to the natural foraging nature of the horse, the patch foraging strategy adopted by horses allows them to select a better than average option from a diverse resource (Prache *et al.,* 1998), foraging bouts on preferred options are interrupted in order to sample others as found by Goodwin *et al.* (2005b). Presumably, this was the case in the present study as the horses were unknowing of the taste of the favoured options until sampled; once tasted, many probably returned to their preferred option of plain water. However, this assumes that

the drinking behaviour of horses is the same as their foraging behaviour, as there is little literature in terms of studying the behaviour of horses toward multiple sources of water at once, and so there is uncertainty in this comparison. The tendency of the horses in both Goodwin *et al.* (2005b) study, and the present study, to favour the familiar option is most probably explained by neophobia, as previously discussed.

4.4.6 Other factors known to affect water intake

Other measurements were also taken to identify if any external factors were influencing water intake. It is known that weather conditions can alter the thirst response; however, in this trial, neither air temperature nor humidity appeared to impact the amount drunk. The lack of relationship between the total amount consumed per repetition and both air temperature and humidity show that although these factors have been found to influence water intake, they did not do so in this trial. It is explained by the NRC (2015) that water intake can increase by as much as 79% when horses are exercised in high air temperatures of 33-35^{o}C combined with high humidity; part of the reasoning behind conducting this trial in the summer months was so that the horses might be more inclined to drink and therefore we would see high water intakes. However, although high humidity readings of 100% were recorded, air temperatures only reached 22^{o}C, with an average of 17.2^{o}C. These air temperatures still fall within the horse's thermoneutral zone of between 5^{o}C and 30^{o}C, according to Kentucky Equine Research (2011b). When in the thermoneutral zone, horses do not have to work to raise or lower their body temperature; therefore, they would not be sweating in order to cool themselves, despite all horses undergoing a 30-minute standard exercise test consisting of moderate exercise, none of the horses displayed signs of sweating. This would mean that the horses would have still maintained a good hydration status at the

beginning of the trial periods; they would not have lost any fluids by sweating and so their thirst response would not have been activated as a result of changes in plasma osmolality. It is stated by Sufit *et al.* (1985), that a significant amount of fluid must be lost via sweating for plasma osmolality to increase and therefore stimulate the thirst response, which would not have been possible in the trial conditions. As temperatures in this trial were not high enough for the horses to be out of the thermoneutral zone, and only moderate exercise was undertaken by the horses, who were all in a good state of fitness owing to the current stage in the competition season, it is not surprising that we did not see any correlation between the weather conditions and water intake.

In some instances, water temperature has been found to affect water intake, but in this trial, there appears to be no relationship between the total amount consumed per repetition and the temperature of the water. This can be explained by the ambient air temperatures; though in cold air temperatures horses prefer to drink warm water (Kristula and McDonnell, 1994), McDonnell and Kristula (1996) found that in temperatures of 15-29^{o}c, the temperature of the water had no effect on water intake, and ponies drank similar volumes of icy and warm water. As air temperatures in this trial fall within the range of McDonnell and Kristula's (1996) study, it would explain why no relationship between water temperature and water intake was seen.

When the amount of hay consumed by each horse was examined, a significant positive relationship between hay intake and total fluid intake was found. It is known that horses drink peri prandially, so the inclusion of forage was thought to stimulate the thirst response. There has been limited research done on the relationship between hay and water intake in short time frames; however, studies on forage and water intake conducted over longer time frames have seen that forage does

stimulate the thirst response. Sweeting and Houpt (1987) recorded hay intake increased water intake in a week-long study. Danielsen *et al.* (1995) recorded higher water intakes the morning after the forage was given, as did Pagan *et al.* (1998). The pH of the fluid was found to relate to fluid intake; plain water was the most alkaline of the treatments and was significantly different to the two flavoured treatments. It is known that horses can show an aversion to acidic solutions; aversion to a sour solution of acetic acid has been recorded by Randall *et al.* (1978), although this was at a much lower pH of 3.1. It seems that acidic solutions are bitter in taste, aversion to bitterness has been apparent in trials by Merkies and Bogart (2013), and Merkies and Carson (2011); as the acidity of the fluid increased, the more rejection seemed to be displayed by the horses. It is unlikely that the very slight differences in pH between the plain water treatment and the two flavours was what caused the horses to reject the flavours; as the studies discussed above involve solutions with a much lower pH. The flavoured treatments in this study were not acidic enough to have a bitter taste, at a pH of 6.7, the solutions would still have what would be deemed a sweet taste, and it must be that the horses simply did not find this sweet taste palatable rather than it being due to an aversion to bitterness. Similarly, the horse's palate would not be sensitive enough to detect such small differences in pH as were in this trial.

To conclude, the significant preference for the plain water treatment can almost certainly be attributed to the lack of palatability of the flavoured treatments; though these flavours were selected based on success in feed trials, they have not been successful when added to water. It may be that horses' flavour preferences in water differ to that of feed.

Chapter 5: General Discussion

Dehydration in horses is one of the main constraints on performance and can occur more easily than generally realised, particularly when travelling horses to and from competitions. Butudom *et al.* (2002), Nyman *et al.* (1996), Schott and Hinchcliff (1998), Marlin *et al.* (1998), and Marlin and Nankervis (2002) suggest the best way to encourage voluntary rehydration in horses is the addition of electrolytes to water or feed, as the changes in the osmolality of the blood plasma is what triggers the thirst response. However, some horses are reluctant to consume electrolytes in either feed or water so other ways in which to stimulate drinking would be a useful addition to the task of maintaining good hydration status. This study sought to determine if flavouring water could stimulate horses to drink. The flavours used here were synthetic cherry and banana, both flavours are known to be palatable to horses in feed, although their use in water had not previously been tested.

The results from Trial 1 where no preferences were detected between the 3 concentrations of either cherry or banana might suggest that neither flavour was particularly palatable to horses when dissolved in water. This could be due to the actual flavour or the fact that when dissolved the synthetic flavour did not taste anything like banana or cherry and therefore was not particularly palatable to the horses.

The lack of intake could also be attributed to other factors. For example, the weather conditions and temperature of the water could have played a part; it is well documented that horses prefer luke-warm water in cold conditions (Butudom *et al.,* 2004; Kristula and McDonnell, 1994; McDonnell and Kristula, 1996), had the flavour concentrations been added to warm water, the choices may have been different. Similarly, the exercise undertaken by the 6 horses prior to each repetition was not

sufficiently vigorous to cause any dehydration so the thirst response was not really triggered. Though there was some variation noted, that may suggest individual preferences, it was not a strong enough response to cause a significant result. As the power calculation for trial 1 proved the sample size was sufficient to detect differences, the main conclusion here must be that horses did not have any preference for a particular concentration of either banana or cherry. The forage intake was found to relate to fluid intake of the banana concentrations, but the cherry concentrations showed no such relationship; as much of the literature suggests that forage stimulates the thirst response, it is unclear why it did not do so for the cherry concentrations.

The findings of trial 2 confirmed the findings of trial 1 that the flavours of banana and cherry were not palatable when added to water; the preference of plain water over the two flavoured water solutions is significant and shows that the flavours of banana and cherry were not effective in encouraging the horses to drink. Trial 2 was also designed to have sufficient power to detect any differences and as none were found this can be regarded as an accurate assessment of the palatability of banana or cherry flavoured water. While it is clear that the SET used in these trials did not cause any dehydration and therefore did not stimulate the horses to drink any more than they would do when at maintenance, all 12 horses consumed enough plain water to maintain their daily water requirements. Therefore, it suggests that the thirst response was sufficiently activated to encourage the horses to drink; and they chose to consume plain water.

While the results from these two trials suggests that plain water is preferred by horses, it might be appropriate to test other flavours, which might work better when dissolved in water than the banana or cherry flavours used here. Apple flavour has

been found to be palatable when added to water (Mars *et al.,* 1992), and fenugreek has been found to be palatable in a number of feed trials (Danel and Merkies, 2009; Randall *et al.,* 1978), so it would be worth trialling these in water to see if they encourage drinking. Similarly, the use of natural flavours may be more effective, the banana and cherry flavours used in this trial were synthetically manufactured and it maybe they did not accurately represent the natural flavouring. While this trial has been a valuable first look into the influence of cherry and banana flavours at increasing water intake in horses, it should not deter further work and the exploration of a wider range of flavours which may indeed prove useful in increasing water intake in horses.

Chapter 6: Conclusions

As plain water was preferred over the banana and cherry flavoured waters, it is clear that these synthetic flavours were not effective in encouraging horses to drink. However, horses have been known to display neophobia towards electrolyte solutions, and some do not find them palatable at all; if a flavour that was palatable in water could be found, then adding it to an electrolyte solution in order to encourage intake could be the solution to combating dehydration. Therefore, further research is required in order to ascertain if, and what, flavours are palatable in water; a wider study is also recommended, to gain more of an idea of what is universally liked or not liked.

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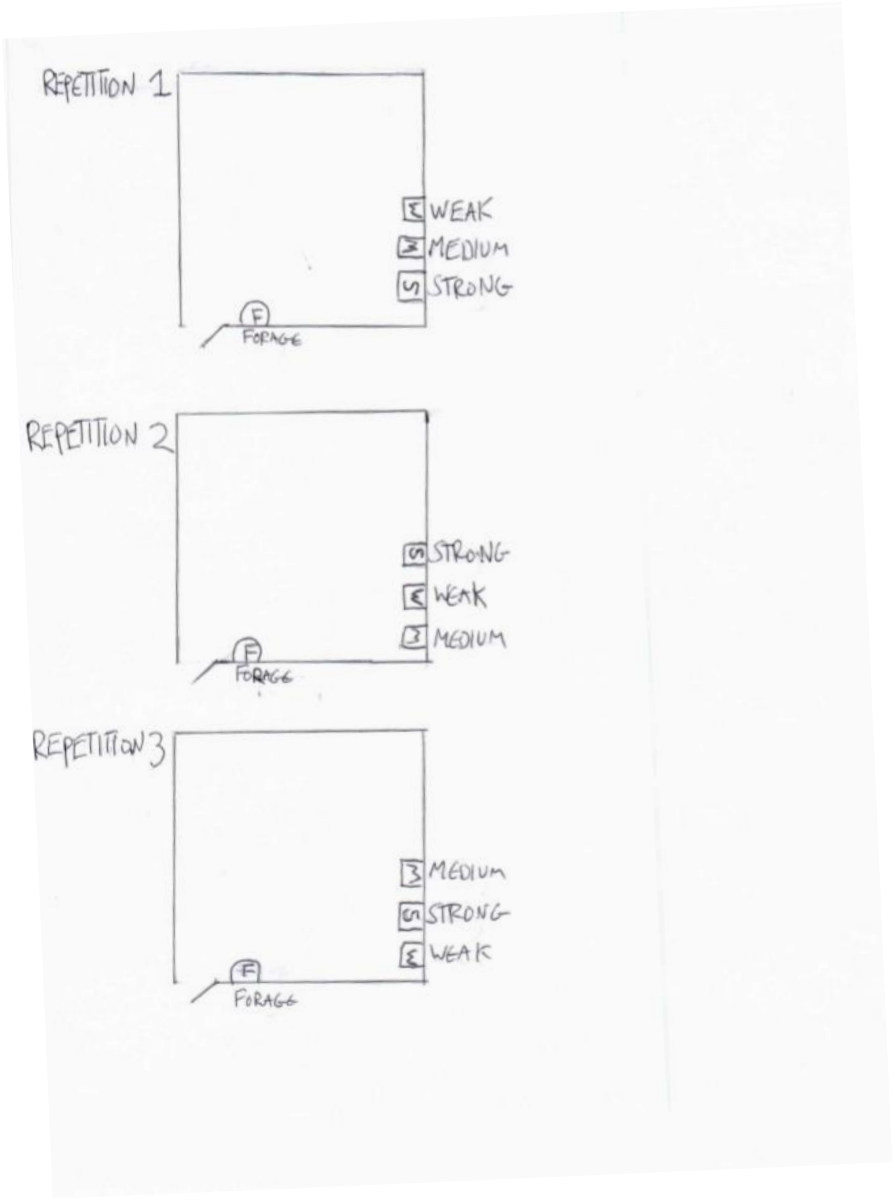
Appendices

Appendix 1 – A diagram showing the different bucket orders and stable layout for trial 1a and trial 1b

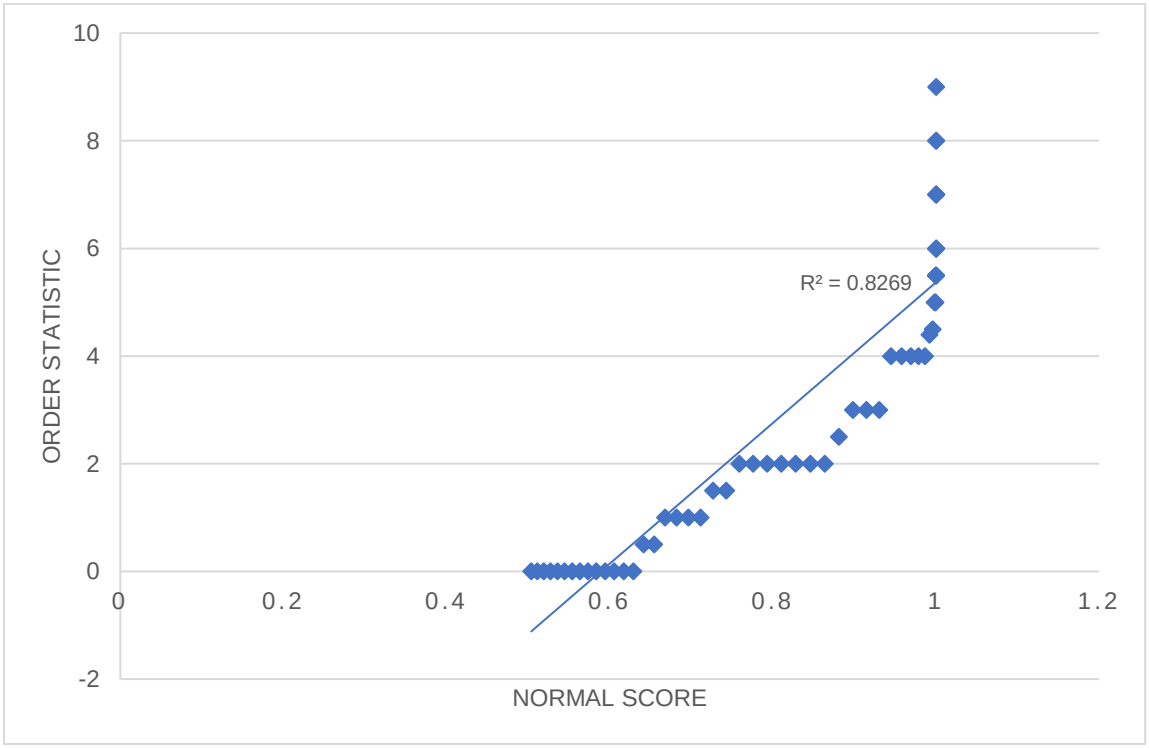


Appendix 2 – The Henneke body condition scoring system used to asses the condition of horses in trial 1

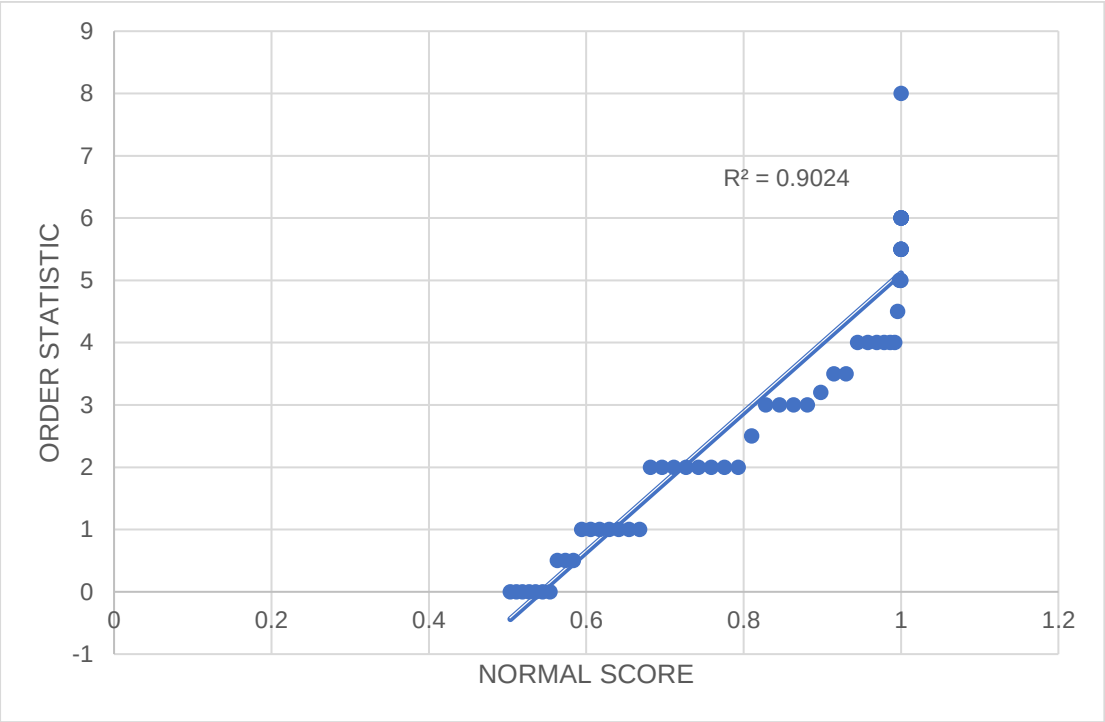
	CONDITION	NECK	WITHERS	BEHIND SHOULDER	RIBS	TOP LINE	TAILHEAD
1	POOR	Bone structure easily noticeable	Bone structure easily noticeable	Bone structure easily noticeable	Ribs protruding prominently	Spinous processes projecting prominently	Tailhead, lower pelvic bones, and hip joints projecting prominently
2	VERY THIN	Bone structure faintly discernible	Bone structure faintly discernible	Bone structure faintly discernible	Ribs prominent	Slight fat covering over base of spinous processes	Tailhead prominent
3	THIN	Neck accentuated	Withers accentuated	Shoulder accentuated	Slight fat over ribs. Ribs easily discernible	Fat buildup halfway on spinous processes, but easily discernible.	Tailhead prominent but individual vertebrae not visible. Hip joints appear rounded, but are still easily discernible.
4	MODERATELY THIN	Neck not obviously thin	Withers not obviously thin	Shoulder not obviously thin	Faint outline of ribs discernible	Peaked appearance along back	Prominence depends on conformation. Fat can be felt. Hip joints not discernible
5	MODERATE	Neck blends smoothly into body	Withers rounded over spinous processes	Shoulder blends smoothly into body	Ribs not visible but easily felt	Back is level	Fat around tailhead beginning to feel soft
6	MODERATELY FAT	Fat beginning to be deposited	Fat beginning to be deposited	Fat beginning to be deposited	Fat over ribs feels spongy	May have a slight groove down back	Fat around tailhead feels soft
7	FLESHY	Fat deposited along neck	Fat deposited along withers	Fat deposited behind shoulder	Individual ribs can be felt with pressure, but noticeable fat filling between ribs	May have crease down the back	Fat around tailhead is soft
8	FAT	Noticeable thickening of neck	Area along withers filled with fat	Area behind shoulder filled in flush with body	Difficult to feel ribs	Positive crease down the back	Fat around tailhead very soft
9	EXTREMELY FAT	Bulging fat	Bulging fat	Bulging fat	Patchy fat appearing over ribs	Obvious crease down the back	Bulging fat around tailhead

Source: https://www.baileyshorsefeeds.co.uk/body-condition-scoring

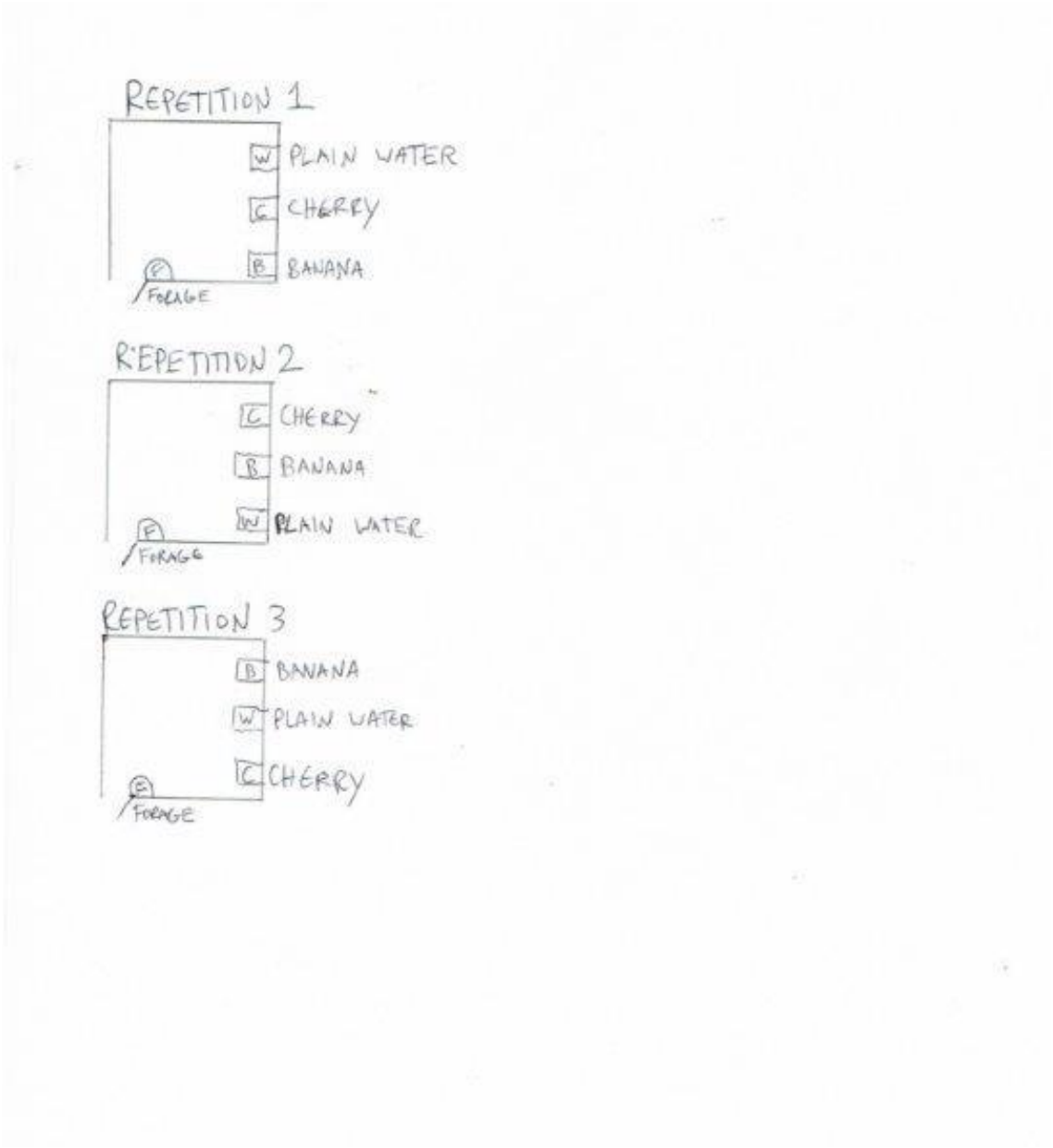
Appendix 3 – Q-Q plot showing the distribution of data for all 3 concentrations across all repetitions of the Banana flavoured water in trial 1a



Appendix 4 - Q-Q plot showing the distribution of data for all 3 concentrations across all repetitions of the Cherry flavoured water in trial 1b



Appendix 5 – A diagram showing the different bucket orders and stable layout for trial 2



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Appendix 6 - Q-Q plot showing the distribution of data for all 3 treatments across all repetitions in trial 2