AGE-RELATED CHANGES IN TASTE AND GUSTATORY RESPONSE AND FEEDING BEHAVIOUR IN THE STABLED HORSE

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Abstract

There is a paucity of research relating to the anatomy and physiology of gustation and olfaction in the horse. Moreover, whilst an age-related decline in gustation and olfaction has been recorded in humans, no such study has been conducted in the horse. The horse is reliant on gustation and olfaction to make appropriate decisions relating to both short and long term diet selection and thus, any compromise in function, has implications for food intake and potentially welfare.

The principal aim of this study was to establish if, and to what extent, taste and gustatory responses are affected by age in the horse. Horses were allocated to the age groups Young (2-5 years), Middle (8-14 years) and Old (16 plus years) for the study of taste (n=18) and to Young (4-6 years), Middle (10-14 years) and Old (16 plus years) for the study of gustation (n=18). Individual taste responses and gustatory responses (taste in the absence of additional olfactory cues) were identified using two-choice preference testing and monadic testing. Statistical analysis was conducted using Minitab 14.0 and behaviour data was analysed using The Observer 5.0 (Noldus, Netherlands).

No effect of age on taste response or gustatory response was recorded. Differences in intake and behaviour between the control and test feed were identified in both studies, although inclusion rate had no significant effect on intake or behaviour. Individual side preference was recorded. This is due to the lack of positive or negative consequence related to ingestion of test feed and the lack of horses in the later stages of senescence being allocated to the study.

Differences in behaviour between age groups recorded during the taste trial were analysed using lag sequential analysis. Consistency in behaviour was found to increase with age, with more fragmented behaviour recorded in Young subjects. Side preference was recorded in individuals in both studies and is suggested to be the result of limitations in study design; namely the lack of the test chemicals to evoke a behavioural response.

The methods employed in this study failed to identify a significant effect of age on taste or gustatory response. Given the increasing longevity of the domestic horse, further study with more appropriate techniques is warranted.

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"You can lead a horse to water…

but you can't make him sink!"

A.A. Milne

Contents

Figures

Tables

Chapter 1:

Literature Review

There has been limited research conducted into the anatomy and physiology of gustation and olfaction in the horse. No study of the effect of age on gustatory and olfactory function has been conducted on horses. Discussion as to whether age affects gustatory and olfactory function in the horse is reliant on comparative studies in humans and other animals.

Animals assess their external environment, physical and chemical, through changes perceived by the sensory systems. Vision, audition and touch are used to assess the physical environment, whilst olfaction and gustation evaluate the chemical environment. Olfaction and gustation are intrinsically linked, combining to determine the perception of flavour. The decision to swallow a potential food source is ultimately based on the detection of acceptable gustatory cues (Rawson, 1990).

Olfaction, gustation, vision and texture play an initial role in food selection; the decision to ingest a feed is ultimately influenced by gustatory cues. Hence, the gustatory and olfactory systems are involved in nutrient acquisition and toxin avoidance (Scott, 2001). Consequently feeding behaviours, and hence diet selection, are induced and controlled by changes in gustatory and olfactory response. Gustation and olfaction are additionally involved in sexual behaviour, territorial behaviour, mother-infant interactions and diet-induced thermogenesis (LeBlanc and Labrie, 1997).

The gustatory and olfactory systems are involved in activities fundamental to the survival and proliferation of a species. Any compromise in function of the gustatory and olfactory systems is suggested to have implications for appropriate perception of gustatory and olfactory cues and hence, diet selection.

As discussed throughout this chapter, gustatory and olfactory functions are compromised by many factors such as: changes in morphology and physiology at receptor, neural and cognitive level; dietary deficiencies; medication; and age. The response to perceived cues may be subject to individual variation as a result of prior experience and individual variation. This study investigates whether, and to what extent, gustatory and olfactory responses are affected by age in the horse. There is a consensus of opinion regarding changes in gustatory and olfactory response within life stage and, hence, changes are hypothesised to occur in the horse.

1.1 The role of gustation and olfaction in the horse

As gustation and olfaction are so intrinsically linked, discussion of gustation and olfaction combined will be referred to as taste, unless stated otherwise.

Horses are general feeders, obtaining nutrients from a wide range of sources. The ability of herbivorous mammals to select for nutrients, and to detect and avoid toxic plants through awareness of the aposematic qualities of plants (Molyneux and Ralphs, 1992; Cassini, 1994; Provenza *et al.*, 2000), would have played a fundamental role in the survival of the horse. The horse is reliant on taste to inform the decision to ingest a potential food source, with gustation ultimately influencing the decision to swallow (Rawson, 1990). The ability to make appropriate ingestion decisions is essential in the horse due to their lack of ability to vomit, and once a food source is consumed, the decision cannot be reversed. Any reduced function in taste response with advancing age may increase the risk of toxin ingestion in the horse.

The diet formulation of the modern horse aims to provide the exact nutritional requirements for the purpose intended. There is an assumption of 100% intake of the ration provided. The overall acceptance of a food involves the determination of the physical properties and chemical properties of the food. Taste plays an important role in mammalian feeding behaviour (Campbell *et al.*, 1997). Foods are developed with the intention of them being consumed consistently over time and the influence of taste on the palatability complex, and consequent intake of the feed, cannot be overlooked (Amerine *et al.*, 1976). Controversy exists over the definition of palatability. Rook *et al*. (1997) define palatability as the short-term response to a feed, excluding the influence of post-ingestive consequences, learning or conditioning on palatability. Rook *et al.* (1997) suggest that the definition presented by Forbes (1995), which states that palatability is the overall sensory impression an animal receives from a food, inclusive of postingestive consequences, learning and conditioning, is more appropriate to acceptability rather than palatability. Provenza and Balph (1989) are in agreement with Forbes (1995), suggesting that palatability is related to and dependent on, postingestive consequences.

The definition of Rook *et al.* (1997), relating palatability to organoleptic properties alone, makes palatability extremely difficult to measure as all animals are subject to influence from conditioning and learning. The selection of food based on short-term palatability alone would have limited survival benefit throughout evolution. A relationship between a sweet taste and calorific value and a bitter taste with toxins has been suggested to influence diet selection (Drewnowski, 2000). It may be that a preference for sweet substances and aversion to bitter substances is based on learning of postingestive consequences, and may convey an evolutionary benefit (Rook *et al.*, 1997). The definition of palatability in the literature is used in an ambiguous manner, generally referring to acceptability.

Palatability has been proposed to be a characteristic specific to an individual as opposed to a food (Rook *et al.*, 1997). This is a result of variation between individuals and variation within individuals to foods when presented twice. This is attributed to changes in the animal's physical and mental state (Rook *et al.*, 1997; McFarland, 1999). The use of intake as an indicator of palatability may be affected by inconsistencies in food selection when presentation is replicated (Rook *et al.*, 1997). Rogers (1990) states that hunger and palatability may act independently of each other and may account for variation in preference within individuals.

1.1.1 Controls of feeding behaviour in the horse

The motivation to feed is driven by the need of an animal to seek and eat food in order to maintain its organised state (Forbes, 1995). Feeding motivation competes with other needs such as reproduction and drinking and sufficient nutrients must be obtained in one meal to justify energy expenditure and meet requirements. Control of intake must enable an animal to eat until satiated but to ignore food when a different motivation is prioritised.

Food motivation is dependent upon changes in the internal state and food availability in the environment (Toates, 1980). Motivation changes as a result of several responses that are functionally related, rather than in response to one specific fluctuation in a stimulus (Manning and Stamp-Dawkins, 1998). Feeding behaviour is influenced not only by the acceptability of a feed, but also by the need to acquire nutrients and avoid toxins. Feeding behaviour is influenced both in the short term, through hedyphagia and in the long term, through awareness of postingestive consequences, with taste playing a fundamental role in both aspects.

Kyriazakis *et al*. (1999) propose that animals have developed feeding behaviours that identify not only the properties, but also the nutritional basis of the food.

This work supports the view that animals respond to long-term changes in the nutritional environment. The point at which animals react to changes in their nutritional environment by changing their feeding behaviour is not known.

Feed intake in the horse is controlled by several factors. The breakdown products of carbohydrate and fat digestion and volatile fatty acids from the caecum and large colon influence meal frequency (Ralston and Baile, 1983). Oropharyngeal and external stimuli also play a role.

The duration an animal spends feeding is dependent on exogenous factors such as oropharyngeal characteristics and external stimuli and endogenous factors such as satiety, hunger, age and nutritional requirements (Toates, 1980). Studies in dairy cows suggest that meals are biologically relevant units of short term feeding behaviour, as opposed to visits (Tolkamp *et al*., 2000). The probability of cows ending and starting a meal was increased with meal length and interval between meals. The feeding behaviour of pigs is characterised by meals, separated by long intervals, with these meals being clusters of short eating bouts, interrupted by drinking or moving in and out of the feeder (Morgan *et al*., 2000). Feeding behaviour, and consequently intake, is subject to satisfying both short term feeding motivations and meeting nutritional requirements.

The study of the control of feed intake in the horse has proposed several mechanisms that regulate diet selection. It may be that these factors work in conjunction with each other, the extent of influence on feeding choice altering with physiological state and the type of diet presented.

The number of swallows a horse performs is suggested to influence food intake in the horse by way of an oropharyngeal metering system (Houpt, 1990). This

supports previous findings that sham feeding of horses through an oesophageal fistula terminates feeding at the same rate in those horses without a fistula (Ralston, 1984).

Ralston *et al*. (1983) found that the administration of glucose, cellulose or volatile fatty acids into the stomach or caecum did not affect intake until after they had been absorbed, suggesting postabsorptive mechanisms are more influential on feed intake in the horse, than chemoreceptors in the gastrointestinal tract.

1.1.2. Disruptions to feeding behaviour in horses

Alterations to intake as a result of changes in feeding behaviour may present as increased intake (hyperphagia), decreased intake (hypophagia) or ingestion of abnormal food stuffs (Ralston, 1984). Such changes in intake have implications for bodyweight, in terms of increased or decreased body weight, but also for increased susceptibility to specific dietary excesses or deficiencies. In the horse this is of importance, not only with regard to welfare, but also to the potential loss of performance.

Due to the increased control of food provision in the stabled horse over the free-living horse, obesity should be preventable. There is a tendency in this country for horses to receive feed in excess of their requirements. Horses receiving *ad libitum* access to feed have been shown to maintain a level of weight, classed as obese (Ralston, 1984). This may be an evolutionary mechanism to cope with seasonal variation in food supply.

Under-eating is observed in two forms: firstly, anorexia, reduced food intake in the presence of disease; and secondly, hypophagia, reduced food intake without the presence of disease, for example following a stressful period. Under-eating generally is associated with a period of maximal nutritional need and, hence, is of great importance to welfare and performance. Poor dental condition reduces food intake in the horse, with an apparent increase in dental problems with age in the horse (Ralston, 1996; Hintz, 1999 b; Ralston *et al*., 2001).

1.1.3 Factors influencing taste response in the horse

Perception of, and response to, a taste cue is influenced by both changes in the internal and external environment of the animal, in addition to prior experience and learning. Due to a lack of research in the horse relating to these areas, it has been necessary to rely on comparative studies in humans and other animals. Endogenous factors suggested to affect taste response in humans are genotype (Prutkin *et al*., 2000; de Castro, 2001), gender (Brand and Millot, 2001), medication (Schiffman *et al*., 1999) and pregnancy (Kuga *et al*., 2002; Kolble *et al*., 2001). Exogenous factors include changes in the feeding environment, social facilitation and gustatory and olfactory cues. Additionally, learning and prior experience has been found to influence taste response in horses (Houpt *et al*., 1990), in addition to humans (Brennan and Keverne, 1997; Bernstein, 1999; Jansen and Tenney, 2001; Mennella *et al*., 2001) and sheep (Arsenos *et al*., 1999). Taste response may be influenced by external and internal changes alongside prior experience.

Taste preferences and aversion are influenced by genotype, which may account for individual variation in taste responses between, and within, species. The influence of the *in utero* and postnatal environment may strengthen or weaken these genotypic derived responses, in addition to developing them.

Research in humans has shown that gender may determine olfactory abilities, with women being superior to men (Brand and Millot, 2001). This difference has been suggested to be due to anatomical and / or physiological differences at the cognitive level between genders (Brand and Millot, 2001).

Administration of medication, predominately anti-inflammatory and antimicrobial drugs, has been shown to affect gustatory function in humans (Schiffman *et al.*, 1999; Schiffman *et al.*, 2000). Schiffman *et al.* (2000) demonstrated a negative effect of certain medications when applied to human subject's tongues. These effects were mainly detected as bitter, metallic or sour tasting and affected other tastant detections including citric acid and sodium chloride.

Gustatory and olfactory function is affected by pregnancy in humans (Kolble *et al.*, 2001; Kuga *et al.*, 2002). Kolble *et al.* (2001) investigated olfactory and gustatory function in women in the first trimester of pregnancy, compared with olfactory and gustatory function in women in a known phase of their menstrual cycle. From this study it was concluded that toxic odours were more aversive during pregnancy and gustatory sensitivity was also lower with pregnancy. Kolble *et al.* (2001), proposes that such changes may enable the olfactory system to protect against toxins, whereas a reduced gustatory sensitivity enables a wider sourcing of food and an increase in electrolytes. The value of an decrease in gustatory sensitivity is questionable due to an increased likelihood of toxin ingestion. Furthermore, no such study has been conducted in the horse.

Exogenous factors affect feeding behaviour in the horse as a result of changes in the external environment. These may include external disturbances such as visual and auditory cues as well as olfactory and gustatory cues. As discussed above, a response to olfactory and gustatory cues may combine with endogenous factors to regulate short and long term diet selection.

Horses are socially facilitated, demonstrated by their grazing in herds. An increase in feed intake is observed when other horses are eating, or observed eating (Houpt, 1990).

Prior experience of a feed in the horse influences taste response in two ways: firstly, through either a conditioned preference or aversion to a previously encountered feed; and secondly, from learned associations between postingestive consequences and taste cues.

Prior exposure to a flavour in lambs has been shown to influence food selection, with lambs demonstrating a preference for a previously accepted coconut-flavoured straw over plain straw (Villalba and Provenza, 2000). Increased exposure to feed may increase preference, and hence, conditioning, with time (Jansen and Tenney, 2001).

1.1.4 The combined effects of endogenous and exogenous factors and prior experience on taste response

The motivation to acquire information from experience is fundamental to learning (Phillips, 1993). Taste plays an important role in the learning of feeding behaviour in terms of developing preference or aversion. The exploratory behaviour of young animals is part of the learning process, the effect of ingestion of certain plants altering the internal state of the animal (Provenza, 1995; Provenza *et al.*, 2000). From birth, foals exhibit behaviour that involves sampling food sources in their environment, nibbling grass from the first few days of birth onwards concurrently with the dam. This behaviour initially occupies 10% of a twenty-four hour period, increasing to 60% at 6 months old (Houpt, 1990). Similar selection of specific plants for ingestion is learnt from observation of the mother's food selection in lambs (Provenza and Balph, 1989). It is suggested that through observation and social facilitation, sampling behaviour and learning of postingestive consequences, the horse is able to learn appropriate diet selection.

Changes in the internal state of the animal as a result of physiological changes, such as lactation or negative consequences of ingestion, will influence learning. Learning, particularly in animals that select from a wide forage source, such as the horse, largely influences feeding behaviour. The duration of learning in ruminants has been shown to vary depending upon the extent of an animal's deficiency or the post-ingestive consequences (Kyriazakis *et al.*, 1999). Kendrick (1997) states that learning in young ruminants may persist for 1-3 years; Kyriazakis *et al.* (1999) state that learnt behaviour will disappear within one week. This contradiction needs to be interpreted with caution due to differences in the taste cues and postingestive consequences used to investigate learning.

Kyriazakis *et al.* (1999) emphasise the role of the internal state of the animal and its influence upon learnt feeding behaviour in animals. Short-term fluctuations in the internal state of the animal do not alter feeding behaviour, though it is uncertain what degree of deviation within the internal state is tolerated before learning occurs. Animals select food on properties and nutritional basis (Kyriazakis *et al.*, 1999).

The influence of learning on the development of taste response *in utero* and in the neonate has been recorded in several mammalian species. Work by Schaal *et al*. (2000) found that human foetuses were able to learn odours from their pregnant mother's diet, showing a preference for the odour immediately after birth and at four days old. Experience of chemosensory information in the foetus was considered normal (Schaal *et al.*, 2000).

An additional study of prenatal and postnatal exposure to flavours has been shown to influence flavour learning in human infants (Mennella *et al.*, 2001). Previous exposure to commonly accepted aversive flavours, through amniotic fluid and breast milk, resulted in less negative facial responses when exposed to these flavours. It was concluded by Mennella *et al.* (2001) that such early exposure to flavours contributes to cultural differences in flavour preferences. Marlier *et al.* (2000) conducted a similar study looking at how olfactory preferences in neonates were influenced by consumption of flavours during pregnancy. Prior exposure to anise during pregnancy resulted in a neonatal preference, compared to unexposed neonates who were indifferent or showed an aversive response. The influence of pre and postnatal exposure in the horse has not been explored, but may be an important factor in the development of individual variations in preference.

The work of Provenza *et al.* (2000) has demonstrated aversion learning in lambs when a novel odour resulted in immediate toxicosis. These lambs subsequently had a more reduced intake of this food, than those that were not induced, leading to the conclusion that aversive responses were transient and depended upon the postingestive consequences of consumption. Without postingestive consequence, odour alone was not sufficient to prevent intake. If toxicity can be detected by odour alone, then intake may be decreased. The sampling behaviour of herbivores requires postingestive toxic effects to prevent further intake. Difficulties may arise in attributing a consequence to a specific food type due the horse selecting from a range of sources.

The ability of horses to learn to avoid a substance that has post-ingestive consequences has been shown by Houpt (1990). For an aversion to be learned, the consequence needed to occur immediately following ingestion, with no learning occurring if postingestive consequences arose thirty minutes post ingestion. This finding suggests that horses are at risk from chronic poisoning. In addition to the time period between ingestion to postingestive consequences being influential on learning, when the negative consequences were associated

with a more palatable feed learning was also less effective (Houpt, 1990). Increased potency of the reinforcer has also been shown to increase the rate of learning and extent of conditioning in rats (Ackroff *et al*., 2001).

The extent of postingestive consequences has also been shown to influence the rate of learning in ruminants (Kyriazakis *et al.*, 1999). Additionally, when unacceptable tastes were paired with calorific reinforcement in rats, a preference for the aversive taste was not learned (Forestell and LoLordo, 2000). Associations are not only influenced by the degree of consequence, but also the orosensory cues which form part of the association.

The ability of animals to select an appropriate diet based on taste or olfactory cues is reliant upon detection of, and appropriate response to, orosensory characteristics of a food and postingestive consequences. The ability of horses to select energy based on associations between taste cues and postingestive consequences has been recorded (Cairns *et al*., 2001). Additionally, Houpt *et al*. (1990) have demonstrated the ability of horses to associate taste with negative consequences of ingestion. Villalba and Provenza (2000) demonstrated that lambs rapidly developed a preference for a novel flavoured food if an increase in the amount ingested was accompanied by an increase in the amount of starch directly infused into the rumen. Lambs were concluded to be able to select a novel food with an associated energy reward. Awareness of postingestive consequences, influences taste response, and consequently intake.

1.1.5 Previous research into taste response in the horse

There has been limited research into taste function in the horse, with no study of whether, and to what extent, taste is affected by age in the horse. Studies have predominantly concentrated on flavour preferences in feed due to the

commercial value of such knowledge. As a result of the need to identify taste response in order to determine preference, these studies prove invaluable.

A study by Randall *et al*. (1978) aimed to provide a benchmark for the comparison of taste responses in the horse with comparative studies in other species. Using two-choice preference testing, Randall *et al.* (1978) studied the taste response of horses to the four basic tastes of sweet, salty, sour and bitter. Taste cues were presented in water, against an unflavoured water control. In order to determine taste response, consumption from each container was measured, with intake of more than 60% from one container indicating a preference. No preference or indifference was recorded if the horse selected less than 60% from one container.

Randall *et al.* (1978) identified taste thresholds for the sweet taste, sucrose, as 1.25g/100ml. Preference for the sucrose was evident at 2.5g, 5.0g and 10g/100ml. At 20g/100ml, no preference for the sucrose was shown suggesting a reduced preference for sucrose, rather than an inability to detect it. From this study (Randall *et al.*, 1978) it was concluded that horses possess similar taste sensitivity to sucrose as sheep and pygmy goats (Goatcher and Church, 1970 a).

Sodium chloride was used to determine taste response to salty substances in the horse (Randall *et al.*, 1978). Taste thresholds were identified at an inclusion level of 0.63g/100ml, with an aversion recorded. The salty solution increased in aversion with increasing sodium chloride inclusion rates. These findings support those of previous trials in cattle and normal goats (Goatcher and Church, 1970 b).

Acetic acid was used to study taste response to sour solutions in the horse, with taste thresholds determined at 0.16ml/100ml (pH 3.1), recorded as an aversive

response. Horses were found to possess a similar response towards acetic acid as normal goats and sheep (Goatcher and Church, 1970 b, c).

When presented with the bitter solution, quinine hydrochloride, horses exhibited a taste threshold at 20mg/100ml, recorded as an aversive response. Horses were found to be less sensitive to bitter solutions than pygmy goats (Goatcher and Church, 1970 a).

The study by Randall *et al.* (1978) found, as expected, a preference for sucrose and aversion to bitter, sour and salty solutions in the horse. Preference varied with inclusion rates and individuals. An aversion towards sucrose was recorded in one horse when 10mg/100ml sucrose was presented. All other horses demonstrated a preference at this inclusion level. This suggests the rejection of sucrose in one individual was the result of either increased taste sensitivity, though this was not recorded towards other solutions, or an aversion to sucrose. Similar variation in preference within species has been recorded in calves, with sucrose evoking responses ranging from indifference to pronounced preference in individuals (Kare *et al*., 1965).

Young horses were concluded to have similar taste preferences to ruminants (Goatcher and Church, 1970 a). There have been numerous water-based trials in ruminants and domestic animals that allow comparison of taste responses (Goatcher and Church, 1970 a, b, c, d).

Animals are more sensitive to the inclusion of chemicals in water than in dry feeds (Goatcher and Church, 1970 e), although taste response in feed will have more influence on diet selection and palatability than water composition. It was not possible to compare data from dry feed trials with those from water trials, as there was no method to extrapolate such data.

The inclusion of flavours into dry horse feeds is a common practice, aimed at increasing palatability or masking unpleasant flavours, with the ultimate aim of increasing intake (Pollack and Burton, 1991). Numerous studies have been conducted in an attempt to determine flavour preference in the horse due to the commercial value of such knowledge, stated by Lewis (1995) to have more of a marketing role than positive effect on food acceptance. It is recognised in human nutrition that food acceptance and hence, flavour inclusion, is essential for optimum health (Ennis, 1998). A summary of findings of flavour preference in the horse is presented in Table 1.1. Extrapolations of results need to account for differences in test chemicals used to represent the flavour, study design and individual variation between horses.

A preference for fruit flavourings may reflect the accepted preference for sweet substances in horses, with lemon oil also increasing intake in the horse (Betz *et al*., 1979). A study by Goodwin *et al*. (2005 b) found a preference for ginger over a range of fruit, spice and herb flavours. These studies reflect a wide range of preference in the horse; however it is assumed that preference will be subject to individual variation.

Table 1.1 Summary of studies of flavour preference and relative acceptance in the horse

Authors	Preference ranking
Cairns *et al.* (2001)	Mint > garlic
Kennedy *et al*. (1998)	Cherry > apple > citrus > teaberry > control
Pollack and Burton (1991)	Control = apple[2] = orange[1] > peppermint[1] = carrot = wheatfeed syrup
Hintz *et al*. (1989 b)	Control = peppermint
Burton and Price (1983)	Control > apple > alfalfa > caramel > anise molasses

[1]preferred at low inclusion levels

[2]preferred at high inclusion levels

1.1.6 Previous research into olfactory response in the horse

Studies into olfactory response in the horse have previously focussed on the role of olfaction in stallion behaviour (Saslow, 2002). A study by Bonde and Goodwin (1999) found behaviours changed in response to novel and aversive odours. Horses showed increased interest in coriander, cumin, orange oil, sage and ginger; exhibiting a preference for coriander and ginger. From the increased interest in odours presented, it is assumed that horses possess the olfactory receptors to be able to detect them.

1.2 Ageing in the horse

From birth, all biological systems are subject to development, maturation, then senescence (Corso, 1981). The widely accepted view that gustatory and olfactory responses decrease with age in humans (Chauhan *et al.,* 1987; Murphy and Gilmore, 1989; Stevens and Cain, 1993; Finkelstein and Schiffman, 1999; Kaneda *et al.*, 2000; Mojet *et al*., 2001; Mojet *et al*., 2005) may also be applicable to the horse. Age comparisons, estimated from physiological changes and average life span of humans and horses, are difficult to determine due to the variable influences of physical and psychological features on development and maturity (Corso, 1981). In the horse, age comparisons are subject not only to individual variation but also variation between breeds, such as longevity in pony breeds. Comparisons of anatomical changes and physiological changes indicate that horses mature more rapidly than humans (Lewis, 1995) (see Table 1.2).

Table 1.2 Suggested age comparisons between the human and the horse

Horse age (years)	Human age (years)
0.5	6
1	12
2	15
10	40
20	70

(Adapted from data, Lewis, 1995; King, 2003)

Taste function in humans is affected by several factors, including gender, genotype, medication and pregnancy (see Section 1.1.3). Alterations in taste function with life stage are stated to be a consequence of normal ageing (Schiffman, 2000). Changes in peripheral sensory receptors (Corso, 1981), neurological pathways and cognitive function (Mojet *et al.*, 2001) have been suggested to alter taste sensitivity with age. Nutritional deficiencies as a cause and effect of reduced taste function may exacerbate further a loss in taste sensitivity with advancing age (Rolls and Drewnowski, 1996; Rolls, 1999). Studies of gustatory and olfactory function have predominately focussed on the effects of advancing age rather than development. No study of the effect of age on gustatory or olfactory response has been conducted in the horse.

Predicted development and decline in equine taste status and changes in gustatory physiology can be made from comparisons of taste status and physiology with different life stages in humans (see Table 1.3 and Figure 1.1). From studies in humans (Corso, 1981), development of a sweet preference and bitter and sour aversion is predicted from birth to two-years in the horse. An associated increase in taste bud per papillae number may influence this development. In neonates, a sweet preference maintains feeding motivation, and bitter and sour aversion ensures toxin avoidance (Drewnowski, 2000).

Taste function is stable from maturity up to 60 years in humans; consistency in taste function is predicted from four to 16 years in the horse. A gradual decline in the taste bud per papillae complex is stated to be insufficient to affect taste response in humans during this period (Corso, 1981).

From 60 years onwards, taste function declines in humans (Corso, 1981). The most pronounced alteration in taste function in the horse is proposed from twenty-years onwards. The equivalent human age is associated with

physiological changes at receptor, neural and cognitive level. Whilst an animal is ageing, it does not mean it is senescing; the onset of the deleterious affects of age will vary between individuals.

1.2.1 The effects of age on the gustatory and olfactory systems

Gustation and olfaction rely upon the detection of specific chemicals in the environment by chemoreceptors. Gustation is the detection of chemicals present in a solution, olfaction being the detection of airborne chemicals. These senses are functionally similar and intrinsically linked, resulting in flavour perception.

It is widely accepted that taste function in humans is subject to development, maturation and senescence (Corso, 1981; Chauhan *et al*., 1987; Murphy and Gilmore, 1989; Stevens and Cain, 1993; James *et al*., 1997; Finkelstein and Schiffman, 1999; Kaneda *et al*., 2000; Pelchat, 2000; Mojet *et al*., 2001; Mojet *et al*., 2005). An age related decline in taste response in dogs (McCune, 2001) and rats (Cicala and McMichael, 1964) has also been recorded.

Changes in taste response in humans is suggested to reduce intake through lack of appetite stimulation and increase the risk of toxin ingestion through lack of appropriate recognition of taste cues and previous consequences of ingestion (Bernstein, 1999).

Table 1.3 Predicted changes in taste function and physiology as a result of age in the h

Horse Age (years)	Human Age (years)	Status of Human Taste Function	Physiological Chan
0	0	sweet preference and bitter aversion (Mela, 2000)	29% of papillae fe
0.5	6	development of bitter and sour aversion (Corso, 1981)	taste bud numbers
1	12		papillae numbers r
3	18		
4	21	taste function stable (Schiffman, 1992)	
5	24		
6	27		reduced papillae w
7	30		(Corso, 1981)
8	33		
10	39		
12	45		
14	51		
16	57		
17	60	taste function declines (Schiffman, 1992)	
18	63		
19	66		
			85% of papillae les (Corso, 1981)
20	69		Increased salivary
20+	70+	severe decline in taste function (Schiffman, 1992; Rolls, 1999; Mojet *et al.*, 2001)	impaired adaptatio reduced replaceme *et al.*, 2001)
			Sensory specific sa

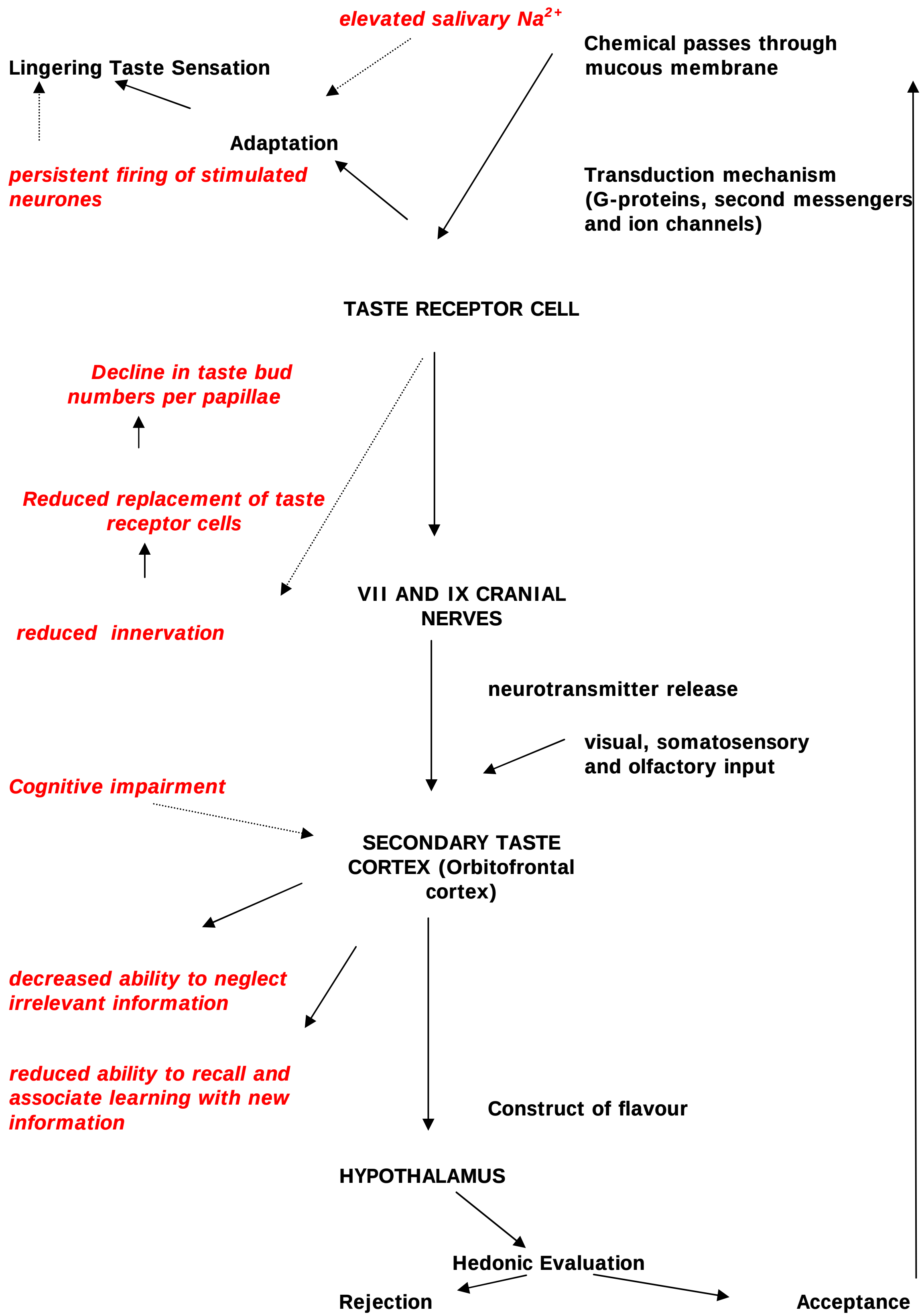


Figure 1.1 Predicted changes in taste function and physiology as a result of age in the horse. Italicised and red font denotes age effects.

The cellular and tissue structure of the horse's gustatory system is similar in architecture and keratinocyte ultrastructure to other mammals (Pfeiffer *et al*., 2000). Differences in papillae structure and taste pore distribution between horses and cows have been recorded (Chamorro *et al.,* 1986). The tongue of the horse has a more gustatory role, with the incisors having a tearing role. This is in contrast to the cow, where the tongue has a more mechanical role, rather than a gustatory role.

There has been no research into the morphology and physiology of the olfactory system in the horse. There has been limited research into the role of flehmen in the horse (Weeks *et al*., 2002); but there has been no study of the morphology and physiology of the olfactory system in the horse, in relation to taste. It is assumed to be no different from other mammalian olfactory systems. The mammalian olfactory system is comprised of three main organs; the main olfactory system, the accessory olfactory system and the chemaesthetic capabilities (Rawson, 1990). This study focuses on the main olfactory system.

1.2.1.1 Effects of age on the receptor level

Gustation

The horses' tongue is a mobile, muscular organ sited on the floor of the mouth, 12-20cm in length (Pfeiffer *et al.*, 2000) and covered in a mucous membrane, preventing drying or osmotic damage and involved in transduction. Prior to contact with the taste receptor cell, the chemical must pass the dense mucous substance filling the apical pit of the taste bud. Human salivary composition alters with age; increased salivary sodium levels distorting taste thresholds (Mojet *et al.*, 2001). The tongue shows no atrophic changes in size or area, as found in other bodily tissues with age (Corso, 1981).

Taste receptor cells are highly differentiated neuroepithelial sensory cells distributed across the dorsal surface of the equine tongue, the epiglottis and the palate (Chamorro *et al.*, 1986). Clustered into papillae, taste receptor cells provide information on physical and chemical changes in the environment. Papillae have two functions; gustatory or mechanical (Chamorro *et al.*, 1986) and are subdivided into five types; filiform, fungiform, vallate and foliate papillae with a line of circumvallate papillae separating the oral and pharyngeal regions of the tongue.

Age related changes in the taste bud number per papilla complex are evident in humans with a development in taste bud number papillae occurring in childhood (Segovia *et al*., 2002; Temple *et al*., 2002), through to a dramatic decline from seventy years onwards (Corso, 1981). In rats, maturation and increased distribution of taste receptor cells throughout the tongue and oral cavity has been recorded within the first postnatal days (Harada *et al*., 2000). Early maturation of taste buds on the soft palate was proposed to be the result of stimulation of the soft palate by milk in pre-weanling rat neonates, indicating their functional significance in suckling behaviour. Numbers of taste buds located on the soft palate (126.7 $\pm$ 23.7 to 203.7 $\pm$ 8.0) and fungiform papillae (110.3 $\pm$ 13.7 to 184.3 $\pm$ 26.3) increased from birth until one week. Circumvallate (1.0 $\pm$ 1.4 to 588.7 $\pm$ 57.5) and foliate papillae (3.0 $\pm$ 4.2 to 247.0 $\pm$ 77.2) continued to develop until rats were 8-9 weeks old.

A preference for sweetness and aversion to bitter and sour recorded in human neonates (Mela, 2000) is suggested to develop further through childhood and adolescence. Harada *et al.* (2000) do suggest that such changes in preference may also be influenced by cognitive maturity and social factors in humans.

Olfaction

The human olfactory system is stated to be anatomically complete before birth (Rawson, 1990), although further developments in morphology are recorded. Perception via the olfactory epithelium *in utero* and in the postnatal period is suggested to influence later preference (Mennella *et al.*, 2001). It is assumed that the olfactory epithelia of the horse are not subject to development in the postnatal phase.

Whilst the olfactory system has been subject to limited study in the horse, it is suggested that due to the large surface area for olfactory epithelium, the horse possesses a high sensitivity to olfactory cues (Saslow, 2002). The mammalian olfactory organ is a moist mucosal layer comprised of epithelium and supporting cells, with Bowman's glands continually producing glandular secretions to ensure the surface remains moist (Rawson, 1990). Whilst there is no uniform thickness of the epithelial layer in the mammalian olfactory organ, increased thickness correlates with increased numbers of sensory neurones.

Olfactory receptor cells have a life span of one month; the number of new cells being proportionate to those replaced. In rats, age is associated with a decline in olfactory receptor cell numbers, and an increased life span of mature neurones, compromising olfactory function (Goto *et al.*, 2001).

The cell bodies of the sensory neurones are positioned centrally within the epithelium, with cilia projecting to the epithelial surface (Rawson, 1990). The cilia increase the receptor cell surface area, increasing the potential for interaction with odours. For the odours to bind to the olfactory receptor cell they must first pass through the mucosal layer. Increasing thickness in the mucosal layer will affect the binding ability of the odour.

The olfactory epithelium of humans in infancy and early childhood is highly vascular. With increasing age, a regression in intraepithelial vessels occurs, ultimately resulting in the olfactory epithelium becoming avascular. A subsequent decline in olfactory cells has been proposed to be the result of accumulation of metabolic waste (Corso, 1981).

1.2.1.2 Effects of age on the neuronal pathways

Gustation

Reduced replacement of taste receptor cells with age may reduce taste function, with dietary deficiencies in zinc suggested to contribute to greater rates of cell apoptosis over renewal (Goto *et al*., 2001; Rudolf *et al*., 2001; Kitagoh *et al*., 2002). Zinc deficiency in the horse results in a decline in food intake and growth rate (Lewis, 1995; Frape, 1998). It has been stated by Schiffman (2000) that whilst age-related declines in taste function are irreversible, supplementation with zinc may reduce the extent of loss. Additionally, neurological decline resulting in reduced innervation may compromise differentiation through lack of cues for specialisation (Mojet *et al.*, 2001) and hence affect the rate of renewal.

Complete adaptation to a specific taste following continuous exposure occurs in one to five minutes in humans (Tortura and Grabowski, 2000). Taste adaptation is the result of reduced permeability to sodium ions in the membrane surrounding the generator region of the cell. Mojet *et al.* (2001) suggest that age affects adaptation due to prolonged firing of neurones, resulting in the lingering stimulus, consequently increasing the period prior to adaptation.

Olfaction

Physiological changes in the olfactory system in elderly human subjects have been recorded (Hirai *et al*., 1996). It is not known whether alterations to the neuroepithelia are the result of physiological age following prior disease or

exposure to irritants, or chronological disease (Corso, 1981; Rawson, 1990). The distribution of human olfactory receptor cells declines with age and is suggested to be a result of a reduced rate of cell renewal. Ageing results in a marked loss in olfactory fibres (97.3% in 17-91 year old humans) (Rawson, 1990).

It has been demonstrated that a reduction in olfactory receptor cell distribution compromises olfactory function little and hence, losses attributable to age may occur elsewhere in the olfactory system. Alzheimer's disease in humans reduces olfactory sensitivity through cognitive impairment (Lehrner *et al*., 1997).

Losses in olfactory function, if applicable to the horse, increase the likelihood of inhalation of chemicals that may have a detrimental effect on the olfactory system, and increase the risk of inappropriate diet selection (Drewnowski, 2000). Age-related losses in olfactory function result in an increased acceptance of novel foods and foods with unpleasant aromas in humans (Pelchat, 2000). Schiffman (2003) has shown that alongside the decline in the sense of smell with age in humans, hedonic response changes with potential implications on diet choice.

1.2.1.3 Effects of age on cognitive function

Gustation

Of the primary taste cortex, 6% of neurones are responsive to taste and 30% of neurones are involved with oral-related sensory and motor activity. The remaining 64% are of an undetermined role, indicated in the selection and digestion of foods (Rawson *et al.*, 1990). A construct of flavour is formed in the secondary taste cortex (orbitofrontal cortex), where visual, somatosensory and olfactory inputs converge. The hypothalamus is responsible for hedonic

evaluation, arousing excitation and inhibition with appetitive and aversive stimuli respectively (Rawson *et al.*, 1990).

The gustatory pathway is an ascending pathway, travelling in the direction of the brain. Mojet *et al.* (2001) propose impaired cognitive processes with age reduce taste sensitivity through: distortion of perception from spontaneously firing neurones; persistence of neural activity after stimulus; decreased ability to neglect irrelevant information; and reduced ability to recall and associate learning with new information. The horse is assumed to have the same neuronal pathways for taste as found in other mammals (Chamorro *et al.*, 1986) and may be subject to impaired cognitive function with age.

Olfaction

The olfactory nerve proceeds to the main olfactory pathway and onto the olfactory bulb. From here, the olfactory nerve branches to the amygdala, the piriform cortex, the olfactory tubercles and the hippocampus. It is within the orbitofrontal cortex that olfactory input combines with gustatory input to form a flavour complex (Rawson *et al.*, 1990).

As discussed in Section 1.2.1.2, Alzheimer's disease in humans reduces olfactory sensitivity through cognitive impairment (Lehrner *et al*., 1997). The impairment of cognitive function with age results in difficulties in measuring the extent of and cause of changes in gustatory and olfactory function.

A decline in olfactory acuity has been found from 20 years onwards in humans (Corso, 1981). A further reduction in acuity from 50 years is followed by a rapid decline from 80 years onwards. Reduced cognitive ability has implications for adaptation, from lingering perception of odours and a decreased ability to ignore irrelevant stimuli (Rolls, 1999; Mojet *et al.*, 2001). An inability to associate

learning with new information or reduced recall ability may compound olfactory receptor cell losses with age, affecting odour perception and flavour construct at cognitive level. There is limited knowledge of age related decline in cognitive function in the horse and such influences on olfactory function cannot be fully excluded from affecting learned aversions or preferences.

1.3 Determining taste and olfactory response in the horse

The effect of taste and olfactory stimulants on a living organism can be determined from changes in behavioural or electrophysiological responses. The study of morphology may indicate function. Approaches to identifying individual taste response are discussed in the following section.

1.3.1 Electrophysiological determination

Electrophysiological determination aims to identify gustatory response from innervations of nerves, specifically the glossopharyngeal nerve that innervates the posterior region of the tongue and chorda tympani that innervates the anterior and posterior region of the tongue (Segerstad and Hellekant, 1989 a, b).

Electrophysiological determination has been claimed to be more sensitive than determination of taste response from changes in behaviour (Segerstad and Hellekant, 1989 a) yet electrophysiological determination fails to provide a true representation of taste response in the 'whole' animal as no influence of prior learning or the environment is exerted, as in behavioural studies. Increased sensitivity of electrophysiological determination in comparison to behaviour changes is supported by findings in calves towards aspartame (Segerstad and Hellekant, 1989 a, b). An ability to identify aspartame was recorded with stimulation of the chorda tympani, with no response being recorded in the glossopharyngeal nerve. Investigation of taste response determined by two-choice preference testing in calves failed to identify a discriminatory response towards aspartame. Whilst the individuals investigated through two-choice preference testing may all have been taste blind, the finding does suggest increased sensitivity in electrophysiological determination does not always correlate with a behavioural change. This is due to the vast array of exogenous and endogenous cues, in addition to prior learning, which are present during behavioural studies. As stated earlier, an electrophysiological response only indicates whether a cue is detected, it provides no indication of the direction of preference.

Although the study of electrophysiological responses can be seen to have the limitation of their invasive nature, due to ethical considerations, benefits can be derived from this type of approach. Direct stimulation of the taste receptor region removes compounding factors such as food characteristics and experience and enables the sensitive areas of the tongue to be accurately recorded. Consistency between electrophysiological findings and behavioural findings has been confirmed in the calf by Hellekant *et al.* (1994), whereby glycine was found to elicit the strongest sweet response both from electrophysiological responses in the CT nerve (Segerstad and Hellekant, 1989 a), the glossopharyngeal nerve (Segerstad and Hellekant, 1989 b) and from behavioural responses (Hellekant *et al.*, 1994). No such correlation between behaviour and electrophysiological responses was recorded in the calf in response to aspartame.

Determination of electrophysiological responses was not appropriate for this study due to the highly invasive nature of the procedure (Segerstad and Hellekant, 1989 a). Nottingham Trent University ethical committee would not support an invasive study of this nature and, if approved, a Home Office Licence would be required.

1.3.2 Determination of taste and olfactory response through behavioural changes

To determine the response of an animal to a stimulus, the simplest method is to allow the animal to respond to this stimulus and to measure the resulting behavioural response and the duration of response (Manning and Stamp-Dawkins, 1998). This can be applied to the determination of taste response through changes in behavioural response and the resulting feed intake.

Behavioural taste thresholds, determined from the proportion of intake of a flavoured feed over an unflavoured feed (control), enable comparison of taste and smell function among species (Goatcher and Church, 1970 e). The testing procedure used influences greatly the reliability and comparative ability of such studies. Previous trials on flavour preference have analysed intake to determine taste response, without accounting for behavioural changes. Taste is involved in motivating and regulating ingestive behaviours in the horse and consequently changes intake in response to the acceptance of a feed.

1.3.2.1 Two-choice preference testing

Two-choice preference testing has been used to measure choice in animals, when one choice is presented with two options. Using two-choice preference testing, food preference has been studied in a range of species including horses (Houpt *et al.*, 1978; Pollack and Burton, 1991; Kennedy *et al.* 1998; Cairns *et al.*, 2001), ruminants (Goatcher and Church, 1970 a), cats (Chaffin and Beaver, 1993) and cockatiels (Matson *et al.*, 2000). Two-choice preference testing measures the consequence, and thus the duration, of behaviour aimed towards a specific stimulus (Lawrence and Illius, 1997), for example orosensory cues. This behavioural response can be used to determine the motivation to move towards or away from a stimulus. Changes in motivation in relation to food is related to cue strength, in addition to energy state (Toates, 1980), thus, preference may

be seen as an operant response to a stimulus, for example the association between a flavour and, in the short term, its hedonistic characteristics, or in the longer term, its postingestive consequences. Thus, it is suggested by Kyriazakis (1997) that the animal is presented with one choice with two options, the first being the source of food and the second being the rate of consumption (Kyriazakis, 1997), in order to satisfy short and long term goals. Choice is dependent upon the animal possessing the ability to detect the cues and being motivated to respond to the cues (Toates, 1980).

Two-choice preference testing relies on the assumption that when two identical choices are presented simultaneously, equal amounts will be eaten from both. Hence intake can be seen to be 0.5A + 0.5B, when A and B represent each choice. It is possibly the simplicity of this approach that has led to its widespread acceptance as a tool for measuring choice (Lawrence and Illius, 1997). Emmans (1991) suggests that a second theory can be proposed, that if given a choice of two identical feeds, selection will be total selection from one choice only (1A + 0B or 0A + 1B). No indication is given as to the direction of selection, or factors that are influencing such choice. If such information were available as to which factors determine preference, prediction of food intake would be achievable (Tolkamp *et al.*, 1998 a). Animals commonly select from both choices in order to satisfy preference (Emmans, 1991), therefore the theory put forward by Lawrence and Illius (1997) appears more plausible. This supports the selection of food based on both hedyphagia and postingestive consequences; a combination of feeds is preferred to a food source in isolation. Additionally a move from one option (A) to the other option (B) may be the result of inspective exploration in response to a change in A or an avoidance of B (Toates, 1980). Selection is influenced by variation between and within animals, and consideration of the dynamic nature of the animal is necessary (Emmans, 1991).

In a discussion relating to preference testing, it is necessary to address issues regarding the term 'preference'. A constraint of preference testing is that it provides limited choice and often is not reflective of the choices an animal is faced with in its natural environment (Lawrence and Illius, 1997). It may be more appropriate to discuss preference in terms of 'choice', as the actual preferred option of the animal may not be available, hence a choice is made.

Two-choice preference testing – operant response

An additional dimension that can be added to the traditional approach of measuring taste response through two-choice preference testing is to study how cost affects consumption. Costs are incorporated into the choice test by placing an obstruction between the motivated subject and the goal (Cicala and McMichael, 1964). This obstruction, such as an aversive tastant (Cicala and McMichael, 1964) or bar press (Johnson and Collier, 2001), has been used as a measure of motivation to select or avoid a gustatory or olfactory stimulus in rats. This degree of suppression or increase in consumptive behaviour can be used as an indicator of motivation and hence, the extent of gustatory or olfactory response.

The aim of this operant approach is to measure the motivation to select for a high value feed, through a higher cost approach. A limitation of this approach has been identified in rats (Johnson and Collier, 2001), whereby the rate of eating decreased towards the preferred feed when costs were increased. Costs were implemented by the rats bar pressing to gain access to the food choices. This decline in preferred food intake when costs increase may indicate that the preferred choice was not essential to the rats, so taste is less influential on choice than, for example, nutritional requirement. The incorporation of cost into the determination of taste response may be seen to provide results contradictory to the accepted view that palatability increases intake (McArthur, 1993).

T-maze apparatus

A variation of the two-choice preference test is the T-maze, whereby the animal has free choice between two options; the spatial distribution between the two choices is greater. This has the implication that sampling times between the two choices are greater than two-choice preference testing. Choices made in T-mazes are subject to the win-stay strategy, where a positive result from initial choice prevents further exploration of alternative sources in the short term, and fixing of positional choice in the longer term (Hosoi *et al*., 1995). The win-stay strategy is supported by the memory of a successful pathway to food (Gillingham and Brunel, 1989), where a positive response influences the following foraging expedition. In addition to the win-stay strategy, choice within a T-maze may be influenced by the lose-shift strategy, where a negative experience drives the animal to select a new choice at the onset of the next foraging expedition (Hosoi *et al.*, 1995). The win-shift strategy may also be influenced by the completion of one food source leading to searching for an alternative food source. The exhibition of choice is influenced by positive and negative experiences of the previous foraging expedition.

In addition to the win-stay strategy, spontaneous alteration may account for inconsistency in choice when two options are presented, alteration in choice occurs when cues remain consistent. It is suggested by Toates (1980), that the reversal of a choice may be the result of boredom relating to the immediately prior option, and attention is then directed to the second option. The animal is said to be responding on the basis of the previous experience, selecting the opposite option to that selected previously. An additional longer term consideration is the distinction between the animal moving towards an option due to exploration or moving away from the option due to avoidance (Toates, 1980).

Although the T-maze can be seen to have its advantages in identifying long-term responses to feeding preferences, it is inappropriate for the identification of taste response in the short term. Sampling is essential to enable the animals to distinguish between the two feeding choices, limited in a T-maze by greater spatial distribution. This may ultimately result in taste response being influenced by the win-stay strategy or win-shift strategy, or spontaneous alteration, rather than short-term taste response. The horse may still be subject to these strategies during two-choice preference testing, the close proximity of the feeding choices aims to reduce this.

Two-choice preference testing is regularly used as a tool to measure choice between two options. It is a successful technique when the animal is motivated to changes its behaviour in response to a cue. Evidence of side preference in relation to two-choice preference has been found in birds (Matson *et al*, 2000), horses (Hawkes *et al*., 1985), cats (Chaffin and Beaver, 1993) and sheep and goats (Hosoi *et al.*, 1995) but may be the result of insufficient motivation to result in a change in behaviour between container positions.

Different approaches have been implemented to attempt to counteract such cues, including daily reversal of test food / chemical positioning in horses (Hawkes *et al*., 1985), sheep and goats (Hosoi *et al*., 1995) and pigs (Kare *et al.*, 1965) placing of the preferred test food on the least preferred side in pigs (Cicala and McMichael, 1964) and cockatiels (Matson *et al.*, 2000) and randomised tastant positioning in humans (Mojet *et al.*, 2001). Richter and Campbell (1940) failed to allow for the influence of side preference on choice in rats by consistently presenting the tastant in the same position. No discussion of the influence of side preference is provided and hence, results should be interpreted with caution.

Bottle tests – water tests

The presentation medium used to measure taste response has been shown to influence the sensitivity of subjects to a tastant, stated to be the result of confounding flavours from 'mixture suppression' (Stevens and Cain, 1993). Water is stated to increase sensitivity to flavours compared to flavour presentation combined with food (Randall *et al.*, 1978). Bottle tests have been successfully used in pigs to identify taste reactions to a range of tastants (Kare *et al.*, 1965). Due to the slower rate of intake of water in comparison to concentrate feeds, the test solutions are placed for a longer period of time, for up to 24 hours. In order to reduce the influence of side preference on choice, reversal of position may be required every three hours (Kare *et al.*, 1965). Additionally, experimental design requires consideration of the influence of ambient temperature on evaporation rate from the test solution.

As stated above, test solutions have been suggested to be more sensitive than incorporation into a feedstuff (Kare *et al.*, 1965; Randall *et al.*, 1978). Taste plays such a fundamental role in foodstuff selection that it is maybe more appropriate to test in the latter manner.

1.3.2.2 Monadic

Monadic testing has previously been used to measure the effect of flavour inclusion on intake in the horse; variations in the usefulness of this technique have been reported. Pollack and Burton (1991) found monadic intake decreased when the least preferred flavoured test feeds were presented, whilst Hintz *et al*. (1989 b) found flavour presentation had no effect on intake. These findings need to be interpreted with caution, due to variation in basal feed and flavours presented.

Monadic testing can be discussed in terms of assessing acceptability, rather than preference, as the absolute values of a feed are determined, not its comparative value. Due to the delay in time, comparisons of findings between days are limited and results should be interpreted with caution. The use of monadic testing may be subject to a further limitation if group intake of a test solution is compared to group intake of a control. This causes results to be subject to both inter and intra individual variations and offers limited reliability (Matson *et al.*, 2000).

1.3.2.3 Determination of olfactory response

Studies on olfactory response may take two approaches, either limiting olfactory cues, or presenting additional olfactory cues. Prior research into olfactory function in the horse has been more in relation to the influence of olfaction on sexual behaviour (Saslow, 2002) than feeding behaviour.

Reducing influence of olfactory cues

Several studies have attempted to reduce the influence of olfactory cues on sexual behaviours in horses (Anderson *et al.*, 1996). Mentholatum® has been indicated as a suitable substance to mask odours, through liberal application to the nostrils and application to a cotton cloth within a leather muzzle (Anderson *et al.*, 1996). A limitation of this technique is that control of olfactory cues by mentholatum does not eliminate olfactory cues, only distorts odours detected. It would be inappropriate to adopt this method for the study of the effect of olfactory cues on feeding behaviour, due to the distortion to both olfactory and gustatory response, rather than removal of cues. Although the study by Anderson *et al.* (1996) found olfactory cues to influence stallion behaviour, results must be interpreted with caution. Olfactory cues were not blocked, indicating asnomia, but masked.

Trichloroethylene has been used to inhibit olfactory stimuli in the horse, when studying the effect of olfactory cues on stallion sexual behaviour (Wierzbowski, 1959). Trichloroethylene works by desensitising olfactory responses.

Nose plugs have been used in humans to reduce olfactory stimuli during identification of gustatory thresholds (Schiffman *et al.*, 1979 a). This is infeasible in the horse due to its reliance on air intake through the nasal cavity.

Presenting additional olfactory cues

Bonde and Goodwin (1999) studied the response of horses to several odours presented in cotton bags, with cotton wool acting as the control. The latency to approach each odour, presented as a two-choice preference test alongside a control, and the behavioural response was recorded. Such studies are subject to error due to additional olfactory cues present in the stabling environment.

Chaffin and Beaver (1993) have studied olfactory response in cats using paired odour testing. Validity of this test is reliant on consistency of air change and quality and may be difficult to employ to study olfactory response in the stabled horse. The influence of olfactory cues from the external environment on olfactory response has been achieved in cows (Corley *et al.*, 1999). Odours were blown over the feeding choice and changes in intake were recorded to study the effect of olfactory cues on food preference. A similar method was used to study the influence of olfactory cues on food preference in the dog (Houpt *et al.*, 1978). Chronic asnomia was medically induced, lasting for approximately six months. The discriminatory olfactory abilities between asnomic and intact dogs were determined using two-choice preference testing.

Corley *et al.* (1999) established a technique for assessing the influence of olfactory cues on feeding preference in Holstein cows. Cows were presented

with two identical feeding choices, presented left and right, simultaneously. Odorants were distributed over a feeding area at a rate of 4.5 $Lmin^{-1}$. Using airflow regulators, the odour was distributed using a standard compressor, passing into the two feeding containers. Baffles were fitted to each container side in an attempt to prevent the carryover of odour between containers. A potential limitation of this technique is the same for any two-choice preference test, the influence of positional cues on intake. A further limitation is the potential for cross contamination that may result during sampling behaviours.

1.3.2.4 Determination of gustatory and olfactory response from behaviour

Behavioural decisions and rate of performance of behaviours are constantly required by animals in order to satisfy feeding motivation, these decisions motivating behaviour (Mason *et al.*, 1997). An animal's short-term preference may not reflect its long-term preference, with short-term choices not always being in the individual's long-term interests (Wathes, 1997). Individual changes in preferences may be influenced by day-to-day experiences in addition to preferences and aversion irreversibly established early in life from maternal influences (Kendrick, 1997). Wathes (1997) proposes that it is necessary for a model preference for a species to be determined, along with the range in variation and dynamic relationships.

The availability of environmental choices enables an animal to develop its own behavioural repertoire and physiological responses in order to satisfy its own requirements (Wathes, 1997). Preference testing enables an animal to perform a response, thus enabling motivation to be measured by recording the frequency and behaviour of ingestive behaviours (Manning and Stamp-Dawkins, 1998). Changes in behaviour are reliant on the animal making appropriate decisions in relation to endogenous and exogenous causal factors (Toates, 1980). Changes

in activities are associated with costs (Toates, 1980) and if behavioural changes are to be used to determine gustatory and olfactory responses, then the cost of switching behaviour in response to a test cue, must convey a benefit for a change in behaviour to occur.

Manning and Stamp-Dawkins (1998) propose that it is easier to determine feeding behaviour by measuring intake than it is to record the frequency and duration of ingestive behaviours. Ingestive behaviours are motivated by the feeding choices of an individual (Mason *et al.*, 1997). Alteration in the priority of feeding motivation is reflected in changes in behaviour and is dependent on when the animal is tested. It is necessary for a feed trial to be carried out at the normal feeding time, when feeding motivation will be highest.

Mason *et al.* (1997) state that it is necessary to study full behavioural bouts with completion of feeding determined by the animal, as opposed to a time frame, when determining choice. This does not enable the short-term response to a taste to be determined and provided sufficient time is allowed for a choice to be made, data will be distorted as a result of the least preferred choice being consumed following total intake from the preferred choice.

The recordings of short-term behavioural sequences that are easily recognised and distinct enable a taste response to be determined. These descriptive terms constitute the behavioural repertoire of a species in response to a specific motivation, in this study feeding, and are recorded as an ethogram (Manning and Stamp-Dawkins, 1998).

Behaviours were originally defined as short sequences of behaviours that were immutable and performed in exactly the same way, termed fixed action patterns. Manning and Stamp-Dawkins (1998) contradict this definition, stating that not all

behaviour patterns are stereotyped. This allows for individual variation within a group and variation within individuals, enabling comparisons across a species group and individuals.

The recording of ingestive behaviours onto videotape enables the analysis of behavioural patterns to be more objective and accurate (Manning and Stamp-Dawkins, 1998).

The reliability of behaviour analysis enables the determination of an animal's ability to be consciously aware of their environment. Such observations act as an index of awareness (Kendrick, 1997). The ability to detect and respond to changes in the environment, such as light intensity and taste, is observed in all organisms and is not unique to the more advanced species (Kendrick, 1997).

1.4 Study aims

There is a paucity of information relating to gustatory and olfactory function in the horse. Whilst there is a consensus of opinion regarding age-related changes in gustation and olfaction in humans, no such study has been conducted in the horse. Gustation and olfaction are fundamentally involved in the role of nutrient acquisition and toxin avoidance. Any loss of function in these systems has consequences for appropriate diet selection and ultimately welfare. The increasing longevity of the horse (Harris, 1999; Hintz, 1999 a; King, 2000), suggested to rise from 20-25 years to 30-40 years (Hintz, 1999 a), increases the risk of a decline in gustation and olfaction associated with ageing. This study aimed to further knowledge of gustatory and olfactory response and determines any age related change in function.

The principal aims of this study were:

Aim One To investigate if, and to what extent, taste response is affected by advancing age in the horse

- § **Objective** To identify individual taste response in the horse for the purposes of age comparisons
- § **Hypothesis** Taste response will be subject to a decline in function, associated with advancing age

Aim two To investigate if, and to what extent, gustatory response is affected by advancing age in the horse

- § **Objective** To identify individual gustatory response in the horse for the purposes of age comparisons
- § **Hypothesis** Gustatory response will be subject to a decline in function, associated with advancing age in the horse

Aim three **To investigate if, and to what extent, feeding behaviour is affected by age in the horse**

- § **Objective** **To identify individual feeding behaviour during determination of taste response, namely; training, two-choice preference testing and monadic testing**
- § **Hypothesis** **Feeding behaviour will be subject to age-related changes in the horse**

Chapter 2:

General Materials and Methods

The materials and methods outlined in this chapter were used to identify individual taste response (Chapter 4) and gustatory response (Chapter 5) in the horse for the purposes of age comparisons. The general materials and methods were established following a number of pilot trials to validate techniques, the findings of which are discussed in Chapter 3.

2.1 Horses

The study was conducted at the School of Animal, Rural and Environmental Sciences, Nottingham Trent University's Equestrian Centre, Southwell, Nottinghamshire. Approximately 60 horses were stabled on the yard, used for the education of students in equitation and stable management. The experimental design therefore needed to take the role of the horses into consideration.

All the horses used throughout this study received a minimum of one hour ridden or loose schooling daily. Horses were not turned out for the duration of the trial and hence, were stabled for up to 23 hours daily. The horses used for this study were predominately Thoroughbred or Thoroughbred crosses, aged from 2 to 21 years and between 150cm and 180cm high. The infeasibility of a longitudinal study due to time limitations resulted in a cross sectional study of horses allocated to Young, Middle and Old age groups to determine the effects of age on taste and gustatory function. The allocation of individuals is detailed in the materials and methods section, relevant to each study.

Although feeding history is stated to have a significant effect on food intake (Tolkamp *et al*., 1998 a), information relating to the feeding history of horses used in this study was limited, but obtained where available. All horses had previously received concentrate feed, albeit small amounts in the younger animals, and had not been previously used in two-choice preference testing prior to the commencement of this study. It was assumed that the horses had not previously been exposed to the flavour used to determine taste response, artificial orange juice flavouring, or to the flavours used to determine gustatory response, aspartame, sucralose or glycine as they are not commonly incorporated into horse feeds. This aimed to remove the influence of prior conditioning to the flavour on preference.

A link between poor dental condition and reduced intake and feed digestibility in the horse has been suggested (Ralston *et al.*, 2001), if not proven clinically. All study horses were therefore subject to a dental examination by a qualified British Equine Veterinary Association (BEVA) Equine Dental Technician (EDT) within two weeks of each trial commencing. No horses were found to have severe dental abnormalities, however all minor hooks and points were removed.

2.2 Feeding regime

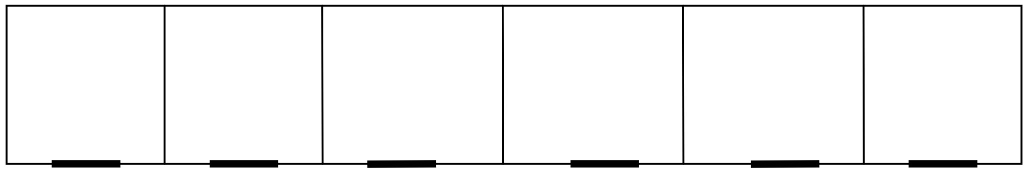
Horses were fed concentrated feeds at 0700h and 1700h each day, with weighed nets or forage, either hay or haylage, presented at 0700h and 1700h each day. The feeds presented as part of the study were given in addition to the normal concentrate feeds received by the horses. Normal concentrate feeds were fed according the manufacturer's recommendations, specific to their energy requirements. The test feeds were presented at 1630h, aiming to ensure feeding motivation was high due to anticipation of food at 1700h, thus resulting in stronger preference responses being exhibited (Tolkamp *et al.,* 1998 b) and attempting to standardise hunger levels. Any remaining forage was removed

from the stable immediately prior to the presentation period commencing, to prevent horses selecting forage over the study feeds.

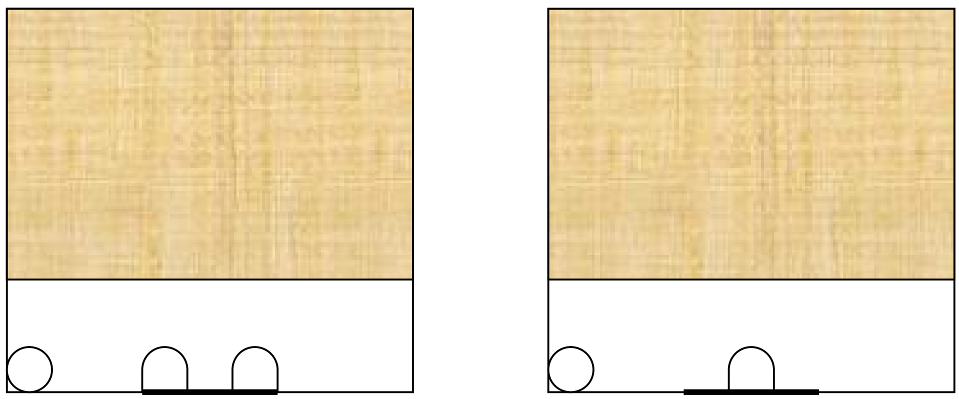
2.3 Housing

Horses were housed individually in loose boxes with water available *ad libitum* (see Figure 2.1 and Figure 2.2). Individual stabling prevented interference from conspecifics and reduced social facilitation from visual cues (Mason *et al.,* 1997), as individuals had no view of other horses eating unless their head was placed over the stable door. All horses were moved into the stables a minimum of three days prior to the trial commencing, to allow them to familiarise themselves with the stables and conspecifics.

Figure 2.1 Individual stable design used for subjects, with the exception of Horse 7 and Horse 8 during 3.1 validation of two-choice preference testing and flavour suitability.



a) Layout of the stable design for the six stables used.



b) Two container presentation **c) Monadic container presentation**

Figure 2.2 Diagrammatic representation of the stables (4.5m by 4.5m) used during all studies, with the exception of Horse 7 and Horse 8 during 3.1 validation of two-choice preference testing and flavour suitability, showing (a) the layout of the stable design and the positioning of the containers during (b) two-choice preference testing and (c) monadic testing.

= feed container **= water container** **= shavings**

In order to prevent stable design influencing the incidence of side preference from positional cues, horses were housed in six stables of identical design, unless stated otherwise. Stables measured 4.5m by 4.5m, with the door positioned centrally. The symmetrical design of these stables enabled the food containers to be positioned centrally within the feeding area, again reducing the influence of positional cues on side preference, and enabled the horses to position themselves perpendicular to the feeding choices.

The six stables formed a row that was isolated from the remainder of the yard and therefore activity on and around the yard could be kept to a minimum during the presentation period. This aimed to prevent external disruptions, such as noise disturbances, affecting the behaviour of the subjects. The close proximity of the stables to each other aided ease of carrying out the protocol, such as placing containers on the door, and thus one individual could carry out the study.

A wood shavings bed covered the rear ¾ of each stable floor, replaced as necessary. Shavings were selected over the more edible bedding option, straw, in order to prevent the consumption of bedding affecting food intake. Wet bedding and droppings were removed in the morning and throughout the day as necessary, thus reducing the effect of ammonia emissions on olfactory response, previously found to affect feeding behaviours in pigs (Wathes, 1997). The front ¼ of the stable area, the area of test food presentation, was bare concrete, aiding the collection of any food spilt by the horses during the presentation period.

2.4 Food preparation and presentation

Hereon in 'test food' refers to the flavoured basal feed, whilst 'control' refers to the unflavoured basal feed. Throughout the trial, feeds were presented in feed containers measuring 39cm X 32cm X 25cm, with a 15 litre dry capacity. Identical black feed containers were used to prevent the influence of visual cues on choice (Hawkes *et al.*, 1985). Feed containers were hooked over the stable door, facing towards the horse. This provided two benefits: firstly, it reduced horses putting their head over the stable door, thus reducing the likelihood of external visual disturbance; and secondly, it enabled a clear view of feeding behaviours to be recorded. Horses had no view of conspecifics whilst their heads were not placed over the door.

To prevent feed preference being affected by residual olfactory cues from previous presentation of the test feed, containers were allocated to two groups at the onset of the study for either test feed presentation or control presentation. Following each presentation period, all containers were thoroughly washed to attempt to reduce residual olfactory cues.

Containers were marked using white paint as either control or test feed accordingly. Marks were placed on the back of the containers and thus were easily identifiable to the researcher, but provided no visual cue to the horse. Control containers were marked with white paint, whilst test feed containers were marked with numbers from 1 to 18, corresponding to the individually numbered horses.

Using clips attached to the back of each container, the containers were secured on to the stable door. The purpose of this was two-fold; firstly, to prevent the containers moving along the door, ensuring the 20cm distance between the left and right container was consistent during two-choice preference testing; and secondly, to prevent the horses knocking the containers off the stable door.

2.4.1 Basal feed

The basal feed comprised of Wheat feed pellets (Hackett Feed Mills, Leicestershire, UK), selected due to the relative acceptance of wheat by horses (Hawkes *et al.*, 1985) and its low nutritive value (Table 2.1). The homogenous characteristics of the pellets, measuring approximately 1.5cm by 0.8cm, prevented the horses selective feeding.

Table 2.1 Nutrient Analysis of Wheat Feed Pellets

Typical Composition	Content (%)
Protein	17
Oil	5
Fibre	7.9
Ash	5
ME-R MJ/kg DM	10.5
Calcium	0.1
Phosphorous	0.9
Magnesium	0.35
Salt	0.1

(Straights direct, 2004)

2.4.2 Flavour selection

In order to determine taste response and gustatory response in horses, it was necessary to select suitable flavours to meet the aims of the study. For a flavour to be suitable to determine either taste response or gustatory response it needed to have no negative impact on the health of the horses, have limited nutritional value to prevent feed selection based on awareness of postingestive consequences and have consistency of characteristics. It was also necessary for the flavours to evoke a change in behaviour, hence intake, in order to determine response. Therefore, horses needed to possess the ability to discriminate between the test feed and control. In addition, the flavour used to determine gustatory response needed to evoke a taste response in the absence of additional feed-related olfactory cues.

2.4.2.1 Flavour to determine taste response

The inclusion of orange flavouring (Pollack and Burton, 1991), citrus flavouring (Betz *et al.,* 1979; Betz *et al.,* 1980) and citrus peel (Lewis, 1995) on dry horse feed has previously been shown to evoke a preferential response in the horse, indicated by an increase in intake. From these studies it may be concluded that horses possess the taste receptors to detect these flavours. However, from the findings of the previous studies, it was necessary to acknowledge variation in the flavour composition, study design and individual variation in the horses. Hence, validation of the artificial orange juice flavouring used in this study to determine individual taste response in the horse is discussed in Section 3.1 and 3.2.

Whilst difficult to obtain a full feeding history of all the horses, orange was selected as a novel flavour as, to the best of the author's knowledge, orange flavouring had not been presented to the horses in the past twelve months. Additionally, orange flavouring is not commonly added to UK horse feeds. The presentation of a novel flavour aimed to avoid prior conditioning to the flavour, which may influence selection. However, as orange is not a natural feed for horses it was necessary to ensure that they had the ability to detect the artificial flavour presented.

2.4.2.2 Flavour to determine gustatory response

The characteristics deemed necessary to identify gustatory response was that the flavour evoked a taste response in the absence of additional feed-related olfactory cues, termed gustatory response. Aspartame and Sucralose were initially studied as potential flavours to determine gustatory response in the horse; however they failed to meet the necessary requirements (see Section 3.3 and Section 3.4). Following an initial study (see Section 3.5), glycine was selected as a suitable flavour to determine gustatory response in the horse.

2.4.3 Test food preparation

Flavours may be added to the basal feed using a range of methods; mixing a liquid flavour with the basal feed immediately prior to presentation (Hawkes *et al.*, 1985) or incorporating the flavour into the feed during the manufacturing stage (Pollack and Burton, 1991; Cairns *et al.*, 2002). Although incorporation of the flavour at the manufacturing stage may increase the uniform distribution of the flavour into the pellets, it was deemed an unsuitable method for this study due to the complexity of different flavour inclusion levels being required. The method of adding the flavour immediately prior to presentation (Hawkes *et al.*, 1985) was adapted to meet the aims of the study.

All the flavours used were provided as white, water-soluble powders. The relevant quantity of flavour powder was dissolved in 40ml-distilled water, to give the appropriate inclusion level ($g.kg^{-1}$). The flavour solutions were vigorously shaken to ensure the powder was completely dissolved in the distilled water.

Food preparation was conducted inside a large, well-ventilated room. This aimed to prevent the loss of flavour due to excess evaporation from exposure to external factors such as sunlight or wind. The basal feed was weighed in batches of 1.5kg and spread over plastic sheets measuring 150cm by 150cm. Each sheet was allocated and labelled as control or flavour, to reduce the likelihood of contamination of the control by residual flavour cues. The allocation of these sheets was consistent throughout the study.

It was necessary to ensure the test solution was equally distributed over the basal feed, to ensure uniform distribution. To meet this objective, 18kg of basal food was spread over large plastic sheets, allocated to the different test feed inclusion rates. Once the basal feed was spread in a thin, even layer over the sheets, the test solution was sprayed onto the basal feed.

The test solutions were sprayed onto the basal feed using 800ml capacity hand operated sprayers (Focus DIY, UK). This procedure aimed to give a uniform covering of the pellets, indicated by a darkening in colour when damp. The feed was turned over by hand at intervals during the spraying process, ensuring all pellets were covered. The sprayed feeds were left for two hours to dry, to prevent changes in texture affecting selection.

An equal amount of distilled water was sprayed over the basal feed to be used as the control (Hawkes *et al.*, 1985), thus preventing any changes in texture, as a result of spraying, influencing selection. The control was subject to the same drying period of two hours, prior to presentation.

2.4.4 Presentation period

As stated previously, the presentation period was conducted at 1630h, for a ten-minute period. All timings were measured using a standard stopwatch. Two approaches to presentation period may be used when determining the effect of orosensory characteristics on intake, finite time period or time to completion.

A finite time period, termed 'closed access', was used in this study to determine taste response and gustatory response in the horse, whereby intake was measured during a specific timed period (Lawrence and Illius, 1997). Previous studies into the effect of food characteristics on intake in the horse have ranged from 10 minutes (Hintz *et al.,* 1989 b) to 30 minutes (Hawkes *et al.,* 1985). It has been previously shown by Hintz *et al.,* (1989 b) that limiting access to a food source does not increase the rate of intake through learning of imminent food removal. Findings by Hintz *et al.* (1989 b) show that in horses the rate of intake is higher during the first five minutes of concentrate feed presentation than in the subsequent five minutes (158.5 $\pm$ 11.8 *versus* 151.1 $\pm$ 12.8 $g.min^{-1}$), with intake averaging 140 to 190 $g.min^{-1}$ during a ten-minute period. It must be acknowledged that intake is subject to inter and intra individual variation and will be affected by numerous factors such as the type and quality of basal feed used, premeal period and dental state. The average meal in the domestic horse is 44 minutes (Hintz *et al.*, 1989 b); therefore it can be stated that a ten-minute presentation period is sufficient time for an exclusive choice to be made, without sensory specific satiety influencing choice (Berridge, 1991). Although motivation may alter during a bout (Mason *et al.,* 1997), in this context feeding motivation,

the horses can be expected to eat continuously during this ten-minute period (Argo *et al.,* 2002), thus reducing the incidence of intrameal intervals.

The time to completion of a predetermined amount (Pollack and Burton, 1991; Cairns *et al.,* 2002; Goodwin *et al.,* 2005 a, b) may also be used to measure the short-term response to the orosensory characteristics of the food. However a significant limitation to this technique may include the disturbance of the horse whilst eating, to identify when the set amount has been consumed. Time to completion does allow for differences in bite-size and chewing rate (Goodwin *et al.,* 2005 a), but this is more applicable to forage intake trials than measurements of short-term response to orosensory characteristics.

For choice to be maintained, it was necessary for each option to be available throughout the presentation period. Total consumption of one feeding choice may result in hunger being measured, rather than preference. 1.5kg of the test feed or control was presented in each container, aiming to ensure a minimum of 20% of food remained if the horse selected from one container choice only (Goodwin *et al.,* 2005 a). This was essential to maintain choice, preventing the exhibition of win-shift strategy (Hosoi *et al.,* 1995), whereby on completion of one feeding choice, the animal moves to the next available option.

To ensure each horse was presented with each container(s) for the ten-minute period, staggered presentation of containers was necessary. Following placement of the first container, or containers, for the first horse, a ten second interval was implemented prior to presentation of the next subject's container(s). This provided sufficient time for the researcher to place the subsequent subject's container(s). If two feed choices were being presented simultaneously, it was ensured that both containers were presented over the

stable door simultaneously, to remove the influence of placement order on selection.

To prevent the presence of the researcher affecting behaviours of the subjects, the researcher left the stabling area immediately following the placing of the final container(s) and waited out of sight. All staff, students and visitors to the yard were made aware of the study, ensuring that activity during the presentation period was minimised. Entry to the stable block during the presentation period was also prevented.

2.5 Study design

To determine taste response or gustatory response, both two-choice preference testing and monadic testing were conducted, as discussed in section 1.3.2.1 and 1.3.2.2 respectively.

The study was conducted over consecutive days, including weekends, to ensure any changes in intake or preference were recorded. Horses were subject to testing only once daily and all horses acted as their own controls.

2.5.1 Testing procedure

In order to study the effect of age on taste and gustatory function, it was necessary to identify taste or gustatory responses in individuals for the purposes of age comparisons. The incorporation of flavours aimed to determine the sensitivity of the horses to the gustatory and olfactory cues from the test feed.

2.5.1.1 Training

Prior to determination of taste response or gustatory response, all horses were subject to an initial training period, which involved the simultaneous presentation of two food containers of an equal quantity of unflavoured basal

feed. The aims of training were two-fold: firstly, to limit neophobic responses to changes in feed presentation method by familiarising the horses with, and ensuring awareness of, two positional feeding choices – left and right; and secondly, training aimed to establish a constant level of intake of the basal feed prior to test feed presentation. To ensure that training met the above aims, validation of training was conducted (see Section 3.1 and Section 3.2).

A decline in total feed intake is a recognised consequence of the introduction of a novel food in horses (Hawkes *et al.,* 1985; Whittemore *et al.,* 2002; Goodwin *et al.,* 2005 a) and ruminants (Tolkamp and Kyriazakis, 1997). The familiarisation of an animal to a novel food source is therefore used to ensure intake has reached equilibrium prior to test feed presentation. Periods of familiarisation have varied from two days (Goodwin *et al.,* 2005 b) to four days (Hintz *et al.,* 1989 b) in horses, seven days (Mueller *et al.,* 1998) in donkeys, and up to 14 days in cows (Tolkamp and Kyriazakis, 1997), possibly reflecting differences in digestive physiology. Equilibrium in intake in the horse has been stated to occur after three (Hintz *et al.,* 1989 b) to five days (Whittemore *et al.,* 2002) following presentation of novel feed; hence the training periods were conducted for a minimum of five days. Training can therefore be seen to have an important role in replacing exploratory behaviour with ingestive behaviours, through awareness of two feeding choices and familiarity with the novel basal feed.

Training can be conducted in one of two ways. One approach is to familiarise the animal to the basal feed and choice of two containers simultaneously, as conducted in this study (Randall *et al.*, 1978; Hintz *et al.,* 1989 b). The second approach is to encourage equal sampling from both containers through the use of a preferred test feed in one container choice. The positioning of this preferred flavour is alternated, until the animal is consistently selecting from both positional choices, used previously in cows (Hellekant *et al.,* 1994) and pigs

(Glaser *et al*., 2000). However, in the author's opinion, the manipulation of behavioural choice through prior exposure to a test flavour has flaws due to conditioning occurring prior to determination of taste response. Moreover, the aims of the current study were to determine short-term response to the orosensory characteristics of the feed and prior conditioning to the test feed would affect this.

During the training period horses were presented with two identical feeding choices, positioned left and right. 1.5kg of the wheat feed pellet basal feed was presented for a ten-minute period, as detailed in Section 2.4. The duration of training is detailed in the relevant materials and methods sections.

2.5.1.2 Two-choice preference testing

Throughout this study, the terms 'left' and 'right' relate to the positioning of the feed containers, as presented to the horse. Left relates to the horse's left positioning and right to the horse's right positioning, not as observed by the researcher.

Each horse was presented with two containers of feed simultaneously, each containing an equal quantity of feed (1.5kg). Containers were identical in colour and design (see Section 2.4) to prevent visual cues influencing choice. The containers were placed 20cm apart, the reasons being two-fold: firstly, to limit energy costs to the horse when selecting between the two containers by ensuring the containers were sufficiently close; and secondly, to prevent 'carryover' of olfactory cues from the test feed influencing selection of the control by ensuring adequate distance between the two containers. To reduce the influence of positional cues of the containers on choice, the position of the control and test feed were reversed daily (Hawkes *et al.,* 1985; Cairns *et al.,* 2002).

During two-choice preference testing all test feeds were presented alongside an unflavoured control. All test feeds were prepared as described in section 2.4.3. The test chemicals and their inclusion rates (gkg^{-1}) are detailed in the relevant materials and methods section of each chapter.

2.5.1.3 Monadic testing

Monadic testing, whereby one container is presented, enabling one feeding choice, by virtue of removal of positioning choice, theoretically should increase the sensitivity of the test compared to two-choice preference testing, as side preference can exert no influence on taste response. Although intake is closely associated with preference, intake in the horse has been shown to decrease little when a previously aversive food is presented monadically (Hawkes *et al.*, 1985). It has been proposed, however, that presentation of a flavour may increase intake when presented monadically (Hintz *et al.*, 1989 b).

Monadic testing, where horses were presented with one feed container, holding 2kg of flavoured basal feed, prepared as detailed in Section 2.4.3. The total food offered in each singly presented container was increased to 2.0kg from 1.5kg, as previously used during training and two-choice preference testing. This was due to a predicted increase in total intake due to a lack of sampling behaviour, as only one option was presented.

Four test feed inclusion levels were presented over eight consecutive days. Inclusion levels were increased over four days, with each previous inclusion level doubled. The reverse presentation order was given over the subsequent four days.

The presentation of the flavours as ascending concentrations during the first four days of monadic presentation aimed to increase the horses' sensitivity to the test

flavour, without evoking a strong aversion. It was proposed that the presentation of a high aversive concentration initially may result in a biasing effect at lower concentrations as a result of a subsequent disproportionate aversive response (Goatcher and Church, 1970 a). Therefore ascending concentrations followed by descending concentrations were employed.

2.6 Measurements

The study design aimed to control the environment and the food choices of the horses during the presentation period, thus ensuring greater accuracy of measurements (Tolkamp *et al.*, 1998 a). Accuracy of food intake and behaviour are thus relatively easily measured in the single housed horse, compared to free-living horses. Feed intake and behavioural changes were measured during all three elements of the study; training, two-choice preference testing and monadic testing.

2.6.1 Measurement of intake

The relationship between the acceptability of a food and the level of intake has been extensively researched in humans; both young subjects and old subjects (Schiffman, 2000).

Changes in feed intake have previously been used as a determinant of food preference or aversion in both the stabled (Betz *et al*., 1979; Betz and Lanter, 1980; Hawkes *et al*., 1985; Hintz *et al*., 1989 a, b; Pollack and Burton, 1991; Holland *et al*., 1998; Cairns *et al*., 2002) and free-living horse (Archer, 1971). Such responses may be used as an indicator of acceptability of a feed (Hawkes *et al.,* 1985).

In order to accurately measure food intake it was necessary to accurately weigh the feed prior to, and following, presentation. All feeds were weighed using electronic scales, accurate to 1g (Hanson, Hertfordshire, UK). The food was weighed prior to spraying with the test flavour and after spraying, once the feed had dried.

Following termination of the presentation period and removal of the containers from the horses, all food remaining in each container was weighed and recorded. In addition, any feed which had been spilt from the container, but not consumed, was weighed and recorded as spillage. All waste feed was disposed of, and not given back to the horses. This was because the feed presented during the presentation period was in addition to the horses' normal feeds, and did not constitute an important part of their nutritional requirements.

The amount of food consumed on a per container basis during training and two-choice preference testing was calculated as follows:

Amount eaten (g) = Total offered in container – Amount remaining in container - (0.5*spillage)

The amount of food consumed on a per container basis during monadic testing was calculated as follows:

Amount eaten (g) = Total offered – Amount remaining – spillage

Differences between individuals and across group were calculated as appropriate.

2.6.1.1 Determination of taste response

Taste response was determined from a significant difference between control and test feed intake during two-choice preference testing to study taste response.

2.6.1.2 Determination of gustatory response

Gustatory response was determined from a significant difference between control and test feed intake during two-choice preference testing to study gustatory response.

2.6.1.3 Determination of side response

Side response was determined from a significant difference between left and right intake during training and two-choice preference testing.

2.6.1.4 Determination of preference during monadic testing

Changes in intake were used as indicators of taste response or gustatory response during monadic testing.

2.6.2 Measurement of behaviour

In addition to behaviour being measured in terms of intake, a physical quantity related to ingestive behaviour (Martin and Bateson, 1986), behaviour was also measured in terms of changes in behaviour in response to the presentation of a test feed or control. Recording of behaviours exhibited during the presentation period enabled changes in feeding behaviours to be analysed in response to test feed presentation and hence taste response or gustatory response could be identified.

The presentation period of each individual was subject to continuous recording. Six monochrome closed-circuit television cameras (Micromark™, UK) were wall mounted, approximately 2m above the floor, providing a view of the individual presentation areas and continuous viewing of each subject (Figure 2.3). The position of the cameras did not enable viewing of the area directly behind the stable door.

Figure 2.3 View of the feeding area recorded by individual CCTV cameras.

Individual cameras were linked to individual videocassette recorders. This allowed recording of the presentation period, for analysis at a later date. Recordings were made onto videocassettes, allocated to each individual horse and labelled clearly. Videocassettes were converted to video files using the Roxio Programme (London, UK). From this format, behavioural responses could be recorded and analysed using continuous sampling via the Observer 5.0™ (Noldus, Netherlands) computer package. All data was exported to Excel, then Minitab 14.0 for statistical analysis.

Recordings of the presentation periods were analysed using the Observer 5.0™ computer package. Observer 5.0™ software is a tool for collecting and analysing observational data. An additional benefit of recording behaviour observations onto tape is that they may be kept as a permanent record and subject to further analysis at a later date.

As behaviour is exhibited as continuous movement and events, it was necessary to identify key behaviours to meet the study aims and to allow uniform analysis of behaviour within and between individuals. An ethogram of 12 mutually

exclusive behaviours was developed from an earlier study (see Section 3.1). Behaviours were allocated to four classes; side, head position, ingestive and locomotory. Frequency and duration of these behaviours was recorded (see Table 2.2).

For ease of measurements, the ethogram recorded behaviours in terms of positional cues, left and right, as opposed to the direction of feeding preference, control *versus* test feed. Consequently it was necessary to convert left and right behaviour to control and test feed behaviour for two-choice preference testing, as appropriate.

A behaviour-indicated preference was determined from a significant preference directed towards either the left container or right container during training and two-choice preference testing, or from the control or test feed during two-choice preference testing alone.

Behavioural classes identified in the ethogram were clustered into attention, indicating whether attention was directed towards the 'left' or 'right' container during training and two-choice preference or the 'monadic' container during monadic testing. Attention directed towards the 'floor' or 'else' related to behaviours recorded away from the presentation area. Head position was recorded as 'in' or 'out'. Ingestive behaviour was recorded from the duration of 'eat' behaviour, whilst non ingestive behaviour was recorded from the sum of 'alert' and 'other' behaviour. Locomotory behaviour was recorded as 'stand' or 'walk' as appropriate.

Table 2.2 The ethogram used to assess feeding behaviour in the horses studied (n=18)

Class	Behaviour	Description	Measure
Side*	Left[a]	The horse's attention is directed towards or in the left container	State
	Right[a]	The horse's attention is directed towards or in the right container	State
	Monadic[b]	The horse's attention is directed towards the single container presented	
	Floor[ab]	The horse's attention is directed away from the feeding area towards the floor	State
	Else[ab]	The horse's attention is directed away from the feeding area	State
Head position	In	The horse's muzzle is positioned below the upper edge of the container, constituting the head in the container	State
	Out	The horse's muzzle is positioned above the upper edge of the container, constituting the head out of the container	State
Ingestive	Eat	The horse is masticating, indicated by side-side grinding movement of the upper and lower jaw	State
	Alert	The horse holds its head rigid, above horizontal and does not masticate	State
	Other	The horse is not masticating or alert	State
Locomotory	Walk	The horse walks around the stable in a four-time gait	State
	Stand	The horse is static and does not move away from the presentation area	State

*The behaviours used to determine side class altered with the mode of presentation. [a] denotes behaviours recorded during training and two-choice preference testing, [b] denotes behaviours recorded during monadic testing.

2.7 Statistical analysis

All statistical analysis was carried out using MINITAB 14.0 (2004). A 95% confidence level was used throughout ($p<0.05$) (Pagano, 2006).

2.7.1 Statistical analysis of intake

In order to determine feed preference, both within individuals and across age groups, intake data were analysed to determine the extent of difference between control intake and test feed intake during two-choice preference testing. In addition, the difference between left container intake and right container intake throughout both the training period and two-choice preference testing was also analysed to determine the extent of side preference in susceptible individuals.

To identify whether an intergroup variation in total daily intake was exhibited during training, one-way ANOVA was used to identify significant differences between the Young and Middle groups, Young and Old groups and Middle and Old groups. To identify whether intragroup variation in total daily intake was significant within age groups, one-way ANOVA was employed, with six horses and nine data points per horse, per group.

One-way Analysis of Variance (ANOVA) was used to identify whether an individual exhibited a significant feed preference or side preference through changes in intake for the one factor with two levels, control and test feed during two-choice preference testing or left and right during training and two-choice preference testing. From this, ANOVA determined whether the individual variation within independent variables was less than variation between individual variables, indicating a difference.

Two-way ANOVA was used to examine the effect of the horses' age as well as test feed presentation on intake. The first factor, age, had three levels; young, middle and old. The second factor, test feed presentation, had two levels; control and test feed. This enabled the interaction between age and response to test feed on intake to be analysed.

Two-way ANOVA was also used to examine the interaction between age and container positioning on intake. Again, the first factor, age, had three levels; young, middle and old. The second factor, container positioning, had two levels; left and right side.

Two-way ANOVA was also employed to determine the interaction between age and intake in response to different test feed concentrations with factor intake, and the factor inclusion, which had three levels.

One-way ANOVA was employed to determine significant differences in total daily intake within groups.

The methodologies detailed above were used to determine significant difference between the independent variables of left and right container positioning.

2.7.2 Statistical analysis of behaviour

Analysis of behavioural changes in response to the test feed presentation aimed to determine the extent of preference, as appropriate, during two-choice and monadic testing. Additionally, behaviour was analysed to identify side preference throughout training and two-choice preference testing. Behavioural changes, in addition to being recorded through changes in intake, were recorded using the ethogram as duration and frequency.

From ingestive behaviour, recorded as 'eat' behaviour, statistical analysis was employed to determine whether age affected ingestive duration. The factor age, with three levels, Young, Middle and Old was analysed using one-way ANOVA to determine whether age effected the duration of ingestive behaviour between age groups.

Significant differences between attention directed towards the left and right container were analysed using one-way ANOVA in individuals. The same approach was applied to the difference between ingestive and non ingestive behaviour, calculated from 'eat' behaviour and the sum of 'alert' and 'other' behaviour respectively. Interaction between the factor of age, with three levels, young, middle and old and the factor of behaviour, with two levels, left and right or ingest and non ingest was calculated using two-way ANOVA.

Two-way ANOVA was also employed to determine the interaction between age and left and right behaviour duration and control and test feed behaviour duration in response to different test feed concentrations as appropriate.

The methodologies to further analyse behaviour using lag sequential analysis is discussed in Chapter 6.

Chapter 3:

Development of techniques for determining taste response and gustatory response in the horse

Prior to the study of the effect of age on taste response (see Chapter 4) and gustatory response (see Chapter 5) it was necessary to conduct preliminary studies to develop the proposed techniques.

3.1 Development of two-choice preference testing and flavour suitability

For an animal to show a preference for a flavoured feed it must possess the ability to discriminate between an unflavoured control and flavoured test feed. The response to the test feed can thus be used to determine preference, aversion or indifference to test feed and hence, taste response. For individual taste response to be identified for the purposes of age comparison, it was necessary to ensure the proposed methodology would enable the aim to be met.

The aims of the initial pilot trial were four-fold: firstly, to ensure training met the appropriate aims; secondly, to establish the discriminatory abilities of horses to an orange flavoured test food in order to identify taste response; thirdly, to study the effects of a delay in test food preparation on taste response; and fourthly, to ensure appropriateness of the ethogram.

For an animal to show a response, it must be able to detect a specific stimulus. It was essential that horses possess the ability to discriminate between the flavoured test feed and an unflavoured control. Variation between and within species has been observed in response to several taste modulators (see Section 1.1.1) with variation between individuals and within individuals also being recorded. The criteria for test flavour selection were that the animal must

possess discriminatory abilities towards the test flavour, not have been previously conditioned to the test flavour and that the test flavour elicited a positive response.

Artificial orange juice flavouring was selected as a suitable test flavour to determine taste response. Orange flavouring has previously been shown to elicit a preference response in horses, when presented at low inclusion levels (Pollack and Burton, 1983). Orange constitutes a novel flavour in equine feed in the UK, though it is commonly used in the USA as a flavour enhancer due to the abundance of citrus peel (Lewis, 1995). The novelty of the flavour aimed to prevent the influence of prior conditioning on taste response. As discussed in section 1.1.4, horses have previously been shown to have a preference for fruit flavours (Betz *et al*., 1979; Betz and Lanter, 1980; Pollack and Burton,1991).

It should be noted that an artificial test flavour was selected, which may bear little resemblance to natural orange juice or to those flavours used in previous studies of flavour preference in the horse. The reasons for selection of an artificial test flavour were two-fold: firstly, an artificial test flavour is assumed to have more consistent characteristics than natural flavourings; and secondly, by selecting an artificial flavour it was possible to use a flavour that had limited calorific value, hence selection would be based on hedyphagia, rather than awareness of postingestive consequences. Although artificial orange juice flavouring is a positive taste to humans, the assumption cannot be made that a horse perceives the flavour similarly (Hellekant and Danilova, 1996).

In addition to selection of a suitable test flavour, it was also necessary to identify suitable inclusion levels, when sprayed on to the basal feed. One of the main aims of this study was to establish the taste threshold of the artificial orange juice flavouring, when presented as a test feed, in the horse. These

threshold levels were to be used as the method of identification of taste response and, hence, the effect of age in the main element of study.

As discussed in section 1.3.2.1, two-choice preference testing can be seen to have several limitations. In the absence of animals being able to communicate their preference directly, two-choice preference testing proves a useful tool to identify taste response.

3.1.1 Study aims

The aims of the study were four-fold; firstly, to ensure training met the hypothesised aims; secondly, to establish discriminatory abilities of horses to an orange flavoured test food in order to identify taste thresholds; thirdly, to study the effects of a delay in food preparation on taste response; and fourthly, to ensure suitability of the ethogram.

3.1.1.1 Development of training

The first element of the study constituted a training period and it was hypothesised that;

1. Equilibrium of feed intake following presentation of the novel basal feed would occur
2. Horses would exhibit sampling behaviour from both feed containers presented
3. Exploratory behaviour would be replaced with ingestive behaviours when presented with a novel method of food presentation and novel basal food
4. Intake would occur around a 50:50 mean when one feeding choice with two identical feed options were presented simultaneously

3.1.1.2 Development of flavour presentation

The second element of this study aimed to ensure that a suitable test flavour was used to identify taste response in the horse. It was hypothesised that:

1. Horses would possess the ability to discriminate between an artificial orange test feed and an unflavoured control.
2. The flavour inclusion levels presented would identify individual threshold levels of artificial orange test feed.

3.1.1.3 Development of test feed preparation

The third element of study aimed to study the effects of varying delays between test food preparation and presentation on taste response.

3.1.1.4 Development of the ethogram

The final element of the study aimed to ensure the suitability of the ethogram of ingestive behaviours used to determine the taste response from changes in behaviour.

3.1.2 Materials and methods

Chapter Two describes the general materials and methods used to achieve the aims, namely housing (see Section 2.3), test feed preparation and presentation (see Section 2.4) and measurements (see Section 2.6). Alterations to the protocol are discussed in the following sections, as appropriate.

3.1.2.1 Horses

Eight horses of similar breeding, seven mares and one gelding, were allocated to this study (see Table 3.1). Horses were selected on the basis of their availability and were aged 3 (n=1), 6 (n=3), 7 (n=1), 9 (n=2), and 15 (n=1) years.

Table 3.1 Details of the 8 horses allocated to the study to develop materials and methods

Horse	Age	Sex	Breed
1	7	Gelding	Irish Draught X Thoroughbred
2	6	Mare	Cob X
3	6	Gelding	Irish Draught X Thoroughbred
4	9	Gelding	Cob X
5	9	Gelding	Thoroughbred
6	3	Mare	Thoroughbred X Warmblood
7	6	Gelding	Cob
8	15	Mare	Thoroughbred X

3.1.2.2 Housing

Horses one to six were housed in identical stables, as outlined in section 2.3. Due to a limit on stable availability, horse seven and eight were housed singly in two stables of identical design to each other, but differing from the remaining six stables (see Figure 3.1). These two stables were Lodden style loose boxes, measuring 1.5m by 2m. The two additional stables used to house Horse 7 and Horse 8 were not of symmetrical design and did not allow for the container(s) to be positioned centrally within the presentation area (see Figure 3.2). As with the block of six stables used for the other subjects, the stables were located in a quiet area of the yard. Activity around the stables was minimised prior to, and during, the presentation period. These stables did not allow viewing of other horses eating unless the head was placed over the stable door.

3.1.2.3 Regime

All horses were subject to the same regime, as outlined in section 2.2. Due to time factors and practicalities of distance, the two horses stabled in the Lodden stable type were presented with their test feeds at 1645h, rather than 1630h.

Figure 3.1 One of the six stables used throughout the study

Figure 3.2 One of the two stables used during the first pilot trial

3.1.2.4 Test Feed Preparation

The test feed was prepared as outlined in section 2.4.3. The artificial orange test chemical (Givaudan Dubendorf Limited, UK) was supplied as a white, water-soluble powder. This flavour has a recommended inclusion rate in humans of 0.1% in water.

3.1.3 Study Design

The study was conducted over 24 days. Of this 24-day period horses were only tested on 18 days, as they were not tested during weekends. During weekend days (days 6, 7, 13, 14, 20 and 21) training was reinforced, where control *versus* control was presented, but measurements of intake and behaviour were not recorded. Test and control feeds were presented in identical black containers, 1.5kg of feed allocated to each, and presented for a ten-minute period.

3.1.3.1 Development of Training

An initial five-day training period was conducted (see Section 2.5.1.1), where two identical feeding choices were presented simultaneously for a ten-minute period (see Section 2.4.4). 1.5kg of wheat feed pellets was presented in each container during the training period, constituting the control to be used throughout the remainder of the study. The training period aimed to meet the objectives set out in section 3.1.1.1.

3.1.3.2 Determination of flavour suitability

Following the training period the orange test food was presented as an increasing dose response over five consecutive days as a two-choice preference test (see Table 3.2). Five inclusion levels were presented, with each inclusion rate being double the previous. This presentation order was replicated over the following five study days, with daily reversal of the control positioning. Inclusion levels were determined from inclusion levels discernable to human taste in water. These levels aimed to represent a threshold level in the horse when combined with the basal feed.

Table 3.2 Presentation order, test feed positioning and inclusion level of the orange test feed to identify taste thresholds

	Day	Left container	Right container
Training	1 to 5	control	control
Replicate 1	8	control	$0.00781 g.kg^{-1}$
	9	$0.01563 g.kg^{-1}$	control
	10	control	$0.03125 g.kg^{-1}$
	11	$0.0625 g.kg^{-1}$	control
	12	control	$0.125 g.kg^{-1}$
Replicate 2	15	control	$0.00781 g.kg^{-1}$
	16	$0.01563 g.kg^{-1}$	control
	17	control	$0.03125 g.kg^{-1}$
	18	$0.0625 g.kg^{-1}$	control
	19	control	$0.125 g.kg^{-1}$

3.1.3.3 Development of test feed preparation

A study of the effects of varying delays between test food preparation and presentation on taste response was conducted. This aimed to ensure the stability of the flavours once applied to the basal feed to enable potential flexibility in test feed preparation prior to presentation. A delay between test feed preparation and presentation was conducted, as outlined in Table 3.3, over three consecutive days, following a two-day reinforcement of training to allow for the weekend.

Table 3.3 Presentation order, test feed positioning and inclusion levels of the orange test feed in order to study the effects of a delay in test feed preparation on taste response

Day	Delay Period (hours)	Left container	Right container
22	2	control	$1 g.kg^{-1}$
23	4	$1 g.kg^{-1}$	control
24	10	control	$1 g.kg^{-1}$

3.1.3.4 Development of the ethogram

Prior to commencement of the initial pilot trial, a preliminary ethogram was constructed from *ad libitum* behaviour sampling (see Table 3.4). The final aim of this study was to ensure the suitability of the ethogram for recording changes in behaviour in order to identify taste and gustatory responses.

Table 3.4 Preliminary ethogram of measure behavioural changes in response to changes in the feeding environment

Class	Behaviour	Description	Measurement
Ingestive	Eat	Horse eats food out of container	State
	Eat floor	Horse eats food off floor	State
	Sniff	Horse sniffs object in presentation area	Event
	Lick object	Horse licks object in presentation area	State
	Lick / chew	Horse's tongue protrudes from its mouth, whilst making small chewing movements	State
Locomotory	Walk	Horse turns away from the presentation area and walks round the stable	State
Visual	Alert	Horse is in an alert position, head raised, ears pricked and is not eating	State
	Head up	Horse has head raised above that which is normal and continues chewing	State
Other	Push	Horse pushes container laterally with its nose	Event
	Spill	Horse proceeds to chew whilst dropping food from its mouth	State

3.1.4 Measurements

Changes in intake and behaviour were recorded to determine preference, aversion or indifference to test feed and hence, taste response.

3.1.4.1 Intake

Intake was measured as detailed in section 2.6.1.

Taste response and side response were analysed as detailed in section 2.6.1.1 and 2.6.1.3 respectively.

3.1.4.2 Behaviour

The behaviour of Horses one to five was recorded during the presentation period using closed circuit television (CCTV) cameras and videocassette recorders as detailed in section 2.6.2. During the presentation period the behaviour of Horses six, seven and eight was recorded by direct observation. This involved the recording of behaviours in real-time, using score sheets.

Analysis of the tape-recorded behaviours was conducted at a later date, whereby the tapes were played using a video recorder and scored onto a check sheet. Behaviour was scored using instantaneous sampling of individuals against an ethogram of ingestive and related behaviours, developed from prior *ad libitum* observations by the author. Behaviours were recorded at one-minute intervals as state or frequency data as appropriate.

The same method of behaviour recording was used during direct observation, as used when analysing the tapes. As Horse six was the only horse not to have its behaviour recorded in the row of six identical stables by CCTV, direct observation was conducted, whereby the observer could see the horse through a slat in the wall of the building directly facing the horse. It was intended that this would cause minimal distraction to the horse. Horse seven and Horse eight, as housed alongside each other but unable to view each other unless their head was placed over the door, were subject to direct observation concurrently. For both methods of behaviour analysis, the same observer was used throughout to maintain consistency.

3.1.5 Statistical analysis

Statistical analysis of intake and behaviour data was conducted using the Minitab 14.0 programme as detailed in section 2.7.

3.1.6 Results and discussion

3.1.6.1 Development of training

All horses showed daily variation in total intake when presented with the novel basal feed, with Horse 2 selecting zero or near zero basal feed intake on days two and three (see Figure 3.3).

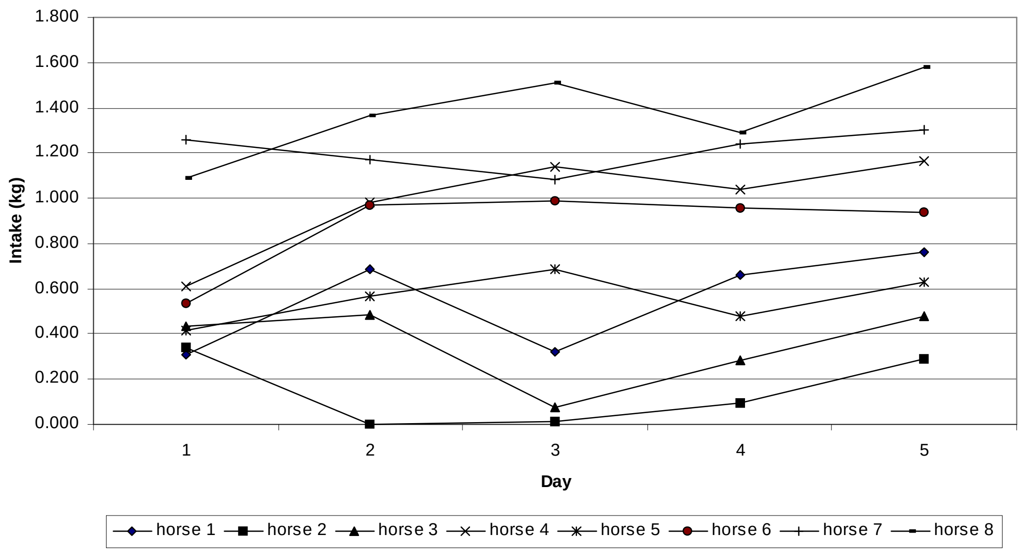


Figure 3.3 Total daily intake per day (calculated from amount eaten from left container plus amount eaten from right container) in all subjects training (n=5 days) showing individual variation in intake

No consistent daily intake was recorded across the 5 day training period, indicated from a significant difference in total daily intake ($F_{(7,32)}$ = 26.04, $p<0.001$). Such variation in initial intake following presentation of a novel feed is consistent with previous findings in the horse (Whittemore *et al.*, 2002) and cow (Tolkamp and Kyriazakis, 1997). It was evident that equilibrium in intake was not achieved prior to the completion of the training period, with intake continuing to increase from day four to five, supporting acceptance of the novel basal feed (Figure 3.4). This supports a previous finding in the horse that acceptance of a novel feed increases following repeated presentations (Whittemore *et al.*, 2002).

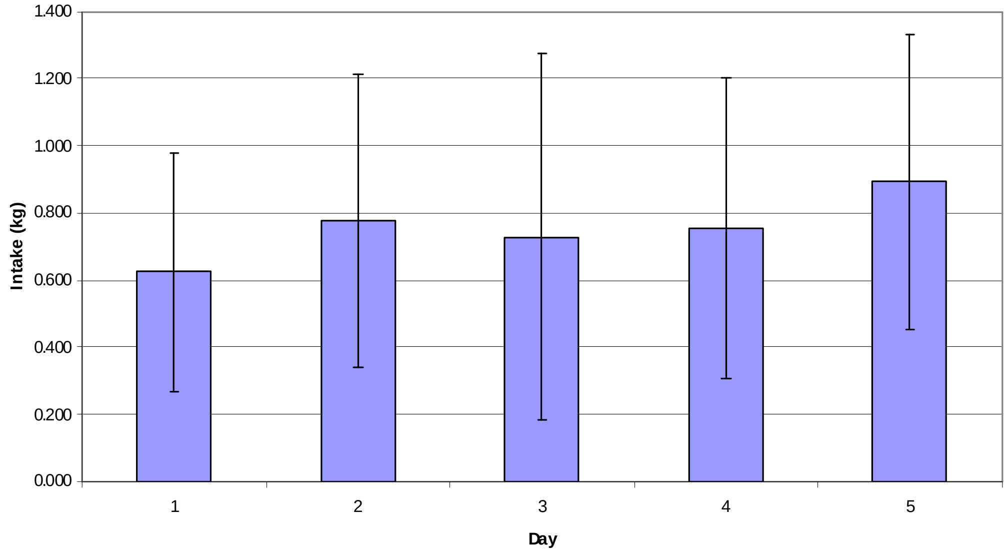


Figure 3.4 Mean total daily intake of all subjects during the training period, indicating increased intake with increased exposure to the novel basal feed (mean ± s.e.m. (g), n=5 days).

To study the validity of the 50:50 assumption when two identical feed choices, left and right, were presented simultaneously, control *versus* control was presented over five consecutive days. No difference between left and right container intake was found when analysed as a group (mean left intake = 407g, s.e.m. = 84.2g: mean right intake = 354g, s.e.m. = 84.0g). Horse 6 was found to have a significant left preference during the training period ($F_{(1,8)}$ = 8.49, p<0.01) consistently selecting for the left container on 5 out of 5 presentations (see Figure 3.5). All horses sampled from both the left and right container, with no horse selecting one hundred percent of intake from one container during a single presentation period.

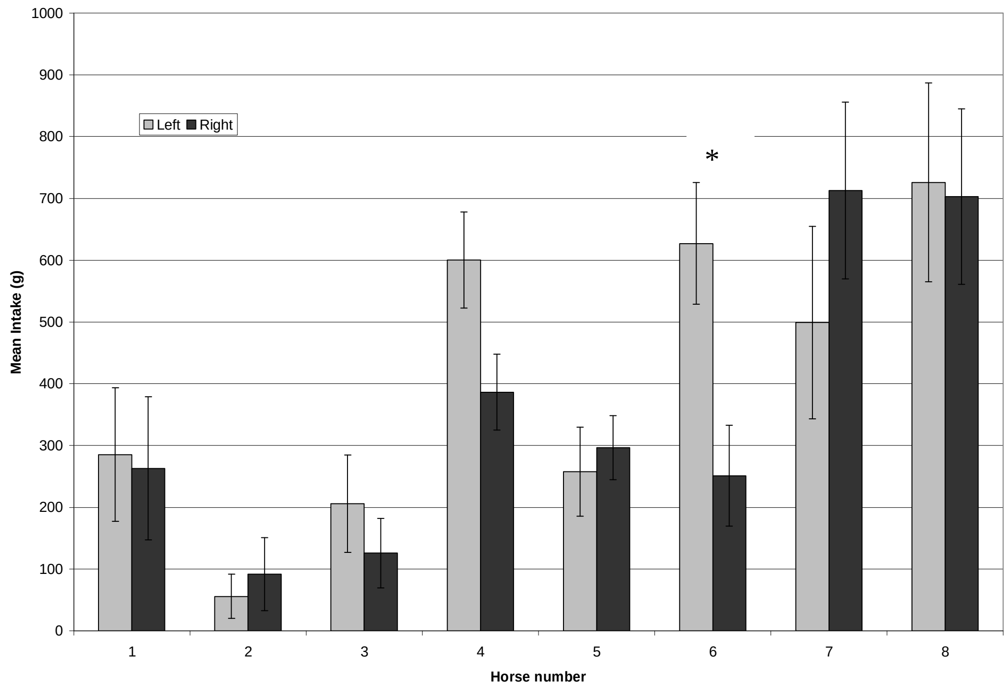


Figure 3.5 Mean individual intake during the training period indicating sampling from both container positions; left and right (mean ± s.e.m. (g), n=5 days).
*** denotes *p*<0.05**

3.1.6.2 Development of flavour presentation

Presentation of the orange test flavour at any inclusion level failed to evoke a taste response in seven of the eights horses studied, with no significant difference between control feed or test feed intake recorded in individuals during the presentation period. Horse 5 demonstrated a significant preference for the orange test feed ($F_{(1,18)}$ = 9.38, $p<0.001$), selecting for the test feed on 9 of the 10 presentations. This finding demonstrates the ability of this individual to discriminate between the test feed and unflavoured control.

The finding of a lack of discrimination between the test feed and the control in the remaining seven individuals suggests either a lack of discriminatory ability to detect the test flavour or indifference towards the test flavour. Further analysis of results suggests that a lack of significant taste response may be due to selection of feed choice based on positional, rather than flavour cues.

A significant side preference was recorded in seven of the eight subjects (see Table 3.5). Six horses exhibited a left preference, whilst Horse 3 exhibited a right preference. An additional theory to account for the lack of taste response is suggested to be that, in addition to an inability to detect the test flavour or an indifference towards the test flavour, there was a greater influence of positional cues over taste response on feeding choice. As discussed above, Horse 5 was the only individual to show a significant taste response. Horse 5 failed to show a side preference. In Horse 5 it was suggested that taste cues influenced feeding choice over positional cues.

A left side preference persisted from the training period in Horse 6. All other horses which showed a side preference during two-choice preference testing had failed to exhibit a significant side preference during the initial training period. The proposed causes of side preference are discussed in detail in Chapter 6.

Table 3.5 Individuals exhibiting side preference during two-choice preference testing (mean ± s.e.m. (g), n=10 days)

Horse	Left Mean ± s.e.m. (g)	Right Mean ± s.e.m. (g)	$F_{(1,20)}$ *value*	*p* value	Preference
1	488 ± 87.0	237 ± 76.0	4.74	0.05	Left
2	761 ± 59.0	87 ± 66.7	33.65	0.001	Left
3	188 ± 58.0	509 ± 59.0	12.16	0.01	Right
4	808 ± 65.9	355 ± 65.1	27.89	0.001	Left
5	424 ± 71.1	392 ± 52.0	0.13	0.723	No preference
6	761 ± 73.7	320 ± 107.0	27.14	0.001	Left
7	704 ± 61.0	309 ± 62.0	20.39	0.001	Left
8	952 ± 71.0	322 ± 34.0	83.13	0.001	Left

Following this study the use of identical stables was deemed necessary. Horse 7 and Horse 8, both of which exhibited a significant side preference, were housed in stables of unsymmetrical design. This resulted in the horses' not standing perpendicular to the feeding area, as observed in Horses 1 to 6 who were housed in symmetrical stables. In order to reduce the influence of stable design on the incidence of side preference, the two stables used to house Horse 7 and Horse 8 were used in no further trials.

An ability to discriminate between the test feed and control was identified in one subject. Indifference or inability to detect the taste cue may have resulted in the direction of intake being influenced by positioning cues rather than taste cues. Side preference was suggested to be more influential on intake than taste cues in some individuals, resulting in distortion of taste response in these individuals.

3.1.6.3 Development of test feed preparation

Increasing the period of time between preparation and presentation had no significant effect on intake. The presentation of a higher inclusion level ($1gk^{-1}$) than previously presented, failed to evoke a significant control or test feed preference in individuals. This element of study was conducted over three days and data generated was insufficient to draw valid conclusions.

3.1.6.4 Development of the ethogram

Insufficient data were generated to conduct chi squared reliably on changes in individual behaviour. The method used to determine eat behaviour did not account for variation in feeding behaviour. Horses which ate with the head remaining in the container throughout were recorded as exhibiting less eat behaviour than those individuals who ate whilst having their head out of the container. This resulted in inaccuracies in interpreting the incidence of eat behaviour between and within container sampling and great variation in eat behaviour being recorded (see Figure 3.6).

Analysis of behaviour failed to identify reliably a single incidence of sniff behaviour. The ethogram also failed to identify the direction of behaviour response towards either the left / right container or the control / test feed container.

The preliminary study identified several errors in the proposed ethogram and the recording methods for determine taste response. Hence, revision and further development of the ethogram was necessary.

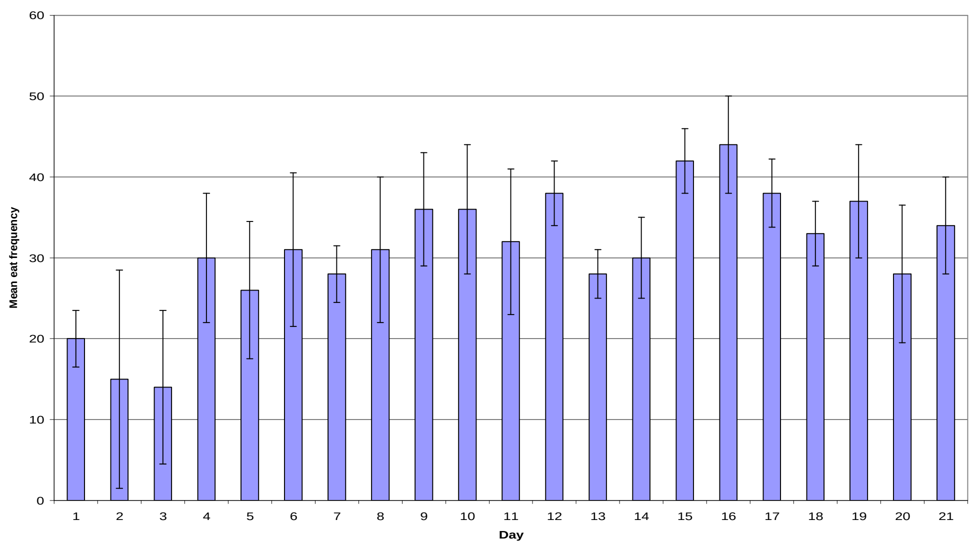


Figure 3.6 Mean eat frequency recorded throughout the study period indicating inconsistency in eat behaviour and variation throughout (mean ± s.e.m. (g), n=21 days).

3.1.7 Conclusions

Several conclusions were drawn from this study. Training enables familiarisation of the horses to a novel basal feed; equilibrium in intake was not reached in the five-day training period. Exposure to the novel basal feed prior to two-choice preference testing enabled exploratory behaviour to be replaced with ingestive behaviours, indicated by increasing total daily intake. Additionally, a training period ensures all horses select from the simultaneously presented feeding choice; left and right. The equal intake from both the left and the right container supports the 50:50 assumption of intake when no choice is presented. The subsequent development of side preference contradicts this.

Whilst it was found that one individual possessed the ability to discriminate between the artificial test feed and an unflavoured control at low inclusion levels, it was apparent that a higher inclusion level was essential to evoke a response in the remaining subjects, provided they possessed the appropriate discriminatory ability. Test feed positioning was reversed daily, yet this alone was insufficient to prevent development of side preference. It was deemed necessary to study taste response through presentation of higher inclusion levels than those used for the identification of taste thresholds. This aimed to ensure taste response was more influential on intake than positional cues. To reduce the influence of stable design on the incidence of side preference it was appropriate to use only stables of symmetrical design for future studies.

Delaying preparation prior to presentation did not appear to affect taste response. This study raised several issues; the suitability of two-choice preference testing in individuals susceptible to side preference and whether a high inclusion level would enable taste response to influence intake more than positional cues.

The initial study of changes in ingestive behaviours identified several shortcomings of the ethogram and method of behavioural sampling. Approaches to more accurately determine taste response from behavioural changes and the method adopted for the main trials have been discussed previously in Section 2.6.2.

3.2 Investigation of the incidence and onset of side preference

From the results of the preliminary study, as discussed in section 3.1, it was deemed necessary that a further pilot study be conducted. The aims of this study were two-fold: firstly, to measure the incidence and onset of side preference in susceptible individuals; and secondly, to study the validity of two-choice preference testing in horses exhibiting side preference.

One of the major findings from the initial pilot trial was the incidence and extent of side preference exhibited in seven of the eight horses studied. It was necessary to explore and attempt to eliminate external factors that may influence choice based on positional cues. Feeding side preference has previously been recorded in one horse (Hawkes *et al.*, 1985) during two-choice preference testing, as a result of which the individual was rejected from the trial. To evaluate the validity of two-choice preference testing in determining taste response, it was necessary to study side preference further.

The incidence of side preference identified in the initial trial resulted in the need to determine taste response through inclusion rates above taste threshold levels. It was therefore necessary to establish inclusion rates to identify taste response.

3.2.1 Study aims

This study aimed to investigate the incidence and onset of side preference, rather than the proposed causes of feeding side preference, as discussed in Chapter 6. It was hypothesised that;

1. There would be a development in side preference when choice is based on positional cues alone
2. The inclusion levels presented would evoke taste response
3. Side preference would be influential on the exhibition of taste response

In order to satisfy these aims, the study was separated into two sections, training and two-choice preference testing, conducted over 19 consecutive days. To meet the first aim, a training period was conducted whereby only positional cues left and right, were presented to the horse. To satisfy aims two and three, a two-choice preference test was conducted.

3.2.2 Materials and methods

Chapter Two describes the general materials and methods used to achieve the aims, namely housing (see Section 2.3), test feed preparation and presentation (see Section 2.4) and measurements (see Section 2.6). Alterations to the protocol are discussed in the following sections, as appropriate.

3.2.2.1 Horses

Four horses were allocated to the study (see Table 3.6). All horses were aged between eight and ten years to remove any potential influence of age on the exhibition of side preference.

Table 3.6 Details of the four horses allocated to the study of the incidence and onset of side preference

Horse	Age	Sex	Breed
1	8	Mare	Thoroughbred X
2	10	Gelding	Thoroughbred X Cob
3	8	Gelding	Irish Draught X
4	9	Gelding	Cob

3.2.3 Study Design

The study was conducted over 19 consecutive days. All feeds were presented in identical black containers, holding 1.5kg of control or test feed, as appropriate, for a ten-minute period.

As stated earlier in section 3.2.1, the study was split into two sections, training and two-choice preference testing. The initial study (see Section 3.1) suggested that the five day training period satisfied the aims of familiarising the horses to the novel basal feed and novel method of food presentation; equilibrium in intake was not achieved. The purpose of this additional training period, increased from five to 13 days, was to establish the incidence and onset period of side preference in susceptible individuals.

Each individual was presented with two identical containers of identical feeding choices, 1.5 kg of control feed, presented simultaneously. This element of study was conducted over 13 consecutive days; from the preliminary study this was an adequate time period for individuals to exhibit consistency in positional choice.

Immediately following the training period a six-day two-choice preference test was conducted. Artificial orange juice flavouring was used as the agent to elicit taste response in the horses. In order to attempt to reduce the influence of side preference on taste response an inclusion rate of $2g.kg^{-1}$ was used with the aim of taste cues being more influential on intake than side preference. The study design allowed for positional cues influencing taste response by the daily reversal of control and test feed positioning (see Table 3.7).

Table 3.7 Presentation order and positioning of the high inclusion rate ($2g.kg^{-1}$) test food

Day	Left container	Right container
14	control	$2g.kg^{-1}$
15	$2g.kg^{-1}$	control
16	control	$2g.kg^{-1}$
17	$2g.kg^{-1}$	control
18	control	$2g.kg^{-1}$
19	$2g.kg^{-1}$	control

3.2.4 Measurements

Changes in intake and behaviour were recorded to determine preference, aversion or indifference to test feed and hence, taste response.

3.2.4.1 Intake

Intake was measured as detailed in section 2.6.1. Taste response and side response were analysed as detailed in section 2.6.1.1 and section 2.6.1.3 respectively.

3.2.5 Statistical analysis

All statistical analysis was conducted using the Minitab 14.0 programme as detailed in section 2.7.

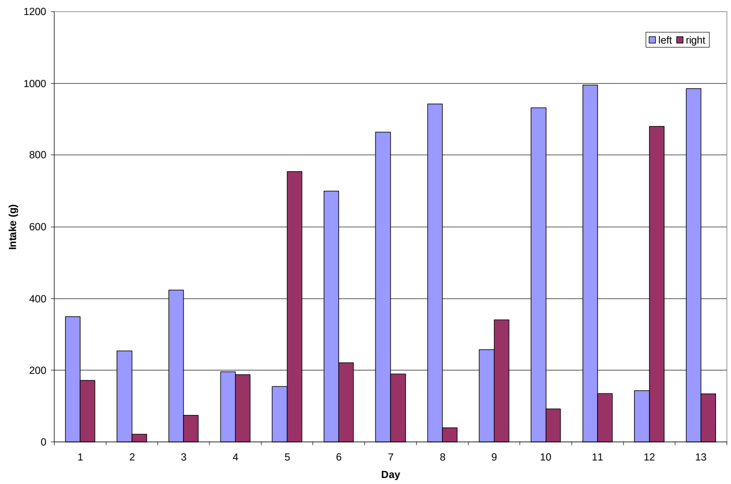
3.2.6 Results and discussion

The initial element of study determined the incidence and exhibition of side preference, with Horse 3 ($F_{(1,24)}$ = 6.16, $p<0.05$) and Horse 4 ($F_{(1,24)}$ = 12.53, $p<0.01$) exhibiting a significant left preference (Table 3.7). The finding of no significant side preference in two of the four horses studied supports the 50:50 assumption of selection falling between around a theoretical mean of 0.5 if no preference is shown.

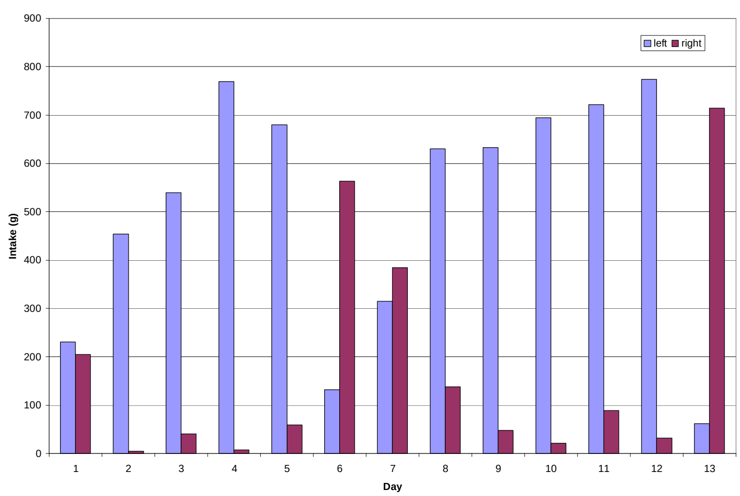
Table 3.8 Individual difference between left and right intake during the training period (mean ± s.e.m. (g), n=13 days)

Horse	Left Mean ± s.e.m. (g)	Right Mean ± s.e.m. (g)	$F_{(1,22)}$ value	*p* value	Preference
1	341 ± 68.9	366 ± 63.1	0.07	0.794	No preference
2	473 ± 73.7	481 ± 46.4	0.01	0.933	No preference
3	554 ± 97.6	250 ± 73.9	6.16	<0.05	Left
4	510 ± 68.7	178 ± 64.2	12.53	<0.01	Left

Determining the onset of side preference in Horse 3 and Horse 4 was complicated due to inconsistency of container selection throughout the study period (see Figure 3.7). In addition to this, sampling of both containers occurred within each intrameal period. Whilst a significant side preference was recorded, both horses continued to sample from both containers throughout the 13 day study period.



a) Horse 3



b) Horse 4

Figure 3.7 Daily left and right intake for (s) Horse 3 and (b) Horse 4 indicating a significant left presentation during the training period (n=13 days). Inconsistency in container selection was recorded for intrameal and intermeal periods.

The increased duration of the training period from 5 days in the first pilot trial (see Section 3.1) to 13 days in the second pilot trial (see Section 3.2) resulted in equilibrium of total daily intake being reached from around day 11 onwards. This indicates that the exploratory behaviour had been replaced by ingestive behaviour in individuals, prior to commencement of the two-choice preference testing on day 10 (see Figure 3.8).

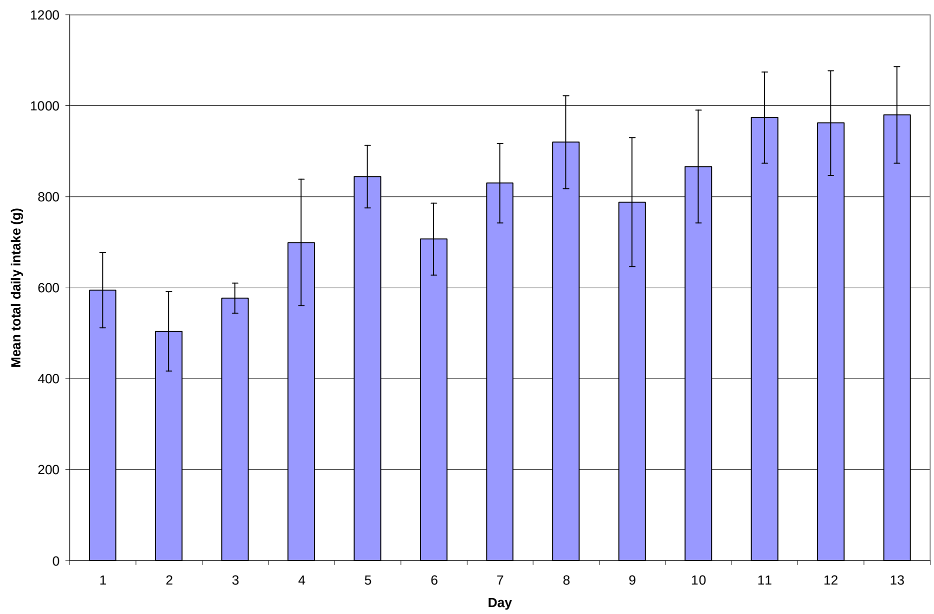


Figure 3.8 Mean total daily intake indicating equilibrium of intake after 13 days of training (mean ± s.e.m. (g), n=13 days).

The second element of study aimed to determine the effects of flavour presentation on side preference. All horses showed a strong preference for the control when presented alongside an orange flavoured test feed (Table 3.9), with the test feed being selected on 1 of 24 presentations. As previously reported in the training prior to the commencement of two-choice preference testing, all horses selected from both feeding choices. A strong aversion to the test feed resulted in container selection being based on taste cues, rather than positional cues.

Table 3.9 Individual differences between the control and test feed during two-choice preference testing (mean ± s.e.m. (g), n=6 days)

Horse	Control Mean ± s.e.m. (g)	Test feed Mean ± s.e.m. (g)	$F_{(1,10)}$ *value*	*p* value	Preference
1	835 ± 29.3	12 ± 4.76	756.2	<0.001	Control
2	985 ± 10.5	18.3 ± 6.53	6141.9	<0.001	Control
3	1151 ± 72.2	103 ± 41.6	158.0	<0.001	Control
4	732 ± 28.5	51 ± 18.8	397.6	<0.001	Control

From these results it was concluded that the horses studied possessed the ability to discriminate between the artificial orange juice flavour test feed and unflavoured control. The preference for the control may have been influenced by wariness towards the novel flavour and associated olfactory cues. Additionally, horses may have exhibited a conditioned preference for the control following prior exposure during the initial training period (Burton *et al.*, 1983; Pollack and Burton, 1991; Kennedy *et al.*, 1998).

The significant left side preference recorded in Horse 3 ($F_{(1,24)} = 6.16$, $p<0.05$) and Horse 4 ($F_{(1,24)} = 12.53$, $p<0.01$) during the training period failed to persist with the presentation of the test feed (Horse 3 ($F_{(1,10)} = 0.25$, $p=0.631$); Horse 4 ($F_{(1,10)} = 0.01$, $p=0.934$). Container selection was based on taste cues rather than positional cues when an aversive flavour was presented.

3.2.7 Conclusions

The presentation of two identical feeding choices, left and right, may result in selection based on positional cues alone. Consistency in both intrameal and intermeal container choice resulted in an increase in the incidence and degree of side preference. The influence of side preference on two-choice preference testing needed to be considered when identifying taste response.

Equilibrium in total daily intake occurred between day 5 and day 10 of training, being subject to individual variation. Equilibrium of total daily intake indicates

acceptance of the novel basal feed and replacement of non ingestive behaviour with ingestive behaviour.

A high inclusion level of the artificial orange test feed evoked an aversive response that was more influential on container selection than positioning cues. This finding supported the earlier result in the first trial of the ability of one horse to discriminate between the test feed and an unflavoured control, whereby a test feed preference was recorded ($F_{(1,18)} = 9.38$, $p<0.001$). The test feed was found to be a suitable flavour to evoke a taste response in the horse.

Following the findings of the preliminary pilot trials (see Section 3.1 and Section 3.2) several adaptations to experimental design were implemented to enable the principal aims of the study to be met.

To enable central positioning of the test feed containers it was necessary for stables to be of identical design, to avoid the influence of stable design on the exhibition of side preference. The use of symmetrical stables enabled the horses to stand perpendicular to the feed presentation area.

The training period enabled the replacement of exploratory behaviour with ingestive behaviours following repeated presentation of the novel basal feed. This resulted in equilibrium of intake being achieved between day 5 and day 10, with all horses selecting from both container positions, left and right, prior to the commencement of the two-choice preference testing period.

It was not possible to identify individual taste thresholds in the horse due to the influence of positional cues on selection in individuals susceptible to side preference. It was necessary for flavour inclusion levels to be sufficiently strong to enable selection to be based on taste response, rather than positional cues.

Use of The Observer Package (Noldus) to record continuous data, rather than instantaneous sampling, aimed to overcome the limitations identified in behaviour analysis identified in the initial study. Adaptations were also made to the ethogram (see Section 2.6.2) to enable taste response to be more accurately determined from changes in feeding behaviour.

3.3 Determination of sucralose and aspartame to determine gustatory response

Prior to commencing the second study to determine the effect of age on gustatory response, it was necessary to identify a suitable test flavour to evoke a gustatory response, in the absence of additional feed related olfactory cues. From this, appropriate inclusion levels to elicit a gustatory response could be determined. The requirements of a suitable test feed to determine gustatory response were three-fold; firstly, horses must possess the ability to discriminate between the test flavour and control and the motivation to respond to the test feed; secondly, the test flavour must evoke a taste response, without olfactory cues and thirdly, the test flavour must have limited nutritional value to reduce the influence of postingestive consequences on feeding choice. It was necessary for the test feed not to provide additional olfactory cues, in addition to those presented by the wheat feed pellets and the housing environment. The artificial sweeteners sucralose and aspartame were initially selected as they were hypothesised to meet the requirements outlined above.

Aspartame (Canderel®) is an artificial non-nutritive sugar substitute that provides 4 calories per gram (Sardesai and Waldshan, 1991), insufficient calorific value to enable selection based on nutritive value alone. Aspartame is perceived as sweet by humans (Schiffman *et al*., 1979 b) and stated to be 160-220 times sweeter than sugar (Sardesai and Waldshan, 1991). Sensitivity to aspartame in elderly human subjects (60-75 years, n=21), in comparison to young subjects (19-33 years, n=21), has been shown to decline with age (Mojet *et al*., 2005).

Catarrhini (Old World primates) possess the ability to detect aspartame (Glaser *et al*., 1995), while *Platyrrhini* (New World monkeys), hamsters (Danilova *et al*., 1998) and pigs (Glaser *et al*., 2000) lack the ability to detect aspartame (Glaser *et al*., 1995). The inability of *non-catarrhini* species to detect aspartame has been attributed to a lack of specific gustatory receptors in these species (Glaser *et al*., 2000; Rawson, 2004). Glaser (2002) suggests that there was no requirement for these species to evolve an ability to detect artificial sweeteners, yet, this does not explain the ability of Old World primates to detect aspartame.

Sucralose (Splenda®) is an artificial sugar substitute and, as with aspartame, the intensity of sweet flavour in humans for sucralose is stated to be 500 times greater than that for sucrose (Sardesai and Waldshan, 1991). Sucralose is a low nutritive polyol (Grotz *et al*., 2003) of sucrose origin. Sucralose evokes a sweet taste response in humans (Sardesai and Waldshan, 1991). Pigs have been shown to have no reaction to sucralose when presented alongside water as a two-choice preference test (Glaser *et al*., 2000) indicating a lack of discriminatory ability in the pig.

3.3.1 Study aims

The principal aims of this study were firstly to establish the discriminatory ability of horses to the proposed test flavours and secondly, to identify appropriate inclusion levels of the proposed test flavours to identify individual gustatory responses.

3.3.1.1 Determination of flavour

The first element of study aimed to ensure horses possessed the ability to discriminate between the proposed test flavour and the unflavoured control.

3.3.1.2 Determination of inclusion level

Following identification of a discriminatory ability, it was necessary to identify the appropriate inclusion levels to evoke a gustatory response in individuals.

3.3.2 Materials and methods

Chapter Two describes the general materials and methods used to achieve the aims, namely housing (see Section 2.3), test feed preparation and presentation (see Section 2.4) and measurements (see Section 2.6). Alterations to the protocol are discussed in the following sections, as appropriate.

3.3.2.1 Horses

Five horses of similar breeding were allocated to the study (see Table 3.10). Horses were selected on the basis of their availability.

Table 3.10 Details of the five horses allocated to the study to identify flavours to determine gustatory response

Horse	Age	Sex	Breed
1	7	Gelding	Thoroughbred X
2	10	Gelding	Thoroughbred X
3	14	Gelding	Cob X Thoroughbred
4	14	Mare	Thoroughbred X
5	12	Gelding	Thoroughbred X

3.3.2.2 Test feed preparation and presentation

The test feed was prepared as outlined in section 2.4. The sucralose (Tate and Lyle, London, UK) and aspartame (Merisant, London, UK) were supplied as a white, water-soluble powder.

3.3.3 Study design

The study was conducted over 12 days. An initial two-day training period, whereby control *versus* control was presented, aimed to familiarise the horses to the novel basal feed and to prevent the establishment of side preference in susceptible individuals. The two day training period aimed to prevent the onset of side preference through presentation of the test feed prior to side preference becoming established. The initial element of study on days 3 to 6 presented sucralose as a decreasing dose response with inclusion levels paired over consecutive days to allow for the reversal of the control positioning. The second element of study on days 7 to 12 presented aspartame as a decreasing dose response; again with pairing of inclusion levels (see Table 3.11).

Table 3.11 Presentation order, test feed positioning and inclusion levels of sucralose and aspartame to identify gustatory response

	Day	Left container	Right container
Training	1	control	control
	2	control	control
Element 1	3	control	5g.kg^{-1} sucralose
	4	5g.kg^{-1} sucralose	Control
	5	control	2.5g.kg^{-1} sucralose
	6	2.5g.kg^{-1} sucralose	control
Element 2	7	control	5g.kg^{-1} aspartame
	8	5g.kg^{-1} aspartame	control
	9	control	2.5g.kg^{-1} aspartame
	10	2.5g.kg^{-1} aspartame	control
	11	control	1.25g.kg^{-1} aspartame
	12	1.25g.kg^{-1} aspartame	control

3.3.4 Measurements

Changes in intake and behaviour were recorded to determine preference, aversion or indifference to test feed.

3.3.4.1 Intake

Intake was measured as detailed in section 2.6.1.

3.3.5 Statistical Analysis

All statistical analysis was conducted using the Minitab 14.0 programme as detailed in section 2.7.

3.3.6 Results and discussion

The main findings of the study are detailed below. Horse 5 was rejected from the study following insufficient consumption of the basal feed over the first four

days of presentation (mean total daily intake – 7g). Consequently, the study was conducted with four horses.

3.3.6.1 Determination of sucralose suitability

No preference for the sucralose test feed was found in individuals (Table 3.12) and changes in inclusion level consequently did not result in any significant changes in intake. A significant side preference was recorded in Horse 2 ($F_{(1,6)}$ = 171.6, p<0.001).

Table 3.12 Individual gustatory responses to the sucralose test feed *versus* the control (mean ± s.e.m. (g), n=4 days)

	Control	Test feed			
Horse	Mean ± s.e.m. (g)	Mean ± s.e.m. (g)	$F_{(1,6)}$ *value*	p value	Preference
1	531 ± 111.0	698 ± 49.1	1.91	0.241	No preference
2	521 ± 188.0	354 ± 155.0	0.04	0.524	No preference
3	471 ± 162.0	560 ± 232.0	0.01	0.776	No preference
4	717 ± 187.0	622 ± 172.0	0.10	0.724	No preference

3.3.6.2 Determination of aspartame suitability

Analysis of intake between the aspartame flavoured feed and the unflavoured control, identified a significant preference for the control in two of the four horses studied (Table 3.12). The lack of a significant difference in intake between the control and the test feed across the group suggests either an indifference to the aspartame or taste blindness. Taste blindness towards aspartame has been recorded in calves (Segerstad and Hellekant, 1989 a, b) with electrophysiological investigation of aspartame finding no response in eight calves, but a response in five other calves. No changes in behavioural response in calves to aspartame were recorded in a later study by Hellekant *et al*. (1994). This supports intraspecies variation in discriminatory ability in the calf and is suggested to be the result of genetic variation (Kare *et al*., 1965). This does need to be questioned that due to the influence of exogenous factors on *in utero*

development and from birth onwards, phenotypic expression may vary. The lack of discrimination from behaviour studies towards aspartame in the calves suggests either taste blindness in all individuals studied, or more likely, lack of sensitivity from behavioural determination of taste response.

Table 3.13 Mean intake response to the control and aspartame test feed in individuals (mean ± s.e.m. (g), n=6 days).

	Control	Test feed			
Horse	Mean ± s.e.m. (g)	Mean ± s.e.m. (g)	$F_{(1,10)}$ *value*	*p* value	Preference
1	501 ± 99.9	745 ± 98.9	3.00	0.114	No preference
2	778 ± 68.5	121 ± 40.6	68.16	<0.001	Control
3	819 ± 58.8	265 ± 73.8	34.40	<0.001	Control
4	383 ± 53.9	535 ± 72.4	2.84	0.123	No preference

A significant difference between left intake and right intake was recorded in Horse 1 ($F_{(1,11)}$ = 10.72, p<0.001). No other individuals exhibited a significant side preference during aspartame presentation. No effect of changes to the aspartame inclusion level on the mean proportion of control eaten was recorded ($F_{(2,9)}$ = 0.05, p=0.95).

3.3.7 Conclusions

A lack of individual preference towards the sucralose test feed indicated an inability of the subjects studied to discriminate between the test feed and an unflavoured control. Although only two test feed inclusion rates were presented, the flavour was easily discernible to the human taster. No previous studies into the ability of horses to detect sucralose have been conducted and hence, it is suggested that sucralose in an inappropriate test chemical to elicit a taste response in the horse.

Individual significant preference for aspartame supports the ability of the study horses to discriminate between aspartame and an unflavoured control. An

ability to detect aspartame is species specific, with pigs being unable to detect aspartame, determined from a lack of behavioural responses and neural responses in the chorda tympani (Danilova *et al*., 2000). A lack of response in individuals may result from intraspecies variation in discriminatory ability in the horse, with such findings of taste blindness recorded in calves (Segerstad and Hellekant, 1989 a, b).

Whilst aspartame elicits a sweet response in humans, this study suggests that aspartame may not be perceived as sweet by the horse. Horses have been shown to have a preference for sweet substances (Randall *et al.*, 1978) and hence, if perceived as sweet, a preference for aspartame would be expected.

Inclusion levels failed to influence gustatory responses. For aspartame to be used to identify individual gustatory responses, higher inclusion levels would be necessary to ensure a gustatory response was recorded in all individuals. An increased inclusion levels would continue to fail to evoke a response in taste blind individuals.

The removal of olfactory cues from the test feed may result in the need for increased sampling behaviour for discrimination to occur. Whilst behavioural analysis is required, it is proposed that increased sampling would result in the gustatory response being distorted towards the indifference / no preference zone of intake.

In conclusion, horses do not possess the ability to identify sucralose and hence, sucralose is an inappropriate chemical to determine taste response. The ability to detect aspartame is subject to individual variation, and this may be the result of inappropriate inclusion levels being presented.

The main finding of this study was the need to further ensure the suitability of aspartame to determine taste response in the horse. The ability to detect aspartame in individuals was necessary; alongside identification of appropriately high enough inclusion levels to determine gustatory response in individuals.

3.4 Determination of suitability of aspartame

Following on from the earlier study of aspartame to determine gustatory response as discussed in section 3.3, a study to establish individual gustatory response was conducted. The rationale of selecting aspartame to determine gustatory response is discussed in section 3.3.

3.4.1 Study aims

The principal aim of this study was to investigate the effect of age on gustatory function in the horse. In order to achieve this aim it was necessary to obtain individual gustatory responses for the purposes of age comparison. In the earlier study, aspartame was found to evoke a gustatory response in two of four horses studied.

3.4.2 Materials and methods

Chapter Two describes the general materials and methods used to achieve the aims, namely housing (see Section 2.3), test feed preparation and presentation (see Section 2.4) and measurements (see Section 2.6). Alterations to the protocol are discussed in the following sections, as appropriate.

3.4.2.1 Horses

As discussed in section 2.3, resource implications restricted each study group to a total of six horses (see Table 3.14). Two horses were assigned to each of three treatment groups, A (“Young”), B (“Middle”) and C (“Old”). This aimed to reduce the influence of age bias and order effect on findings. In this study, Young horses were numbered Horse 1 and Horse 2, Middle horses were numbered Horse 7 and Horse 8 and Old horses were numbered Horse 13 and Horse 14.

Table 3.14 Details of the six horses allocated to the study to identify suitability of aspartame to determine gustatory response

Horse	Age	Sex	Breed
1	4	Gelding	Cob
2	6	Gelding	Cob X
7	14	Mare	Thoroughbred
8	14	Gelding	Thoroughbred X Irish Draught
12	16	Gelding	Thoroughbred
13	18	Gelding	Thoroughbred X

3.4.3 Study design

Each group was subject to three different test elements; training, two-choice preference testing and monadic testing. The study was conducted over 24 consecutive days, with two-choice preference testing and monadic testing used to determine gustatory response in the horse. Training was conducted over a four-day period, whereby control *versus* control was presented simultaneously, with two-choice preference testing being conducted over the next twelve consecutive days, as detailed in Table 3.15.

Monadic testing was conducted on days 17 to 24 whereby an increasing dose response was presented, followed by a decreasing dose response. All horses were subject to the same presentation order. In addition to the inclusion levels presented during two-choice preference testing, an additional inclusion level of $0.25 g.kg^{-1}$ was presented to the horses (Table 3.16). This aimed to enable increased sensitivity to the test feed to be detected when positional cues were removed.

Table 3.15 Presentation order, test feed positioning and inclusion levels during two-choice preference testing to establish gustatory response in individuals

Subject Allocation	Young and Old		Middle and Old		Young and Middle	
Container Positioning	**Left**	**Right**	**Left**	**Right**	**Left**	**Right**
Replicate One	Control	0.5g.kg^{-1}	control	1g.kg^{-1}	control	0.25g.kg^{-1}
	0.5g.kg^{-1}	control	1g.kg^{-1}	control	0.25g.kg^{-1}	control
	Control	0.25g.kg^{-1}	control	0.5g.kg^{-1}	control	1g.kg^{-1}
	0.25g.kg^{-1}	control	0.5g.kg^{-1}	control	1g.kg^{-1}	control
	Control	1g.kg^{-1}	control	0.25g.kg^{-1}	control	0.5g.kg^{-1}
	1g.kg^{-1}	control	0.25g.kg^{-1}	control	0.5g.kg^{-1}	control
Replicate Two	Control	0.5g.kg^{-1}	control	1g.kg^{-1}	control	0.25g.kg^{-1}
	0.5g.kg^{-1}	control	1g.kg^{-1}	control	0.25g.kg^{-1}	control
	Control	0.25g.kg^{-1}	control	0.5g.kg^{-1}	control	1g.kg^{-1}
	0.25g.kg^{-1}	control	0.5g.kg^{-1}	control	1g.kg^{-1}	control
	Control	1g.kg^{-1}	control	0.25g.kg^{-1}	control	0.5g.kg^{-1}
	1g.kg^{-1}	control	0.25g.kg^{-1}	control	0.5g.kg^{-1}	control

Table 3.16 Presentation order, test feed positioning and inclusion levels during monadic testing to establish individual gustatory responses, with the additional inclusion rate (0.25g.kg^{-1})

Day	Inclusion rate (g.kg^{-1})
17	0.5
18	0.5
19	1
20	2
21	2
22	1
23	0.5
24	0.25

3.4.4 Measurements

Changes in intake and behaviour were recorded to determine individual responses to the test feed, for the purposes of age comparison.

3.4.4.1 Intake

Intake was measured as detailed in section 2.6.1.

3.4.5 Statistical analysis

All statistical analysis was conducted using the Minitab 14.0 programme as detailed in section 2.7.

3.4.6 Results and discussion

No significant difference between control and test feed intake was recorded in any of the individuals studied (Table 3.17).

Table 3.17 Individual differences between intake from the control and the aspartame test feed during two-choice preference testing (mean ± s.e.m. (g), n=12 days).

	Control	Test feed			
Horse	Mean ± s.e.m. (g)	Mean ± s.e.m. (g)	$F_{(1,22)}$ *value*	*p* value	Preference
1	635 ± 172.0	676 ± 174.0	0.03	0.868	No preference
2	493 ± 71.9	362 ± 83.8	1.42	0.246	No preference
7	534 ± 132.0	588 ± 127.0	0.09	0.773	No preference
8	604 ± 172.0	658 ± 179.0	0.05	0.828	No preference
12	555 ± 84.8	598 ± 83.1	0.13	0.722	No preference
13	768 ± 136.0	411 ± 120.0	3.87	0.062	No preference

A significant side preference was recorded in three horses during two-choice preference testing (Table 3.18) indicating selection based on positional cues.

Table 3.18 Individuals exhibiting side preference during two-choice preference testing (mean ± s.e.m. (g), n=12 days).

	Left	Right			
Horse	Mean ± s.e.m. (g)	Mean ± s.e.m. (g)	$F_{(1,22)}$ *value*	*p* value	Preference
1	103 ± 38.1	272 ± 53.5	297.4	<0.001	Right
2	272 ± 53.5	584 ± 75.5	11.36	<0.01	Right
7	480 ± 123.0	642 ± 131.0	0.82	0.375	No preference
8	50 ± 6.73	1212 ± 21.1	275.8	<0.001	Right
12	615 ± 79.4	539 ± 87.3	0.41	0.527	No preference
13	575 ± 131.0	604 ± 83.1	0.02	0.855	No preference

3.4.7 Conclusions

Presentation of aspartame during two-choice preference testing failed to evoke a taste response in all horses allocated to this study. This was suggested to be the result of either taste blindness in individuals or an indifference towards aspartame. The presentation of varying inclusion levels and daily reversal of control positioning may have prevented horses making a choice based on taste cues due to inconsistent presentation between meals. Due to the inability of aspartame to reliably evoke gustatory responses in the horse, there was insufficient justification to continue with the trial and an alternative flavour to determine gustatory response was sought.

3.5 Determination of suitability of glycine

Following the conclusion that aspartame and sucralose were unsuitable test flavours to study gustatory responses in the horse, it was deemed necessary to conduct a further pilot trial to identify a suitable test flavour to evoke a gustatory response in the absence of olfactory cues. Glycine meets the requirements of the test flavour, being of low nutritive value, odourless and potentially evoking a preference response in the horse. As discussed in Section 1.1.4, it has been demonstrated that horses have an innate preference for sweet substances (Randall *et al.*, 1978), though this is subject to individual variation. It was hypothesised that glycine would evoke a positive response in the horse.

Glycine is a nonessential amino acid that evokes a sweet taste in humans (Schiffman *et al.*, 1979 b; Glaser *et al.*, 2000; Glaser, 2002; Rawson, 2004). The sweet taste is attributable to the aliphatic side chain of glycine, resulting in higher sensitivity levels over other amino acids studied (Schiffman *et al.*, 1979 b). It cannot be assumed that glycine is perceived similarly in the horse; though, glycine has previously been demonstrated to evoke a sweet response in calves (Segerstand and Hellekant, 1989 a) and felines (Rawson, 2004). The odour of glycine has previously been stated to depress feed intake of hay in sheep (Arnold *et al.*, 1980). Such a finding has to be questioned due to the lack of volatiles released by glycine and may ultimately indicate an aversion to the taste of glycine on intake, rather than olfactory cues.

It has been shown in humans that amino acid thresholds increase significantly with age, most notably to glycine (Schiffman *et al.*, 1979 a; Schiffman and Clark, 1980). Gustatory sensitivity was found to be 2.5 times lower in elderly human subjects (75 to 87 years) than young subjects (17 to 27 years). Schiffman *et al.* (1979 a) attributed this loss in gustatory sensitivity to a decline in the gustatory apparatus with age, consistent with other findings in humans,

discussed in section 1.2.1. To the author's knowledge no such study of the effect of age on amino acid thresholds has been conducted in animals.

3.5.1 Study aims

The aims of this pilot trial were to ensure the suitability of glycine as a test flavour to identify gustatory response in the horse. The aims of this element of study were two-fold. It was hypothesised that:

1. **Horses would possess the ability to discriminate between a glycine test feed and an unflavoured control.**
2. **The inclusion levels presented would be suitable to measure gustatory response in the horse.**

3.5.2 Materials and methods

Chapter Two describes the general materials and methods used to achieve the aims, namely housing (see Section 2.3), test feed preparation and presentation (see Section 2.4) and measurements (see Section 2.6). Alterations to the protocol are discussed in the following sections, as appropriate.

3.5.2.1 Horses

Five horses of similar breeding were allocated to this study. Horses were selected on the basis of their availability (see Table 3.19).

Table 3.19 Details of the five horses allocated to the study to determine suitability of glycine to identify gustatory response

Horse	Age	Sex	Breed
1	9	Gelding	Thoroughbred
2	10	Gelding	Thoroughbred X
3	12	Mare	Thoroughbred X Cob
4	9	Gelding	Cob
5	7	Mare	Thoroughbred X

3.5.3 Study design

The study was conducted over ten consecutive days. All feeds were presented in identical black containers, holding 1.5kg of control or test feed, as appropriate, for a ten-minute period.

The study was split into two sections, training and two-choice preference testing. Training was conducted over four days, as described in section 2.5.1.1. Two-choice preference testing was conducted, whereby glycine was presented as a increasing dose response on days five to seven, followed by an decreasing dose response on days eight to ten (Table 3.20). To reduce the influence of side preference on gustatory response, the control positioning was reversed daily.

Table 3.20 Presentation order, test feed positioning and inclusion levels of glycine during two-choice preference testing.

Day	Left container	Right container
5	Control	$2.5g.kg^{-1}$
6	$5g.kg^{-1}$	Control
7	Control	$10g.kg^{-1}$
8	$10g.kg^{-1}$	Control
9	Control	$5g.kg^{-1}$
10	$2.5g.kg^{-1}$	Control

3.5.4 Measurements

Changes in intake and behaviour were recorded to determine preference, aversion or indifference to the test feed.

3.5.4.1 Intake

Intake was measured as detailed in section 2.6.1.

3.5.5 Statistical analysis

All statistical analysis was conducted using the Minitab 14.0 programme as detailed in section 2.7.

3.5.6 Results and discussion

No preference for the control or the glycine test feed was recorded in individuals (Table 3.21). Individual responses to the glycine at varying inclusion levels support the discriminatory ability of the horse to detect glycine, as discussed below.

Table 3.21 Difference between control and test feed intake during two-choice preference testing, indicating that no significant feed preference was recorded (mean ± s.e.m. (g), n=6 days).

	Control	Test feed			
Horse	Mean ± s.e.m. (g)	Mean ± s.e.m. (g)	$F_{(1,10)}$ *value*	*p* value	Preference
1	500 ± 152.0	669 ± 179.0	0.51	0.490	No preference
2	652 ± 197.0	577 ± 209.1	0.07	0.801	No preference
3	491 ± 132.1	549 ± 137.0	0.09	0.768	No preference
4	833 ± 198.2	694 ± 203.0	0.24	0.633	No preference
5	418 ± 234.3	445 ± 216.6	0.04	0.843	No preference

Analysis of individual inclusion levels found evidence of discriminatory ability. A group preference for the control was recorded when 5g.kg^{-1} glycine was presented alongside the control ($F_{(1,18)}$ = 5.27, p<0.05). Presentation of the inclusion levels 0.25g.kg-1 ($F_{(1,18)}$ = 0.43, p=0.522) and 10g.kg^{-1} ($F_{(1,18)}$ = 2.14, p=0.161) failed to evoke a significant difference in intake between the control and test feed. The second presentation of the 10g.kg^{-1} test feed on day 8 ($F_{(1,8)}$ = 17.40, p<0.01) resulted in a significant difference in intake compared to the first presentation on day 7 ($F_{(1,8)}$ = 0.05, p=0.811), where no preference was recorded.

The ability of individuals to detect the glycine at the 5g.kg^{-1} and 10g.kg^{-1} inclusion levels indicates horses possess the ability to discriminate between a glycine test feed and an unflavoured control. Additionally a variation in preference with inclusion levels suggests that preference increases with inclusion level. An increase in sensitivity to the test feed following repeat presentation at the $10g.kg^{-1}$ inclusion level, suggests repeated presentation of the glycine test feed increases the discriminatory ability.

Gustatory responses were distorted due to the exhibition of a left side preference recorded in four individuals (see Table 3.22). Overall, side preference influenced feeding choice more than gustatory response.

Table 3.22 Individual difference between right and left container intake during two-choice preference testing (mean ± s.e.m. (g), n=6 days).

Horse	Left Mean ± s.e.m. (g)	Right Mean ± s.e.m. (g)	$F_{(1,10)}$ *value*	*p* value	Preference
1	532 ± 157.0	669 ± 179.0	0.20	0.664	No preference
2	966 ± 143.0	262 ± 114.1	14.77	<0.01	Left
3	703 ± 98.1	337 ± 117.0	5.74	<0.05	Left
4	1070 ± 154.0	457 ± 154.0	8.37	<0.05	Left
5	618 ± 26.3	245 ± 121.9	43.90	<0.001	Left

3.5.7 Conclusions

Horses possess the ability to discriminate between a glycine flavoured test food and an unflavoured control. Inclusion levels were found to affect the response shown towards glycine; recorded as a control preference at the 5g.kg-1 inclusion level and a glycine preference at the 10g.kg-1 inclusion level. For glycine to be used to determine gustatory response in the horse, it was necessary to include glycine at levels sufficient to evoke a gustatory response stronger than a side preference. In order to establish individual gustatory responses to glycine for the purposes of age comparison, it was recommended that inclusion levels be raised from $2.5g.kg^{-1}$, $5g.kg^{-1}$ and $10g.kg^{-1}$ as used in the pilot trial to $5g.kg^{-1}$, $10g.kg^{-1}$ and $20g.kg^{-1}$ in the main trial (Chapter 6).

3.6 Summary

Difficulties arose when identifying a suitable test flavour for identification of taste response and gustatory response. From the developmental studies, orange test feed and glycine were suggested to be suitable chemicals.

The exhibition of side preference was recorded throughout the developmental studies and approaches were developed to attempt to minimise the development of selection based on positioning cues. These included the use of identical stable design and inclusion levels above threshold levels to encourage a greater response to taste cues than positioning cues.

The development of behavioural recording techniques resulted in the conclusion that the Observer 5.0 Package (Noldus, Netherlands) would prove useful in collecting and analysing meaningful behavioural data in order to study taste function and gustatory function.

Chapter 4:

The effect of age on taste response in the horse

There is a paucity of information regarding gustatory and olfactory anatomy and physiology in the horse, with many assumptions being drawn from studies conducted on other species. To the author's knowledge, only one study has been conducted on equine taste function (Randall *et al.*, 1978). Numerous studies to determine equine feed and flavour preference have been conducted due to the commercial benefit of such information.

Randall *et al.* (1978) used two-choice preference testing to establish taste thresholds in the horse, whereby a test solution was presented alongside an unflavoured water control. Randall *et al.* (1978) proposed that the use of water as the carrier enabled increased sensitivity to the test chemicals. The limitation of such an approach is that results cannot be extrapolated to thresholds determined using feed as a carrier. Such knowledge of taste response is of relevance to feed companies and has implications for welfare with hypophagia associated with maximal nutritional need and increasing longevity in the domestic horse (Harris, 1999; Hintz, 1999 b; King, 2000).

A decline in taste function as a result of age may compromise the horse's ability to identify and select suitable foodstuffs and avoid toxin ingestion in the short term and appropriate diet selection in the longer term. Whilst the modern domestic horse has most of its food sources selected for it, an inability to recognise or form appropriate associations between gustatory and olfactory cues and postingestive consequences may affect health. Such losses of taste function are of increased importance in free-living horses. As discussed previously (see Section 1.2.1), such losses of taste function have been implicated in loss of appetite in human subjects and hence, may be of relevance in the horse.

The principal aim of this area of study was to establish individual taste responses for the purpose of age comparisons. The findings of Chapter Three support the discriminatory ability of horses to distinguish between an artificial orange test feed and an unflavoured control (see Section 3.1 and Section 3.2). Although the orange flavoured test feed elicited an aversive taste response, this was deemed suitable to establish taste response. The influence of side preference on taste response discussed in Chapter Three supported a high inclusion rate of test flavour in order to overcome positional bias (see Section 3.2). The implication of such findings was that it was unfeasible to determine taste thresholds in subjects exhibiting side preference and therefore taste response was taken as an indicator of taste function. Hence, taste response may be sufficient to override the exhibition of side preference in susceptible individuals.

4.1 Study aims

The principal aim of this study was to investigate the effect, if any, of age on taste function in the horse. In order to achieve this aim it was necessary to firstly obtain taste response in individuals for the purpose of age comparison. Following this, analysis could be conducted to determine whether age does compromise taste function (see Section 1.2.1).

The following hypotheses were therefore proposed,

- A decline in taste function will occur with increasing age in the horse
- Taste response in the horse will be subject to within and across age group variation

4.2 Materials and methods

Chapter Two describes the general materials and methods used to achieve the aims, namely housing (see Section 2.3), test feed preparation and presentation (see Section 2.4) and measurements (see Section 2.6). Alterations to the protocol are discussed in the following sections, as appropriate.

4.2.1 Horses

The infeasibility of a 20-year longitudinal study due to unrealistic time implications resulted in a cross sectional study being conducted. 18 horses aged between two and 22 years were studied. Horses were allocated into three groups (n=6 horses) on the basis of age and availability; Young (2-5 years), Middle (8-14 years) and Old (above 16 years).

Within each age group horses were randomly assigned numbers. Subjects in the Young group were numbered one to six, Middle group subjects were numbered seven to twelve and subjects in the Old group were numbered 13 to 18. Details of the study horses and allocated numbers are listed in Table 4.1. Due to resource limitations, namely stabling availability and camera equipment, it was impractical to study more than six horses in each trial. Each study of the 18 horses was therefore conducted as three separate trials (A, B and C), with two horses from each age group used (n=6 horses) to reduce the influence of age bias and order effect on findings (see Table 4.2).

Table 4.1 Details of the 18 horses allocated to the study of the effect of age on taste response

Horse	Age	Sex	Breed
1	4	Gelding	Thoroughbred X Warmblood
2	4	Mare	Cob X Thoroughbred
3	3	Mare	Irish Draught X Thoroughbred
4	2	Mare	Warmblood X Irish Draught
5	3	Gelding	Thoroughbred
6	5	Gelding	Irish Draught X Thoroughbred
7	14	Mare	Thoroughbred
8	14	Gelding	Thoroughbred X
9	12	Gelding	Thoroughbred X
10	9	Mare	Irish Draught X Thoroughbred
11	13	Gelding	Cob
12	9	Gelding	Cob
13	18	Gelding	Cob
14	18	Gelding	Irish Draught X Thoroughbred
15	16	Gelding	Irish Draught X Thoroughbred
16	21	Gelding	Cob X
17	16	Gelding	Cob X Thoroughbred
18	18	Gelding	Cob

Table 4.2 Details of the horses, allocated to the study on the basis of age, to the three study groups, A, B or C. Numbers were allocated based on age; Young horses were numbered 1 to 6, Middle horses were numbered 7 to 12 and Old horses numbered 13 to 18.

Group	Young & Old	Middle & Old	Young & Middle
A	Horse 1 & Horse 13	Horse 7 & Horse 14	Horse 2 & Horse 8
B	Horse 3 & Horse 15	Horse 9 & Horse 16	Horse 4 & Horse 10
C	Horse 5 & Horse 17	Horse 11 & Horse 18	Horse 6 & Horse 12

4.2.2 Housing

Horses were housed in stables as described in Section 2.3. The use of uniform stable design enabled central positioning of the containers in the presentation area. Each horse was therefore subject to similar external factors, aiming to reduce the influence of positioning cues on container selection.

4.3 Study Design

Each group was subject to three different test elements: training, two-choice preference testing and monadic testing. All horses were subject to the same protocol throughout. Each study was conducted over 29 consecutive days, with two-choice preference testing and monadic testing being used to assess taste response in the horses.

4.3.1 Training

The aims of training are detailed in Section 2.5.1.1. Following the findings of an earlier study (see Section 3.2), it was concluded that a nine-day training period was sufficient time for side preference to be identified in susceptible individuals. Horses were given the choice of control *versus* control during the training period.

4.3.2 Two-choice preference testing

Over the following 12 days, two-choice preference testing was conducted to establish taste response in individuals. The study design aimed to control for presentation order, position and subject (age) order. The study design incorporated three flavour inclusion rates, identified as low, medium and high. Each inclusion rate differed from the previous one by a factor of two. Artificial orange juice flavouring was used to evoke a taste response, following confirmation of the horses' ability to discriminate between an artificial orange juice test feed and control in an earlier study (see Section 3.2). Further discussion of the rationale for using orange flavouring is discussed in Section 3.1.1.2.

Groups A, B and C were subject to identical study design throughout. As discussed above, the study design had to account for three main factors: presentation order, test feed positioning and age effect. A balanced Latin square, with reversal of control positioning on subsequent days (see Table 4.3)

aimed to control for these factors. Replication of results was incorporated into the study design, with the procedure being repeated on the subsequent six days, following the first presentation of all test feed inclusion rates in both left and right positioning.

Table 4.3 Presentation order, test feed positioning and inclusion level of the orange test feed during two-choice preference testing to establish taste response in individuals

Subject Allocation	Young and Old		Middle and Old		Young and Middle	
Container Positioning	Left	Right	Left	Right	Left	Right
Replicate One	control	$1 g.kg^{-1}$	Control	$2 g.kg^{-1}$	control	$0.5 g.kg^{-1}$
	$1 g.kg^{-1}$	control	$2 g.kg^{-1}$	control	$0.5 g.kg^{-1}$	control
	control	$0.5 g.kg^{-1}$	Control	$1 g.kg^{-1}$	control	$2 g.kg^{-1}$
	$0.5 g.kg^{-1}$	control	$1 g.kg^{-1}$	control	$2 g.kg^{-1}$	control
	control	$2 g.kg^{-1}$	Control	$0.5 g.kg^{-1}$	control	$1 g.kg^{-1}$
	$2 g.kg^{-1}$	control	$0.5 g.kg^{-1}$	control	$1 g.kg^{-1}$	control
Replicate Two	control	$1 g.kg^{-1}$	Control	$2 g.kg^{-1}$	control	$0.5 g.kg^{-1}$
	$1 g.kg^{-1}$	control	$2 g.kg^{-1}$	control	$0.5 g.kg^{-1}$	control
	control	$0.5 g.kg^{-1}$	Control	$1 g.kg^{-1}$	control	$2 g.kg^{-1}$
	$0.5 g.kg^{-1}$	control	$1 g.kg^{-1}$	control	$2 g.kg^{-1}$	control
	control	$2 g.kg^{-1}$	Control	$0.5 g.kg^{-1}$	control	$1 g.kg^{-1}$
	$2 g.kg^{-1}$	control	$0.5 g.kg^{-1}$	control	$1 g.kg^{-1}$	control

4.3.3 Monadic Testing

In order to attempt to establish taste response in the absence of positional cues, monadic testing was conducted. Over four consecutive days an increasing dose response was presented to each horse, each presented in the same order. An additional inclusion rate of 0.25g.kg^{-1} was presented monadically to increase the sensitivity of the monadic test. The removal of position cues by monadic presentation theoretically should increase sensitivity to the lower inclusion level, not presented during two-choice preference testing. Each inclusion rate was replicated over the subsequent four days as a decreasing dose response (see Table 4.4).

Table 4.4 Presentation order and test flavour inclusion rates during monadic testing to establish individual taste responses, with the additional inclusion rate of 0.25g.kg^{-1}

Day	Inclusion rate (g.kg^{-1})
22	0.25
23	0.5
24	1
25	2
26	2
27	1
28	0.5
29	0.25

4.4 Measurements

Changes in intake and behaviour were recorded as indicators of changes in taste response, as discussed in Section 2.6.1 and Section 2.6.2 respectively.

4.5 Statistical analysis

Statistical analysis of intake and behaviour was conducted as described in Section 2.7.

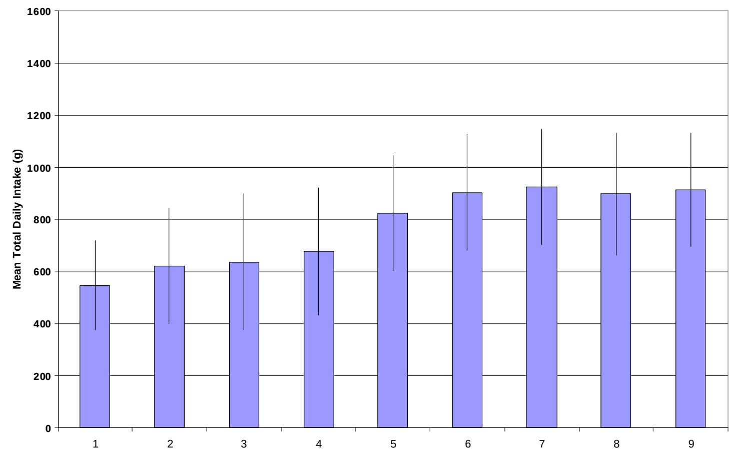
4.6 Results

4.6.1 Training

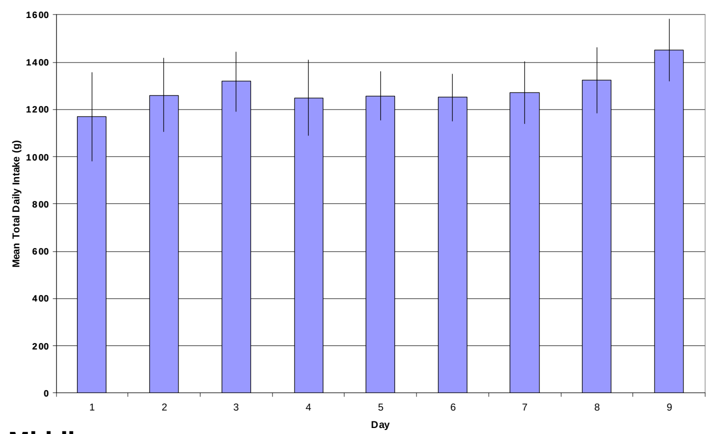
The basal feed was found to be acceptable to all horses following presentation during the training period. The total daily intake, calculated from left plus right intake, reached equilibrium during the training period, prior to commencement of two-choice preference testing on day 10. An effect of age on the total daily intake was recorded, with a significantly lower mean total daily intake found in the Young group, when compared to both the Middle group ($F_{(1,107)}$ = 37.58, $p<0.001$) and the Old group ($F_{(1,107)}$ = 56.88, $p<0.001$) (see Figure 4.1).

A significant difference in total daily intake was recorded between individuals within groups, with the Young group ($F_{(5,53)}$ = 49.05, $p<0.001$) and the Middle group ($F_{(5,53)}$ = 22.91, $p<0.001$) exhibiting more intragroup variation than the Old group ($F_{(5, 53)}$ = 5.04, $p<0.01$).

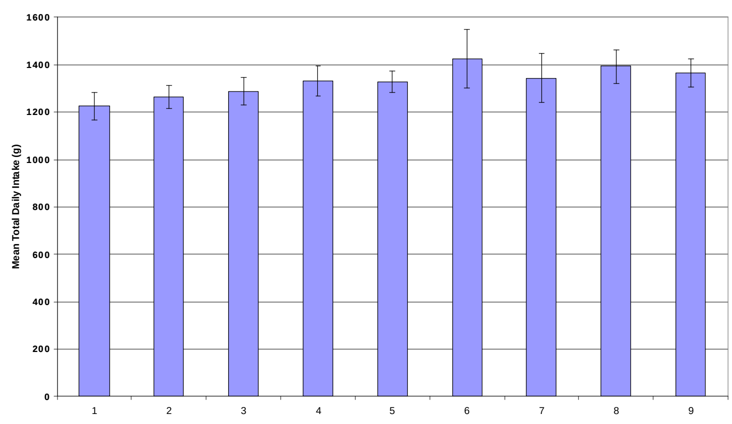
The mean duration of ingestive behaviour, measured as 'eat' behaviour, within age groups reflected the effect of age on total daily intake. Non ingestive behaviour was calculated from the sum of the duration of 'alert' and 'other' behaviours. The Young group had a mean duration of ingestive behaviour of 430 seconds (s.e.m. 61.6, n=6 horses), out of the maximum 600 second period, indicating that ingestive behaviour was interspersed with non-ingestive behaviour. A characteristic time event chart of ingestive and non ingestive behaviour in a Young horse is shown in Figure 4.2. This was significantly shorter than the Middle group ($F_{(1,107)}$ = 24.79, $p<0.001$) and the Old group ($F_{(1,107)}$ = 42.22, $p<0.001$) who exhibited a mean duration of ingestive behaviour of 572 seconds (s.e.m. 19.4, n=6 horses) and 600 seconds (s.e.m. 0.5, n=6 horses) respectively. The difference between the duration of ingestive behaviour between the Old and Middle groups was also significant ($F_{(1,107)}$ = 6.02, $p<0.05$).



a) Young group



b) Middle group



c) Old group

Figure 4.1 Mean total daily intake of the (a) Young group, (b) Middle group and (c) Old group during the training period (mean ± s.e.m. (g), n=9 days). Equilibrium in total daily intake was reached prior to commencement of two-choice preference testing on day 10.

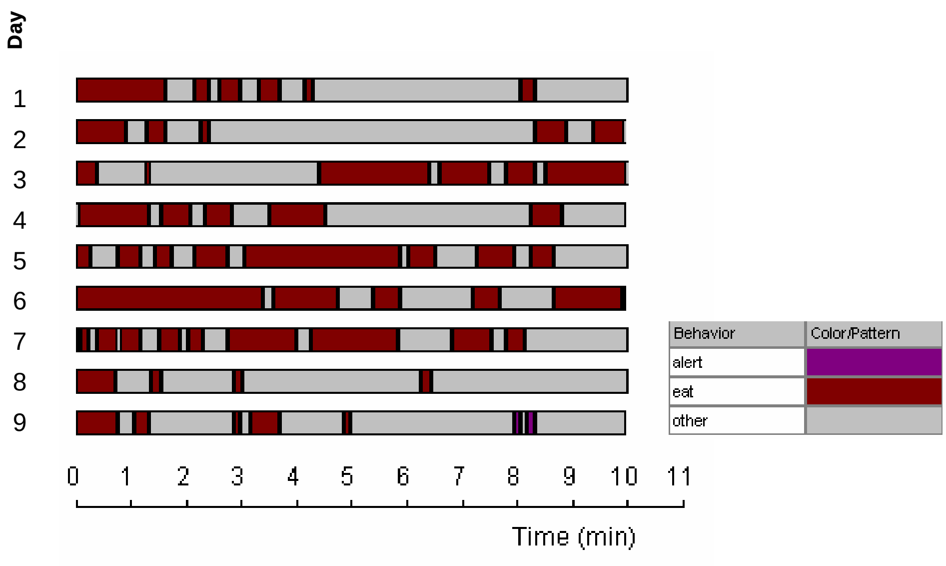


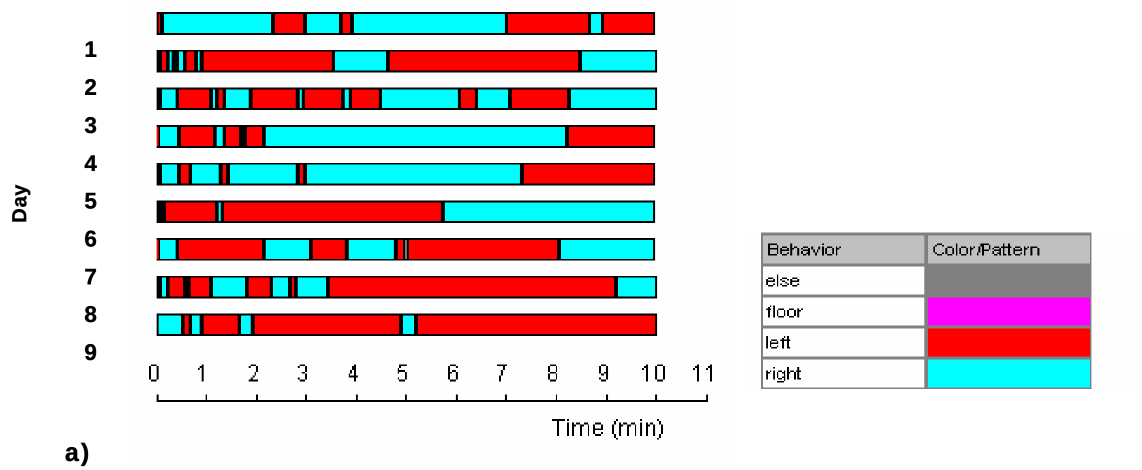
Figure 4.2 Characteristic time-event view ingestive behaviour in a Young horse during training demonstrating inconsistency in ingestive behaviour.

From the difference in left and right intake throughout the training period, side preference was recorded in eight of the 18 horses studied (Young n=3 horses; Middle n=1 horse; Old n=4 horses) (see Table 4.5). An equal distribution of left and right preference was recorded (n=4 for each).

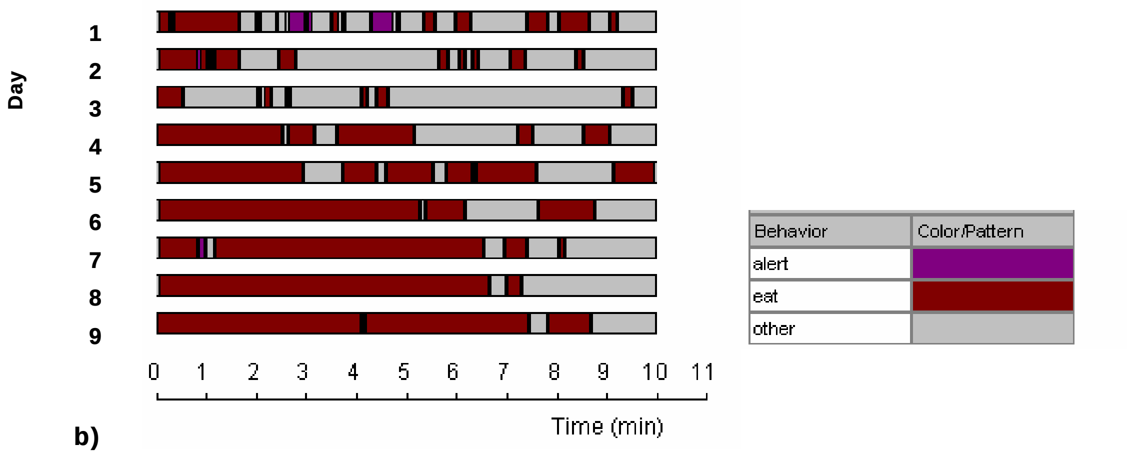
The incidence of intake-indicated side preference was supported by the duration of attention directed towards the left container *versus* the right container in these eight individuals (see Table 4.5). Inconsistency in preference was recorded between intake-indicated side preference and behaviour-indicated side preference in two horses. Analysis of left *versus* right behaviour during training found Horse 4 to exhibit a left preference ($F_{(1,17)}$ = 4.74, p<0.05). This was not supported by intake data ($F_{(1,17)}$ = 1.57, p=0.229). The time event charts for side behaviour (Figure 4.3a) and ingestive behaviour (Figure 4.3b) indicate that whilst Horse 4 was directing attention towards the left container, eat behaviour was not necessarily occurring. Therefore behaviour analysis from duration failed to distinguish between exploratory and ingestive behaviour.

Table 4.5 Individuals exhibiting an intake-indicated and behaviour-indicated side preference during training (mean ± s.e.m. (g/s), n=9 days)

	Intake					Behaviour	
	Left	**Right**				**Left**	**Right**
Horse	**Mean ± s.e.m. (g)**	**Mean ± s.e.m. (g)**	$F_{(1,17)}$ **value**	*p* **value**	**Preference**	**Mean ± s.e.m. (s)**	**Mean ± s.e.n (s)**
1	337 ± 29.5	521 ± 80.3	4.57	<0.05	Right	221 ± 31.3	370 ± 29.0
2	137 ±	245 ± 64.3	2.42	0.14	NP	111 ± 12.9	214 ± 54.0
3	160 ± 55.8	102 ± 22.9	0.93	0.35	NP	176 ± 33.3	101 ± 14.5
4	247 ± 83.3	126 ± 49.3	1.57	0.229	NP	221 ± 44.1	105 ± 30.0
5	502 ± 42.2	777 ± 99.2	6.54	<0.05	Right	232 ± 29.2	364 ± 30.8
6	319 ± 166.0	1161 ± 168.0	12.65	<0.01	Right	110 ± 68.5	490 ± 68.4
7	944 ± 71.0	681 ± 69.0	7.03	<0.05	Left	328 ± 37.5	272 ± 37.4
8	493 ± 63.1	611 ± 123.0	0.73	0.406	NP	281 ± 43.1	316 ± 44.2
9	493 ± 135.0	476 ± 137.0	0.01	0.934	NP	303 ± 83.3	295 ± 84.1
10	491 ± 106.0	531 ± 116.0	0.07	0.8	NP	247 ± 49.9	273 ± 38.1
11	550 ± 172.0	1066 ± 188.0	4.1	0.06	NP	198 ± 68.3	403 ± 68.2
12	554 ± 125.0	813 ± 123.0	2.17	0.16	NP	228 ± 45.5	321 ± 55.0
13	568 ± 70.5	576 ± 59.0	0.01	0.93	NP	288 ± 32.1	312 ± 32.0
14	476 ± 96.3	912 ± 88.2	11.18	<0.01	Right	164 ± 44.4	437 ± 44.4
15	1254 ± 41.0	222 ± 40.5	321.28	<0.001	Left	543 ± 15.4	58 ± 15.6
16	982 ± 168.0	319 ± 163.0	8.01	<0.05	Left	425 ± 76.6	175 ± 76.3
17	820 ± 168.0	589 ± 159.0	1	0.332	NP	340 ± 68.6	254 ± 69.6
18	1164 ± 87.7	95 ± 55.6	105.96	<0.001	Left	550 ± 22.1	51 ± 22.1



a)



b)

Figure 4.3 Time event chart for Horse 4 during training, illustrating (a) the direction of attention and (b) the exhibition of ingestive behaviour. An increase in ingestive behaviour is observed with repeated presentation of the novel basal feed.

An intake-indicated left preference ($F_{(1,17)}$ = 7.03, p<0.05) recorded in Horse 7, was not supported by behaviour-indicated side preference ($F_{(1,17)}$ = 1.12, p = 0.305). This inconsistency in preference is proposed to be the result of exploratory behaviour towards the least preferred right container, with the greatest proportion of intake from the preferred left container (see Figure 4.4). Behavioural recording failed to distinguish between whether the horse was masticating food from the left container, whilst directing attention to the right container and so over recording of right duration may have occurred.

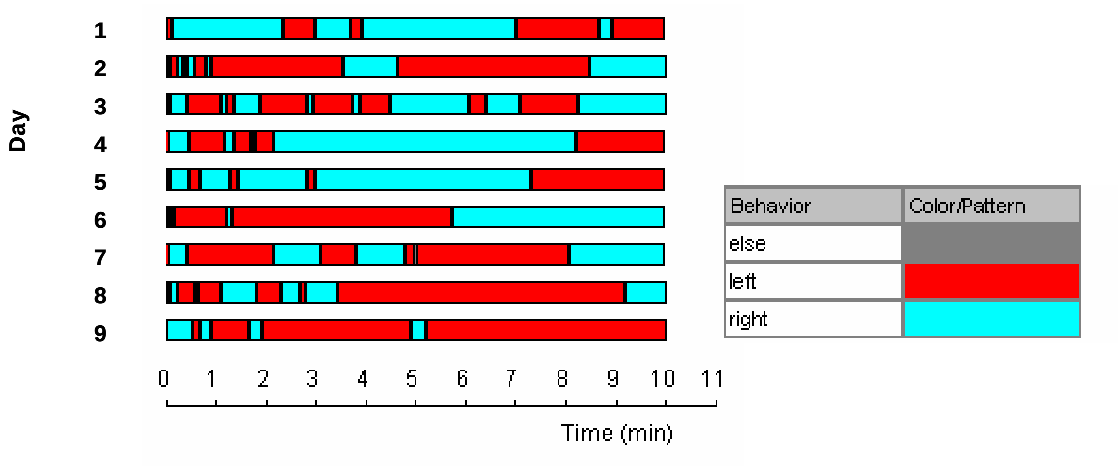


Figure 4.4 Time event chart for Horse 7 during training showing the duration of behaviour recorded towards the two containers and the non feeding area, 'else'.

4.6.2 Determination of taste response by two-choice preference testing

Prior to investigating the effect of age on taste response, it was necessary to identify not only the extent of preference in individuals, but also the direction of preference. Once individual taste responses were established, it was then possible to study if, and to what extent, taste response was affected by age.

A difference in intake between the control and the test feed for the duration of the two-choice preference test period was found in nine of the 18 horses studied (see Table 4.6) (Young n=5 horses; Middle n=2 horses; Old n=2 horses). A preference for the control in these nine horses is evidence that these horses possess the ability to discriminate between the control and the artificial test feed and are motivated to alter their behaviour in response to the taste cues.

A significant difference between the duration of behaviour directed towards the control feed and the duration of behaviour directed towards the test feed was found in 10 individuals, all exhibiting a control preference (see Table 4.6). Horse 17 was the only horse to exhibit a behaviour-indicated control food preference ($F_{(1,23)} = 5.79$, $p<0.05$) not supported by control intake ($F_{(1,23)} = 2.85$, $p=0.105$).

Table 4.6 Individuals exhibiting an intake-indicated and behaviour-indicated difference between test feed and control during two-choice preference testing (mean ± s.e.m. (g/s), n=12 days)

	Intake					Behaviour		
	Control	Test feed				Control	Test feed	
Horse	Mean ± s.e.m. (g)	Mean ± s.e.m. (g)	$F_{(1,22)}$ value	p value	Preference	Mean ± s.e.m. (s)	Mean ± s.e.m. (s)	F(va
1	1087 ± 46.7	109 ± 28.0	322.32	<0.001	Control	492 ± 23.2	69 ± 196.5	1
2	902 ± 47.7	4 ± 1.8	346.22	<0.001	Control	592 ± 1.8	7 ± 1.5	6
3	503 ± 63.3	46 ± 20.5	47.09	<0.001	Control	342 ± 44.4	54 ± 36.9	3
4	1054 ± 45.1	153 ± 108.0	371.04	<0.001	Control	544 ± 16.0	36 ± 658.9	6
5	1161 ± 36.7	129 ± 30.9	463.42	<0.001	Control	533 ± 13.5	65 ± 587.9	5
6	586 ± 201.0	919 ± 194.0	1.41	0.248	NP	235 ± 83.8	364 ± 1.2	1.
7	857 ± 128.0	886 ± 129.0	0.03	0.871	NP	315 ± 40.9	284 ± 0.28	0.
8	517 ± 135.0	609 ± 134.0	0.23	0.634	NP	266 ± 71.1	332 ± 0.63	0.
9	1008 ± 26.5	23 ± 11.1	1175.08	<0.001	Control	580 ± 7.8	18 ± 2736.2	2
10	816 ± 110.0	541 ± 83.9	3.95	0.059	NP	345 ± 41.8	242 ± 3.3	3.
11	563 ± 117.0	863 ± 120.0	3.19	0.088	NP	234 ± 51.7	366 ± 3.3	3.
12	1038 ± 75.0	503 ± 85.1	22.24	<0.001	Control	374 ± 40.1	222 ± 7.5	7.
13	1143 ± 82.8	192 ± 81.9	66.91	<0.001	Control	492 ± 27.3	107 ± 99.0	9
14	1355 ± 16.5	44 ± 12.9	3920.49	<0.001	Control	575 ± 5.6	21 ± 5355.2	5
15	990 ± 185.0	488 ± 185.0	3.68	0.068	NP	399 ± 76.7	201 ± 3.3	3.
16	780 ± 200.0	600 ± 203.0	0.4	0.535	NP	345 ± 88.0	231 ± 0.5	0.
17	918 ± 173.0	506 ± 172.0	2.85	0.105	NP	409 ± 63.9	191 ± 5.8	5.
18	881 ± 182.0	533 ± 183.0	1.82	0.191	NP	362 ± 80.6	237 ± 1.2	1.

A significant preference for the control feed was found ($F_{(1,30)}$ = 32.20, *p*<0.001), calculated from the mean control and test feed intake for individuals, whilst age was found to have no significant effect on feed preference ($F_{(1,30)}$ = 1.21, *p*=0.312). No significant interaction between the factor of feed preference and the factor of age was found ($F_{(1,130)}$ = 2.35, *p*=0.113).

The effect of age on control preference was the result of an overall control preference recorded in the Young group ($F_{(1,142)}$ = 95.36, *p*<0.001) and the Old group ($F_{(1,142)}$ = 46.16, *p*<0.001), whilst the Middle group exhibited a less strong control preference ($F_{(1,142)}$ = 10.83, *p*<0.01).

In order to study the extent of preference and hence the effect of age on taste response (TR), rather than the direction of preference, individual control or test feed preference was used to determine the direction of greatest intake. For horses not exhibiting a significant feed preference, the greatest intake was calculated as the proportion of intake around 0.5, when calculated from the mean total daily consumed. A proportion of control intake of more than 0.5 supported a greater intake of control feed and a proportion of intake of less than 0.5 supported a greater intake of test feed (see Table 4.7). From hereon in, results presented refer to preferred *versus* non-preferred, rather than control *versus* test feed (see Table 4.8).

The analysis of the extent of preference, rather than direction of preference, within age groups recorded a highly significant difference between preferred *versus* non preferred intake (see Figure 4.5a; Figure 4.5b) and the duration of behaviour directed towards the preferred *versus* non preferred container (see Figure 4.6a; Figure 4.6b).

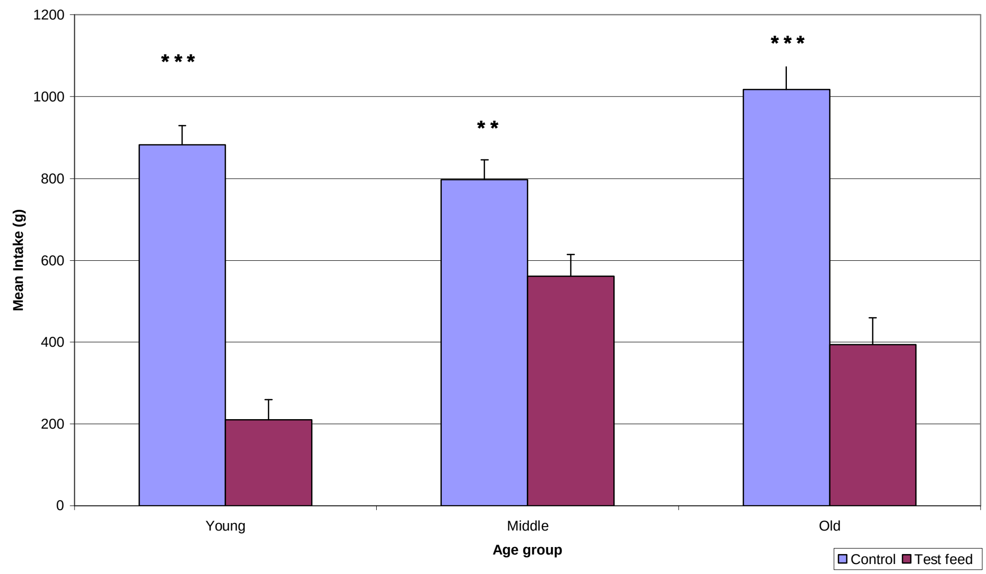
Table 4.7 Individual preferences towards the control and test feed and preferred and non-preferred feed during two-choice preference testing (mean ± s.e.m. (g), n=12 days)

	Intake					
	Control	Test feed		Preferred	Non preferred	
Horse	Mean ± s.e.m. (g)	Mean ± s.e.m. (g)	Gustatory Response	Mean ± s.e.m. (g)	Mean ± s.e.m. (g)	$F_{(1,22)}$ valu
1	1087 ± 46.7	109 ± 28.0	0.88	1087 ± 46.7	109 ± 28.0	322.32
2	902 ± 47.7	4 ± 1.8	0.99	902 ± 47.7	4 ± 1.8	346.22
3	503 ± 63.3	46 ± 20.5	0.86	503 ± 63.3	46 ± 20.5	47.09
4	1054 ± 45.1	153 ± 108.0	0.94	1054 ± 45.1	153 ± 108.0	371.04
5	1161 ± 36.7	129 ± 30.9	0.89	1161 ± 36.7	129 ± 30.9	463.42
6	586 ± 201.0	919 ± 194.0	0.39	919 ± 194.0	586 ± 201.0	1.41
7	857 ± 128.0	886 ± 129.0	0.53	886 ± 129.0	857 ± 128.0	0.03
8	517 ± 135.0	609 ± 134.0	0.44	609 ± 134.0	517 ± 135.0	0.23
9	1008 ± 26.5	23 ± 11.1	0.97	1008 ± 26.5	23 ± 11.1	1175.08
10	816 ± 110.0	541 ± 83.9	0.59	816 ± 110.0	541 ± 83.9	3.95
11	563 ± 117.0	863 ± 120.0	0.39	863 ± 120.0	563 ± 117.0	3.19
12	1038 ± 75.0	503 ± 85.1	0.63	1038 ± 75.0	503 ± 85.1	22.24
13	1143 ± 82.8	192 ± 81.9	0.82	1143 ± 82.8	192 ± 81.9	66.91
14	1355 ± 16.5	44 ± 12.9	0.96	1355 ± 16.5	44 ± 12.9	3920.49
15	990 ± 185.0	488 ± 185.0	0.66	990 ± 185.0	488 ± 185.0	3.68
16	780 ± 200.0	600 ± 203.0	0.60	780 ± 200.0	600 ± 203.0	0.4
17	918 ± 173.0	506 ± 172.0	0.68	918 ± 173.0	506 ± 172.0	2.85
18	881 ± 182.0	533 ± 183.0	0.60	881 ± 182.0	533 ± 183.0	1.82

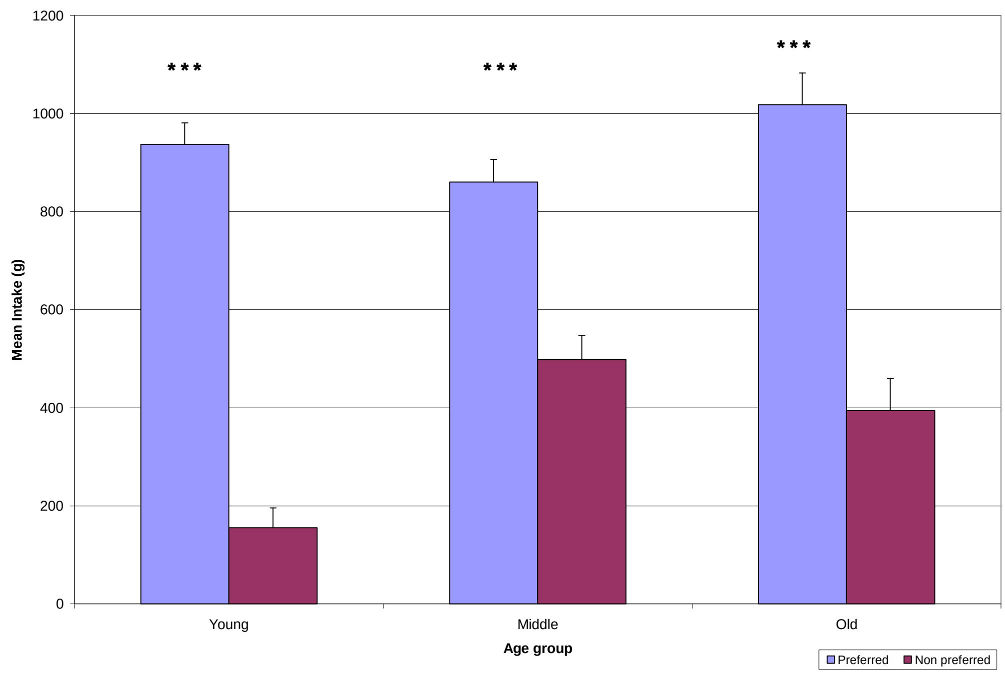
*NP denotes no preference

Table 4.8 Effect of age on mean control *versus* test feed intake and behaviour compared to preferred *versus* non preferred intake and behaviour (mean ± s.e.m. (g/s), n=12 days)

		Control	Test feed			Preferred
Age group		Mean ± s.e.m. (g/s)	Mean ± s.e.m. (g/s)	$F_{(1,142)}$ value	*p* value	Mean ± s.e.m. (g/s)
Young	Intake (g)	882 ± 47.4	561 ± 53.1	10.83	<0.001	937 ± 43.9
	Behaviour (s)	456 ± 21.9	99 ± 20.3	142.83	<0.001	478 ± 19.5
Middle	Intake (g)	797 ± 48.1	561 ± 53.1	10.83	<0.01	860 ± 46.2
	Behaviour (s)	352 ± 22.5	244 ± 22.3	11.68	<0.01	380 ± 21.3
Old	Intake (g)	1018 ± 64.1	394 ± 65.8	46.16	<0.001	1018 ± 64.1
	Behaviour (s)	430 ± 27.1	165 ± 26.5	11.68	<0.001	430 ± 27.1

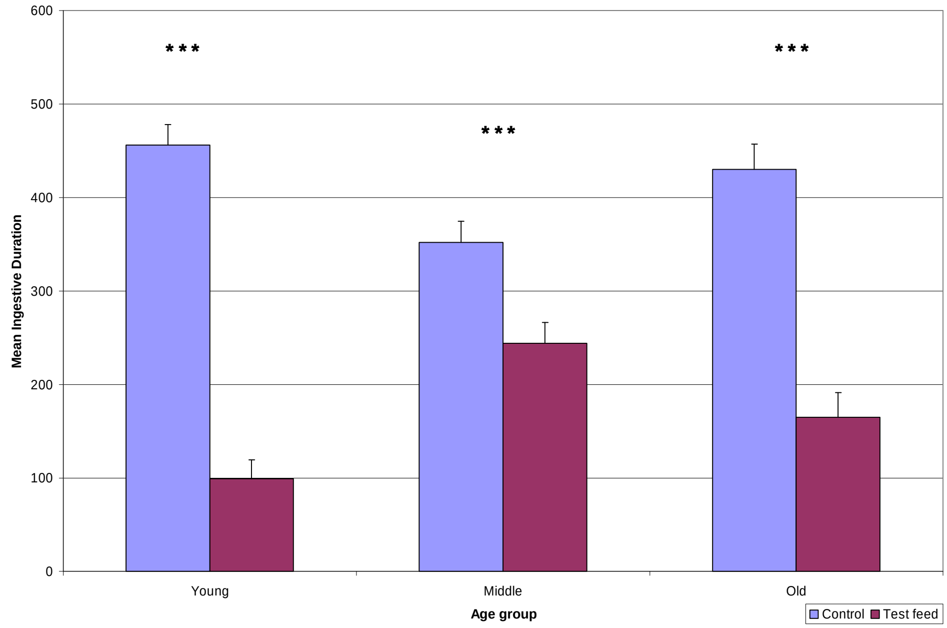


a) Mean daily control and test feed intake for each age group during two-choice preference testing.

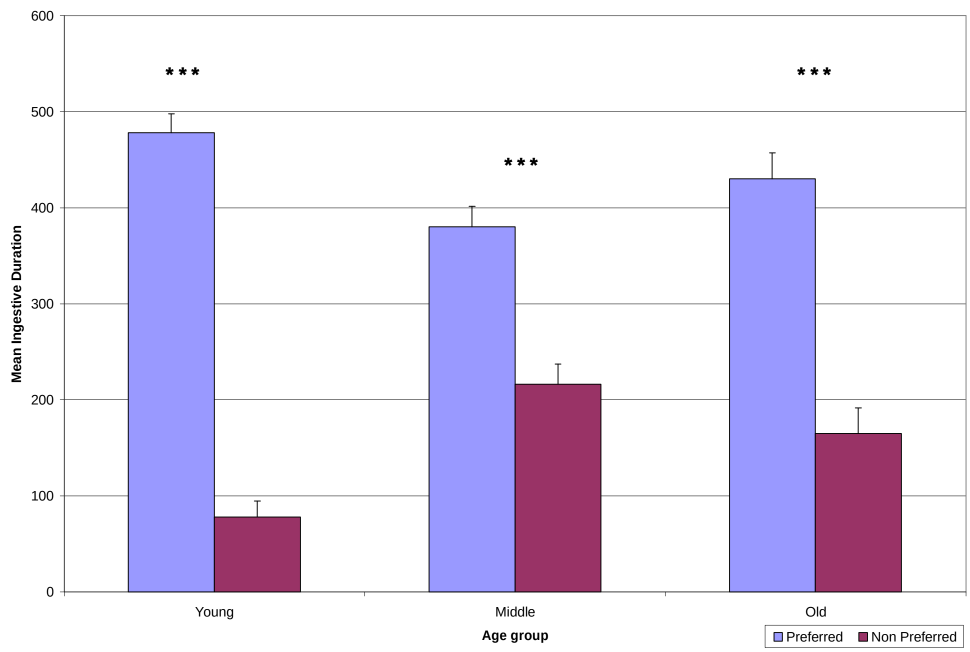


b) Mean daily preferred and non preferred feed intake for each age group during two-choice preference testing.

Figure 4.5 The effect of individual variation on the extent of feed preference when comparing control *versus* test feed intake and preferred *versus* non preferred feed intake, indicated by a stronger response to the test feed when preferred *versus* non preferred was analysed.
***denotes $p<0.05$, ** denotes $p<0.01$ and *** denotes $p<0.001$**



a) Mean daily duration of behaviour towards the control and test feed for each age group during two-choice preference testing.



b) Mean daily duration of behaviour towards the preferred and non preferred feed for each age group during two-choice preference testing.

Figure 4.6 The effect of individual variation on the extent of behaviour duration when comparing control *versus* test feed duration and preferred *versus* non preferred duration, indicated by a stronger response to the test feed when preferred *versus* non preferred was analysed.
***denotes $p<0.05$, ** denotes $p<0.01$ and *** denotes $p<0.001$**

Analysis of the main factor of variation in inclusion level was found to have no significant effect on the proportion of preferred feed eaten ($F_{(2,207)}$ = 1.31, p=2.71). The main factor of age was found to have a significant effect on intake ($F_{(2,207)}$ = 8.85, p<0.001). No significant interaction between age and inclusion level on intake was found ($F_{(4,207)}$ = 0.58, p=0.681). No significant effect of inclusion level on intake ($F_{(2,9)}$, p>0.05) or behaviour duration ($F_{(2,9)}$, p>0.05) was recorded within individuals.

Analysis of the effect of inclusion level on preferred *versus* non preferred taste response, calculated from the proportion of preferred feed consumed from total eaten, found no significant effect on intake across all age groups (Young $F_{(2,69)}$ = 0.31, p=0.738; Middle $F_{(2,69)}$ = 0.55, p=0.582; Old $F_{(2,69)}$ = 0.37, p=0.695; see Table 4.9). No significant main effect of flavour inclusion level on preferred *versus* non preferred intake ($F_{(2,207)}$ = 1.31, p=0.271) was recorded, whilst a significant effect of age on preferred *versus* non preferred taste response was recorded ($F_{(2,207)}$ = 0.58, p<0.001). No significant interaction between the factor of inclusion level and the factor of age ($F_{(4,207)}$ = 0.58, p<0.681) was recorded.

Table 4.9 The effect of variation in inclusion rate on preferred *versus* non preferred intake and behaviour (mean ± s.e.m. (g/s), n=12 days)

		Preferred	Non preferred			Preferred	N
Age group	**Inclusion level ($g.kg^{-1}$)**	**Mean intake ± s.e.m. (g)**	**Mean intake ± s.e.m. (g)**	**$F_{(1,46)}$ value**	***p* value**	**Mean duration ± s.e.m. (s)**	**M s.**
Young	0.5	853 ± 79.0	182 ± 70.5	40.09	<0.001	445 ± 36.9	8
	1	854 ± 82.9	529 ± 93.9	44.79	<0.001	484 ± 33.4	9
	2	945 ± 118.0	454 ± 123.0	98.7	<0.001	231 ± 38.5	3
Middle	0.5	920 ± 73.2	184 ± 82.4	0.38	0.539	358 ± 40.1	2
	1	859 ± 73.5	473 ± 71.2	14.28	<0.001	390 ± 31.5	2
	2	976 ± 118.0	442 ± 118.0	8.62	<0.01	393 ± 39.7	2
Old	0.5	1038 ± 74.2	98 ± 58.6	8.23	<0.01	399 ± 50.7	2
	1	868 ± 86.4	492 ± 94.2	10.25	<0.01	403 ± 49.7	1
	2	1135 ± 96.5	286 ± 101.0	36.91	<0.001	488 ± 39.3	9

A significant difference between the duration of walk in the Young group was found, when analysed against the Middle and Old group ($F_{(2,213)} = 9.99$, $p<0.001$). An apparent increased exhibition in walk behaviour was recorded in the Young group, with 155 incidences of 'walk' behaviour recorded, in comparison to 75 incidences of 'walk' in the Middle group and 0 incidences in the Old group. The high occurrence of walk behaviour recorded in the Young group was influenced by Horse 3 (mean stand duration 533 s, s.e.m. 13.2s; mean walk duration 66 s, s.e.m. 13.2s), accounting for 93 of the 155 incidences. Horse 3 walked on average for one minute every ten-minute presentation period.

A significant intake-indicated side preference was recorded in five individuals (left = 4; right = 1), with behaviour-indicated side preference supporting these findings (see Table 4.10). Further discussion of side preference is given in Chapter 6.

Table 4.10 Individuals exhibiting a significant side preference from intake and behaviour during two-choice preference testing (mean ± s.e.m. (g/s), n=12 days)

	Intake					Behaviour	
	Left	Right				Left	Right
Horse	Mean ± s.e.m. (g)	Mean ± s.e.m. (g)	*p* value	$F_{(1,22)}$ value	Preference	Mean ± s.e.m. (s)	Mean ± s.e.m. (s)
1	615 ± 154.0	581 ± 150.0	0.875	0.03	NP	267 ± 67.3	294 ± 67
2	447 ± 136.0	456 ± 142.0	0.964	0	NP	302 ± 88.1	298 ± 88.3
3	277 ± 83.7	272 ± 83.0	0.961	0	NP	196 ± 55.8	199 ± 53.9
4	570 ± 157.0	540 ± 153.0	0.892	0.02	NP	293 ± 77.9	287 ± 77.9
5	679 ± 160.0	611 ± 158.0	0.765	0.09	NP	309 ± 71.6	289 ± 71.8
6	374 ± 168.0	583 ± 1131.0	<0.01	10	Right	139 ± 70.9	246 ± 460.2
7	1015 ± 121.0	728 ± 121.0	0.108	2.81	NP	340 ± 39.4	259 ± 39.5
8	950 ± 68.7	238 ± 175.3	<0.001	64.59	Left	500 ± 37.7	131 ± 97.7
9	504 ± 144.0	527 ± 155.0	0.918	0.01	NP	308 ± 85.3	290 ± 85.0
10	924 ± 87.2	302 ± 432.9	<0.001	20.78	Left	396 ± 31.4	109 ± 191.0
11	579 ± 105.0	846 ± 134.0	0.131	2.46	NP	245 ± 52.8	355 ± 52.9
12	741 ± 111.0	801 ± 116.0	0.713	0.14	NP	244 ± 426	352 ± 42.1
13	787 ± 161.0	545 ± 160.0	0.298	1.13	NP	336 ± 63.1	263 ± 63.1
14	726 ± 197.0	674 ± 199.0	0.855	0.03	NP	302 ± 83.4	295 ± 83.9
15	909 ± 195.0	674 ± 569.0	0.225	1.56	NP	371 ± 79.4	275 ± 229.3
16	1242 ± 93.2	323 ± 90.9	<0.001	81.07	Left	531 ± 48.9	169 ± 44.3
17	646 ± 180.0	779 ± 185.0	0.612	0.27	NP	243 ± 69.9	356 ± 69.7
18	1199 ± 118.0	409 ± 215.0	<0.001	34.3	Left	524 ± 48.1	167 ± 75.3

*NP denotes no preference

4.6.3 Determination of taste response by monadic testing

Monadic presentation of the test flavour at the four inclusion levels failed to elicit a significant change in intake in individuals, or across age groups. A significant main effect of age on monadic intake was recorded ($F_{(2,132)}$ = 10.79, p<0.001), whilst no significant main effect of inclusion level on intake was found ($F_{(3, 132)}$ = 0.16, p=0.926). No significant interaction between the factor of age and the factor of inclusion level was recorded ($F_{(6,132)}$ = 0.42, p=0.863).

A significant difference in intake during the monadic period was recorded between the three age groups ($F_{(2,141)}$ = 11.27, p<0.001) (see Table 4.11). This was attributable to a significant difference in intake between the Young and the Middle group ($F_{(1,94)}$ = 9.72, p<0.01) and the Young and the Old group ($F_{(1,94)}$ = 18.33, p<0.001). No significant difference in intake between the Middle and Old group during monadic testing was recorded ($F_{(1,94)}$ = 1.25, p=0.266).

Table 4.11 Mean daily intake per horse for age groups during monadic testing (n=8 days)

Age group	Mean intake ± s.e.m. (g)
Young	1146 ± 67.9
Middle	1378 ± 53.2
Old	1432 ± 28.4

Although changes in inclusion levels did not influence intake, presentation order was found to affect intake. A trend of increased daily intake was recorded in the Young group throughout monadic testing (see Figure 4.7). Monadic presentation of the test feed saw a decrease in the Young total daily intake from the first monadic presentation on day 22 (mean intake 865g, s.e.m. 243.0g) compared to the mean total daily intake recorded on the final day of two-choice preference testing on day 21 (mean total daily intake 1185g, s.e.m. 108.0g). Such a reduction in intake was largely attributable to Horse 1 (total daily intake day 21 1217g, total intake day 22 173g) and Horse 2 (total daily intake 1197g, total

intake day 22 288g). The behaviour of Horse 2 during the initial monadic period was characterised by fragmented behaviour directed towards the monadic container and away from the feeding area, indicating a high degree of exploratory behaviour following the presentation of the single monadic container (see Figure 4.7).

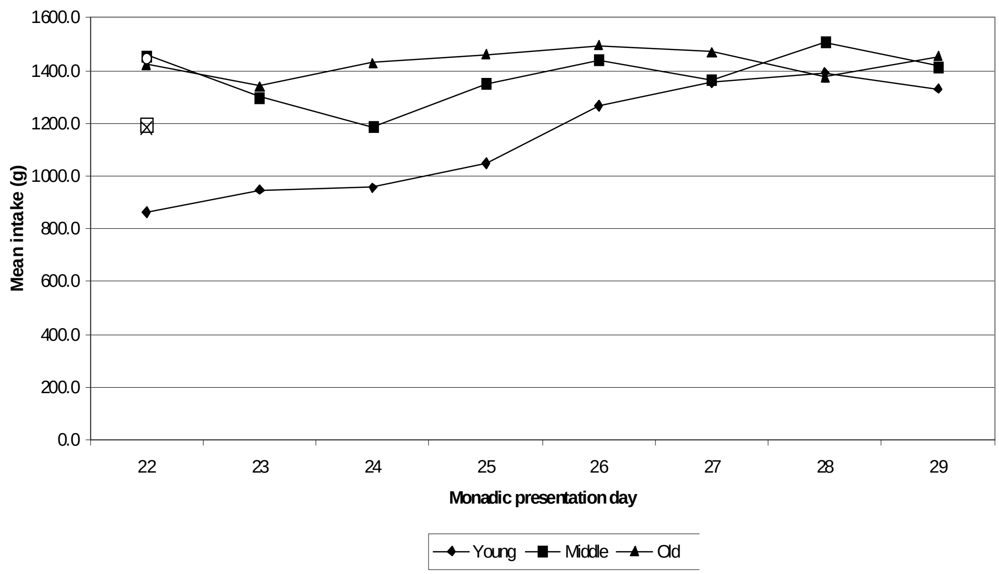


Figure 4.7 Effect of presentation order on mean daily intake during monadic testing.
ˣIndicates the Young group mean total daily intake on day 21, prior to monadic testing
▫Indicates the Middle group mean total daily intake on day 21, prior to monadic testing
°Indicates the Old group mean total daily intake on day 21, prior to monadic testing

The overall lower daily intake in the Young group was attributable to two individuals; Horse 1 (mean intake 683g, s.e.m. 184g) and Horse 2 (mean intake 889g, s.e.m. 889g). Exclusion of these individuals from the mean age group intake increased the mean from 1146g (s.e.m. 75.4g) to 1326g (s.e.m. 47.4g), more comparable to the Middle and Old mean intake. Total daily intake in the Young group increased to equilibrium throughout monadic testing. Therefore, Horse 1 and Horse 2 are proposed to be exhibiting either a neophobic response to the forced presentation of the test feed, or a loss of feeding choice from two

containers presented simultaneously during two-choice preference testing, to one container during monadic testing.

4.7 Discussion

Previous research into the effects of age on taste response has been conducted in humans, with limited research into other animals (see Section 1.2.1). No prior study into the effect of age on taste response has been conducted in the horse. An associated decline in chemical sense functioning with age warrants investigation in the horse due to its relative importance in diet selection and toxin avoidance. This is of increasing importance as a result of improving veterinary care and management techniques increasing the average life span of the pleasure horse (Hintz, 1999 b).

The present study investigated the effects of age on taste response in subjects allocated to three age groups; Young, Middle and Old. Individual taste responses to a novel orange flavour were determined and used to investigate the effects of age on taste response. Taste response was determined from changes in intake and behaviour throughout both two-choice preference testing and monadic testing. It was originally envisaged that a test feed eliciting a positive taste response would be used to determine taste response, but following validation of techniques, the orange flavour was found to result in an aversive response. Aversive cues have been shown to result in a stronger response in cows (Arsenos *et al.*, 1999) and humans (Berstein, 1999), hence taste response was still proposed to be identifiable using a negative taste cue. The study design was based on the assumption that differences in taste sensitivity between equally motivated individuals of different age groups would be indicated by changes in taste response as a result of age.

4.7.1 Training

Analysis of intake and behaviour data found the wheat feed pellets to be acceptable to all horses with daily intake reaching equilibrium prior to commencement of two-choice preference testing. Acceptability increased with

presentation, indicated by an increase in total daily intake and in the duration of ingestive behaviour in all age groups. The findings supported the replacement of non ingestive behaviour with ingestive behaviours. The data obtained during training are discussed further in Chapter 6.

The initially low total daily intake recorded in the Young subjects is contradictory to expected intake. It has been suggested that intake is higher in young horses in relation to their body size due to the demands of growth on nutritional requirements (Campling and Lean, 1983). Reduced intake in the Young subjects in the present study was suggested to be the result of wariness towards the novel basal feed and novel method of food presentation. The Young subjects allocated to this study had previously received little exposure to the stable environment and limited exposure to concentrate feed in comparison to the Middle and Old subjects (pers. Comm. Yard Manager, Anna Gregory). As stated earlier (section 4.7), the study design was based on the assumption that individuals would be equally motivated. From the significant difference in intake between the age groups, it was evident that the Young group exhibited lower feeding motivation than the Middle and Old group. This was supported by a significantly lower duration of eat behaviour recorded in the Young group in comparison to the Middle and Old group.

The lack of side preference recorded in ten individuals supports the assumption that when no choice between foods is presented, intake will occur around a 50:50 mean. A significant side preference was recorded in eight individuals, indicating that a left or right positioning represented a choice in these individuals. Further discussion of side preference is given in Chapter 6.

4.7.2 Two-choice preference testing

Much research demonstrating an effect of age on taste response has been conducted in humans (see Section 1.2.1). The present study aimed to investigate if, and to what extent, age affected taste function in the horse, for which no previous studies have been conducted. It was hypothesised that taste response in the horse would follow a similar pattern of development to that observed in humans and characteristic of most biological systems (Corso, 1981); development, maturation and senescence.

Much research in humans supports changes in the gustatory and olfactory system with age, with the main focus of research on the effect of senescence on morphology and physiology, rather than on the developmental stage (see Section 1.2.1). The gustatory system of the horse is indistinct from those of other species (Pfeiffer *et al.*, 2000), and therefore it is hypothesised that age-related changes in the gustatory system in humans are applicable to the horse. Changes in taste function with age in humans are stated to occur at three levels; taste receptor level (Corso, 1981; Meisami *et al.*, 1998), neuronal level (Mojet *et al.*, 2001) and cognitive level (Mojet *et al.*, 2001). As a consequence of such changes, taste preference, food choices and eating habits have been shown to alter with age in humans (Drewnowski, 2000).

The present study was conducted with 18 horses, allocated to three age groups (n=6 horses), Young, Middle and Old. Two-choice preference testing and monadic testing were implemented to identify taste responses in individuals for the purpose of age comparisons. Whilst two-choice preference testing has been employed previously to determine flavour preference and taste response in a range of animals (see Section 1.3.2.1), the taste responses of several horses in this study influenced as a result of significant left or right container preference.

The selection of feeding choice based on positional cues is discussed further in Chapter 6. It must be noted that the lack of reliability of horses to select based on taste cues rather than positional cues did, in some horses, compromise the reliability of the data.

From analysis of the intake data a more significant preference for the control was recorded in the Young and Old group, compared to the Middle group. When the extent of preference, rather than the direction of preference was studied, a more significant response to the preferred feed was recorded in the Young and Old group, compared to the Middle group. Analysis of the behaviour duration towards the control and test feed and preferred and non preferred feed found a highly significant preference for the control and preferred feed across the three age groups.

Analysis of the effect of variation in inclusion levels of the orange test feed were found to have no significant effect on intake of individuals. As a group, analysis identified a significant difference between preferred and non preferred intake at the three inclusion levels, indicating an ability to discriminate between the test feed and the control at the three inclusion levels. The Young group recorded a consistently high sensitivity towards the three inclusion level, with the Old group demonstrating most sensitivity at the highest inclusion level only. The Middle group recorded the greatest sensitivity to the test feed at the mid inclusion level, followed by the highest inclusion level.

A stronger response towards the test feed in the Young group compared to the Old group would be expected if the findings of this study were to support an age-related decline in taste function. As discussed in Section 1.2, the gustatory and olfactory systems are suggested to function more effectively in young subjects. Therefore, increased sensitivity towards the test feed, based on gustatory and /

or olfactory cues would result in higher sensitivity towards the test feed being recorded as a result of feed choice being based on the organoleptic properties of the food. In addition to food being selected on the basis of taste cues, feeding behaviour is influenced by both previous experience and changes within the horse's external and internal environment.

As stated earlier, the subjects allocated to the Young group had previously had limited exposure to the stabling environment and the presentation of concentrate feed, compared to the Middle and Old group. It was apparent from the significantly lower total daily intake recorded in the Young subjects, in comparison to the Middle and Old group, and the significant incidence of locomotory behaviour recorded in the Young group, in comparison to the Middle and Old group, that the Young group exhibited lower feeding motivation and greater exploratory motivation during two-choice preference testing. This suggests that the assumption that the horses were all subject to the same motivational state was incorrect.

From the findings of this study and previous research, increased sensitivity towards the test feed in Young subjects may be the result of increased wariness towards the test feed following an initial acclimatisation period to the control; a period which was initially characterised by a high level of non ingestive behaviour in Young subjects. This is discussed further in chapter 6. A decline in taste function with age in humans has been reported to decrease neophobia and increase acceptance of novel food stuffs in humans (Pelchat, 2000). This study presented a novel test feed to all the subjects; wariness to novelty is more apparent in young subjects due to a limited period of prior experience. A less significant response towards the test flavour was recorded in the Middle and Old group and may reflect increased prior exposure to, and hence acceptance of, novelty through an increased opportunity for experience and learning. Prior

experience and learning influences taste response and therefore alters feeding behaviour with life stage.

Increased gustatory and olfactory sensitivity in the Young subjects, coupled with wariness towards the novel test feed, may have resulted in a stronger sensitivity towards the test feed being recorded. Evidence of a conditioned preference for the control was recorded across all age groups following repeated exposure to the control feed during training. The significant control preference recorded in nine of the eighteen subjects indicated either a conditioned preference for the control or a dislike towards the orange test feed. The horses not exhibiting a significant preference may have been exhibiting an indifference towards the test feed and control, an ability to discriminate between the test feed and the control or selection of feed based on positioning cues.

The high sensitivity towards the test feed recorded in the Old group does not support the hypothesis that an age related decline in taste function occurs in the horse. A decline in taste sensitivity with advanced years in humans has been attributed to several factors; changes in anatomy and physiology at receptor, neural and cognitive levels, alterations to salivary composition and dietary insufficiencies or excesses. Whilst these factors identified in humans may be influential on taste response in the horse, the inability of horses to communicate detection of a chemical directly results in the need to consider the role of changes in behaviour in indicating taste response also.

The methods used in this study are proposed to be inappropriate to identify taste response in the horse. The inability to effectively identify taste threshold levels resulted in the validation of inclusion levels which identified more substantial changes in behaviour than threshold levels would evoke. An additional factor identified in earlier studies was the selection of positioning cues over taste

response. The inclusion levels used therefore had to be sufficient to evoke a taste response greater than side preference in individuals exhibiting selecting of feed based on container position. It therefore proposed that the inclusion levels used were sufficiently high enough to prevent subtle discrimination between the inclusion levels, resulting in significant preference for the control being recorded in individuals. The inclusion levels presented may have been insufficiently sensitive to enable comparison of an effect of age from an increasing response to increasing inclusion levels. Additionally, the inclusion levels used were easily discernable to the human test through olfactory cues once incorporated with the basal feed. Whilst a concurrent decline in the olfactory system has been recorded alongside a reduced ability to detect olfactory cues in humans (Corso, 1981), avoidance of the test feed based on olfactory cues may have been enabled due to inclusion levels sufficient to enable discrimination between the control and test feed across all three age groups.

Studies on humans suggest that olfactory losses are greater than gustatory losses (Chauhan *et al*., 1987). If applicable to horses, the lack of ability by the Old subjects to detect olfactory cues would result in a reliance on gustatory cues, requiring ingestion to enable discrimination, thus increasing intake and hence, reducing the extent of the taste response.

An additional limitation of this study was the upper age limit of the horses allocated to the Old age group. The horses were allocated to the study on the basis of availability, with the majority of horses available falling into the Middle age range. In humans, the most dramatic decline in taste function occurs from sixty years onwards (Corso, 1981). It is proposed that the study of older subjects than those available for this study would be more likely to be in the latter stages of senescence. The late stages of senescence in humans are characterised by a decline in gustatory receptor cell distribution and cell renewal

(Corso, 1981) and therefore, if the study included older subjects, a more dramatic decline in gustatory and olfactory function would be expected as the gustatory system of the horse is indistinct from other mammalian species (Pfeiffer *et al.*, 2000).

Reduced cognitive function has been indicated as a cause of reduced taste function in humans (Mojet *et al.*, 2001). The horses allocated to the Old group in this present study are suggested not to have compromised cognitive function as again, they were not in the later stages of senescence and no evidence supported such a decline.

In humans, dietary insufficiencies of zinc and copper have been suggested to compromise taste receptor renewal, alongside associated increases in the rate of cell apoptosis with age (Goto *et al.*, 2001). The resultant alterations to taste sensitivity are suggested to result in inappropriate diet selection, resulting in exacerbation of the dietary deficiency. Whilst this is plausible in humans who have direct control over their diet, the human handler controls dietary provision in the stabled horse. The horses used in this study received a diet appropriate to their nutritional needs and therefore the likelihood of a nutritional deficiency occurring was insignificant. There is no research, to the best of the author's knowledge, to suggest an increased risk of zinc or copper deficiency occurring in the aged horse.

The significant side preference recorded in four individuals during two-choice preference testing indicates that food was selected for on the basis of positioning cues, that than taste cues. This may have resulted from an indifference to the test feed or control, or an inability to discriminate between the two feeds. Additionally side preference may develop due to an awareness of no negative or positive outcomes of selecting for the test feed or control; hence a favoured

container position is consistently selected for. The presentation of the test feed removed the influence of container position in food intake in four horses, with a significant control preference recorded in three of these horses. In these individuals selection based on taste cues can be seen to be greater than positioning cues. Additionally, feed intake in these individuals may be influenced by wariness towards the novel basal feed or a conditioned preference for the control, resulting in selection for the control.

As stated earlier, previous exposure to a feed influences food choice and hence, in the context of this present study, taste response. The ability of horses to select an appropriate diet incorporates taste response both in the short term through hedyphagia and the longer term through the association of hedyphagia with postingestive consequences, as previously demonstrated in the horse (Cairns *et al.*, 2001). Therefore an increased experience of novel flavours in the Old subjects may result in rapidity of choice as a result of hedyphagic and euphagic experience, hence exhibiting less sampling behaviour compared to younger subjects.

The use of an artificial test feed to evoke taste response in horses aimed to prevent selection based on postingestive consequences. An ability to associate the orosensory characteristics of the test feed with a lack of postingestive consequences may result in a more rapid acceptance of the test feed and hence, reduced preference for the control. Additionally, feed may be selected based on positioning cues if there is no negative or positive consequence of selecting for the control or test feed.

The relationship between sweet taste cues and the presence of energy, alongside postingestive consequences, reinforces the selection of sweet tasting foods in humans (Cabanac, 1971; Drewnowski, 2000). Although the artificial orange

flavour had a sweet taste to the human researcher, no such assumption can be made of the horses' perception. Certainly, solutions which evoke a sweet taste response in humans do not always correspond with a sweet preference in pigs (Glaser *et al*., 2000). A preference for sweet solutions has been demonstrated in the horse (Randall *et al*., 1978; Hawkes *et al*., 1985). Variation in preference is a common occurrence between and within species, as well as within individuals.

Individual variation in preference may convey the ecological benefit of assisting the avoidance of over exploitation of a resource (Goatcher and Church, 1970 b; Kare *et al*., 1965). In this present study, variation in preference occurred between individuals, within individuals (intermeal variation) and within individuals (intrameal variation). Variation in preference may be influenced by several factors. Allisthesia, the influence of changes in the physiological state on flavour preference is recognised in humans (Epel *et al*., 2001) as influencing short-term preference. Additionally sensory specific and calorific sensory specific satiety in rats (Berridge, 1991) has been demonstrated to alter short-term preference. Longer-term preference is influenced by prior experience and learning.

The ability of a horse to associate orosensory characteristics with postingestive consequences is reliant on both adequate functioning of the gustatory and olfactory systems as well as memory. The capacity for memory has been demonstrated to increase with age; whilst younger subjects retain memory more rapidly, the duration of memory is shorter. It is therefore proposed that the Old subjects possess the ability to retain the memory of a lack of postingestive consequences following ingestion of the test feed during intermeal periods and therefore aversion to test feed declines with increasing exposure. Such enhanced memory ability in Old subjects is reliant on no reduction in cognitive function associated with age. As stated earlier, the horses allocated to the Old group were not believed to be in the late stages of senescence, hence cognitive function should not be compromised in these individuals.

The Middle group demonstrated the most inconsistency in preference from analysis of the effect of inclusion level on intake. As stated earlier, the inclusion levels may have been insufficiently sensitive to enable comparison between different inclusion levels. As with the Young and Old groups, a highly significant preference for the control was recorded across the group, with two Middle subjects exhibiting a significant preference for the control. It is recognised in the horse that if provision of suitable choice is given, they favour multiple choices over one choice (Goodwin *et al*., 2002; Goodwin *et al*., 2005 b). This is suggested to satisfy their requirement for choice as patch strategists.

Whilst the Young subjects exhibited higher levels of non ingestive behaviour and inconsistency in locomotory behaviour, the Old subjects exhibited rapidity of choice due increased acceptance to novelty in the environment, potentially resulting in monotony within the feeding environment. The Middle group, having greater prior experience of novelty than the Young group, may express their desire for choice through intermeal and intrameal inconsistency. The individuals showing indifference to the test feed may therefore be demonstrating a preference for choice, through both avoidance and preference for the test feed, resulting in a taste response nearer 0.5 or selection based on positioning cues. For a horse to discriminate between two feeds it may be necessary to detect the test flavour through comparison of olfactory cues or gustatory cues, prior to a choice being made. Such sampling increases intrameal inconsistency and results in a lesser degree of preference being recorded. Variation in preference within a meal may reflect sampling before an exclusive choice is made, an indifference to the feeds presented or a preference for selection from more than once choice. Increased exposure to the test feed may result in an awareness of a lack of

postingestive consequences and subsequent feed selection may therefore be based on side preference rather than taste response in susceptible individuals.

From analysis of the data it was evident that age had no significant effect on taste response. There was evidence of a significant difference between the Young group and the Middle and Old group with regard to ingestive and non ingestive motivation. This difference in motivational state between the Young group and the Middle and Old group may have resulted in the apparent effect of age on taste response due to an increased wariness towards the novel test feed, rather than a more sensitive gustatory or olfactory system compared to the Middle or Old group.

The methodology used in this study failed to be sufficiently sensitive enough to meet the study aim, to determine if, and to what extent, taste response is affected by age in the horse. The allocation of horses in the late stages of senescence to the Old group would enable greater distinction between the Middle and Old group and potentially, would represent a marked decline in gustatory and olfactory function, thus enabling comparison between age groups.

No significant effect of differences in inclusion level on intake was recorded within individuals. It is therefore suggested that the inclusion levels, which were easily discernable to the researcher via olfactory cues, were too high to enable distinct differences in behaviour between the three levels. Also, the paired presentation of inclusion levels, with daily reversal of the control, would have represented a transitional cue between meals and between the inclusion levels, confounding choice.

4.7.3 Monadic testing

Whilst monadic testing removes the influence of positioning cues on feed choice, it is inappropriate for determining taste response, failing to identify individual or group taste responses. This finding is consistent with those of Hawkes *et al.* (1985) whereby total intake does not vary when the least preferred food is fed alone. Although inclusion levels were presented at concentrations that were avoided when presented alongside an unflavoured control, horses consumed the least preferred test feed when presented monadically.

Consistent with the presentation of the novel basal feed during training, forced choice of the test feed during monadic testing resulted in an initial depression of intake. This is consistent with the findings of Whittemore *et al*. (2002) on presentation of a novel feed. Although the test feed was not novel following two-choice preference testing, it is proposed that a stronger neophobic response was exhibited in the Young group due to the removal of choice, rather than single presentation of the test feed.

4.8 Conclusions

From this study, no conclusion of an age-related decline in taste function in the horse can be drawn. It was apparent from the data that differences in motivational state between age groups resulted in differences in behaviour being recorded, being most pronounced between the Young group compared to the Middle and Old group.

The methodology used to identify taste response in the horse proved to not be sensitive enough to meet the study aim. Increasing the age of horses in the Old group would enable the study of horses in the late stages of senescence, when a dramatic decline in taste function and cognitive function would be hypothesised from similar declines in other mammalian species. Additionally, this would provide more distinction between the Middle and Old group.

The main conclusion from this study was that further analysis using more sensitive methodologies would be necessary before an effect of age on taste in the horse could be confirmed.

Chapter 5:

The effect of age on gustatory response in the horse

There has been no study of the effect of age on olfactory or gustatory function in the horse. However, studies have identified a loss of olfactory sensitivity with age in human subjects, though controversy remains over the magnitude of olfactory loss in relation to gustatory loss (Kaneda *et al.*, 2000; Pelchat, 2000).

The study of gustatory function is complicated due to the intrinsic nature of the gustatory and olfactory systems, which combine to produce a taste response. This study is concerned with the investigation of taste response in the absence of olfactory cues, from hereon in termed the gustatory response. Whilst it is acknowledged that the horses studied will still be subject to olfactory cues from the external environment, this study aimed to remove additional olfactory cues from the test feed, to enable the study of gustatory response.

There is limited information available on the anatomy and physiology of the equine gustatory and olfactory systems. Whilst numerous studies have been conducted to determine feeding preference in the horse, limited emphasis has been placed on the gustatory system in isolation of olfactory cues. Moreover, studies on the influence of olfactory cues on feeding behaviour have not been conducted, with studies of olfactory function focussing on the influence of olfactory cues on stallion behaviour (Wierzbowski, 1959; Anderson *et al.*, 1996). The lack of studies on the influence of gustatory and olfactory cues on feeding behaviour is proposed to be the result of gustation and olfaction being so intrinsically linked to taste response.

5.1 Study aims

The principal aim of this study was to investigate the effect, if any, of age on gustatory function in the horse. This study therefore adapted the methodology used to determine individual taste responses in order to determine individual gustatory responses for the purposes of age comparisons. Moreover, in order to achieve this aim, it was necessary to develop a novel method of assessing gustatory response in the horse. Development of the method to determine gustatory responses is discussed in Sections 3.3, 3.4 and 3.5. Age has been shown to affect gustatory function in humans (Chauhan *et al*., 1987; Murphy and Gilmore, 1989; Stevens and Cain, 1993; Finkelstein and Schiffman, 1999 a; Kaneda *et al*., 2000; Mojet *et al*., 2001; Mojet *et al*., 2005) and a specific loss in gustatory sensitivity towards amino acids has been recorded in elderly human subjects (Schiffman *et al*., 1979 a; Schiffman and Clark, 1980).

The following hypotheses were therefore proposed,

1. **A decline in gustatory function will occur with increasing age in the horse**
2. **Gustatory response in the horse will be subject to within and across age group variation**

5.2 Materials and methods

Chapter Two describes the general materials and methods used to achieve the aims, namely housing (see Section 2.3), test feed preparation and presentation (see Section 2.4) and measurements (see Section 2.6). Alterations to the protocol are discussed in the following sections, as appropriate.

The findings of a previous study (see Section 3.5) validated the non-essential amino acid glycine as a suitable flavour to determine gustatory responses in the horse. Glycine has been previously shown to be detectable in a range of mammalian species (Glaser *et al*., 2000; Glaser, 2002) and in humans evokes a sweet taste response, without the release of olfactory cues. Moreover, gustatory sensitivity to glycine has been shown to decrease with age in human subjects (Schiffman *et al*., 1979 a; Schiffman and Clark, 1980).

5.2.1 Horses

As discussed in section 4.2.1, horses were allocated to the study on the basis of age. For this study, horses were allocated into three age groups (n=6 horses); Young (4-6 years), Middle (10-14 years) and Old (above 16 years). Details of the horses used in this study and their allocated numbers are given in Table 5.1.

Each study of the 18 horses was conducted as three separate trials (A, B and C), with two horses from each age group used (n=6 horses) (see Table 5.2). This aimed to reduce the influence of age bias and order effect on findings. Due to limitations on the number of horses available to study, it was necessary to allocate horses to this study that had previously been used to determine the effect of age on taste response. Three horses were allocated to this study, having previously been used in the study on the effect of age on taste response (Chapter 4). Horse 1, Horse 5 and Horse 9 during Chapter 4 were renumbered Horse 3, Horse 1 and Horse 12 respectively. All horses were subject to a

minimum of four months between studies.

Table 5.1 Details of the 18 horses allocated to the study of the effect of age on gustatory response.

Horse	Age	Sex	Breed
1	4	Gelding	Irish Draught X Thoroughbred
2	4	Gelding	Thoroughbred X
3	5	Gelding	Thoroughbred X Warmblood
4	6	Mare	Irish Draught X Thoroughbred
5	4	Gelding	Thoroughbred
6	5	Gelding	Irish Draught X Thoroughbred
7	12	Gelding	Thoroughbred
8	14	Gelding	Cob X
9	14	Mare	Thoroughbred X
10	14	Mare	Irish Draught X Thoroughbred
11	10	Mare	Irish Draught X Thoroughbred
12	13	Gelding	Thoroughbred X
13	18	Gelding	Thoroughbred
14	18	Gelding	Thoroughbred X
15	16	Gelding	Irish Draught X Thoroughbred
16	16	Gelding	Irish Draught X Thoroughbred
17	16	Gelding	Cob X Thoroughbred
18	18	Gelding	Thoroughbred X

Table 5.2 Details of the horses, allocated on the basis age, to the three study groups, A, B or C. Numbers were allocated based on age; Young horses were numbered 1 to 6, Middle horses were numbered 7 to 12 and Old horses numbered 13 to 18.

Group	Young & Old	Middle & Old	Young & Middle
A	Horse 1 & Horse 13	Horse 7 & Horse 14	Horse 2 & Horse 8
B	Horse 3 & Horse 15	Horse 9 & Horse 16	Horse 4 & Horse 10
C	Horse 5 & Horse 17	Horse 11 & Horse 18	Horse 6 & Horse 12

5.2.2 Housing

Horses were housed in stables as described in Section 2.3. The use of uniform stable design enabled central position of the containers in the presentation area. Each horse was therefore subject to similar external factors, aiming to reduce the influence of position cues on container selection.

5.3 Study design

Each group was subject to three different test elements: training, two-choice preference testing and monadic testing. All horses were subject to the same protocol throughout. Following the persistence of side preference when taste cues were presented during two-choice preference testing (see Section 4.6.2), the training period was shortened from nine days, to four days. The rationale behind the shortening of the training period was to enable horses to have sufficient time to familiarise themselves with the basal feed and enable awareness of two simultaneous feeding choices, without side preference becoming established in susceptible individuals. This study was conducted over 24 consecutive days, with two-choice preference testing and monadic testing being used to assess gustatory response in the horse.

5.3.1 Training

The aims of training are detailed in Section 2.5.1.1. As discussed in section 5.3, training was conducted over four consecutive days

5.3.2 Two-choice preference testing

Over the following 12 days, two-choice preference testing was conducted to establish individual gustatory response. The study design aimed to control for presentation order, position and subject (age) order. The study design incorporated three flavour inclusion rates, identified as low, medium and high. Each inclusion rate differed from the previous one by a factor of two. Glycine was used to evoke a gustatory response in the absence of additional feed-based olfactory cues.

Groups A, B and C were subject to identical study design throughout. As discussed above, the study design had to account for three main factors; presentation order, test feed positioning and age effect. A balanced Latin square,

with reversal of control positioning on subsequent days aimed to control for these factors (see Table 5.3). Replication of results was incorporated into the study design, with the procedure being repeated on the subsequent six days, following the first presentation of all test feed inclusion rates in both left and right positioning.

Table 5.3 Presentation order, test feed positioning and inclusion level of the glycine test flavour during two-choice preference testing to establish gustatory response in individuals for the purposes of age comparison

Subject Allocation	Young and Old		Middle and Old		Young and Middle	
Container Positioning	Left	Right	Left	Right	Left	Right
Replicate One	control	5g.kg^{-1}	control	10g.kg^{-1}	control	2.5g.kg^{-1}
	5g.kg^{-1}	control	10g.kg^{-1}	control	2.5g.kg^{-1}	control
	control	2.5g.kg^{-1}	control	5g.kg^{-1}	control	10g.kg^{-1}
	2.5g.kg^{-1}	control	5g.kg^{-1}	control	10g.kg^{-1}	control
	control	10g.kg^{-1}	control	2.5g.kg^{-1}	control	5g.kg^{-1}
	10g.kg^{-1}	control	2.5g.kg^{-1}	control	5g.kg^{-1}	control
Replicate Two	control	5g.kg^{-1}	control	10g.kg^{-1}	control	2.5g.kg^{-1}
	5g.kg^{-1}	control	10g.kg^{-1}	control	2.5g.kg^{-1}	control
	control	2.5g.kg^{-1}	control	5g.kg^{-1}	control	10g.kg^{-1}
	2.5g.kg^{-1}	control	5g.kg^{-1}	control	10g.kg^{-1}	control
	control	10g.kg^{-1}	control	2.5g.kg^{-1}	control	5g.kg^{-1}
	10g.kg^{-1}	control	2.5g.kg^{-1}	control	5g.kg^{-1}	control

5.3.3 Monadic testing

In order to attempt to establish taste response in the absence of positional cues, monadic testing was conducted. Over four consecutive days an increasing dose response was presented to each horse, each presented in the same order. An additional inclusion rate of 20g.kg^{-1} was presented monadically, aiming to evoke a stronger gustatory response than that observed during two-choice preference testing. Each inclusion rate was replicated over the subsequent four days as a decreasing dose response (see Table 5.4).

Table 5.4 Presentation order and glycine inclusion rates during monadic testing to establish individual gustatory responses, with the additional inclusion rate of 20 g.kg^{-1}, not previously presented.

Day	Inclusion rate (g.kg^{-1})
17	2.5
18	5
19	10
20	20
21	20
22	10
23	5
24	2.5

5.4 Measurements

Changes in intake and behaviour were recorded as indicators of changes in taste response, as discussed in Section 2.6.1 and Section 2.6.2 respectively.

5.5 Statistical analysis

Statistical analysis of intake and behaviour was conducted as described in Section 2.7.

5.6. Results

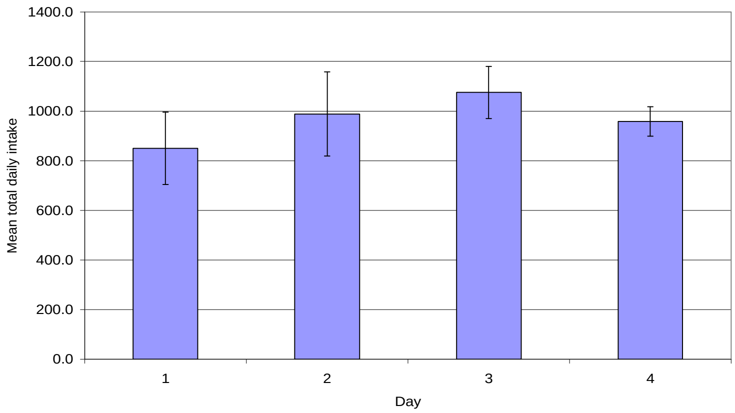
5.6.1 Training

The basal feed was found to be acceptable to all individuals, with relatively consistent intake over the four-day training period. Reduced total daily intake in the Young and Old group on day 4 suggests that equilibrium of intake had not been reached, prior to commencement of two-choice preference testing on day 5 (see Figure 5.1).

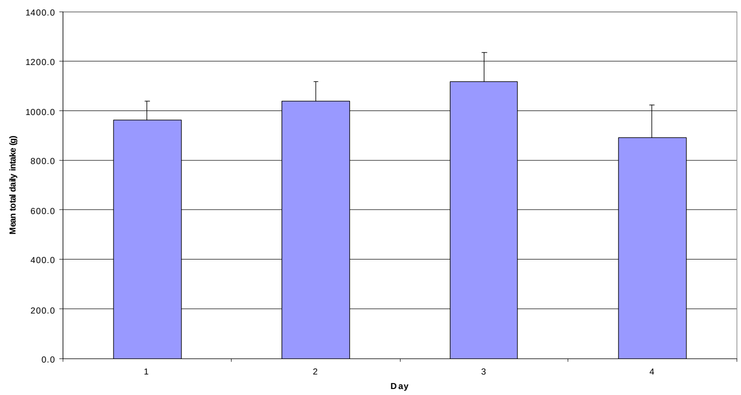
No effect of age on total daily intake ($F_{(2,71)} = 0.65$, $p = 0.528$) was recorded during the training period. The Middle group recorded the lowest mean total daily intake (mean total daily intake 905g, s.e.m. 46.5g, n=6 horses) compared to the Young group (mean total daily intake 968g, s.e.m. 46.5g, n=6 horses) and Old group (mean total daily intake 1003g, s.e.m. 48.4, n=6 horses).

Greater intragroup variation in total daily intake during the training period was recorded in the Young group ($F_{(5,18)} = 9.02$, $p < 0.001$) and Middle group ($F_{(5,18)} = 12.66$, $p < 0.001$), compared to the Old group ($F_{(5,18)} = 5.27$, $p < 0.01$).

Age was found to have no effect on the duration of ingestive behaviour, determined from 'eat' behaviour, between age groups ($F_{(2,71)} = 1.81$, $p = 0.171$). A significant difference between the duration of ingestive behaviour and non ingestive behaviour was recorded across all three age groups (Young ingestive duration *versus* non ingestive duration, $F_{(1,47)} = 3362.83$, $p < 0.001$; Middle ingestive duration *versus* non ingestive duration, $F_{(1,47)} = 119.88$, $p < 0.001$; Old ingestive duration *versus* non ingestive duration, $F_{(1,47)} = 460.95$, $p < 0.001$).



a)



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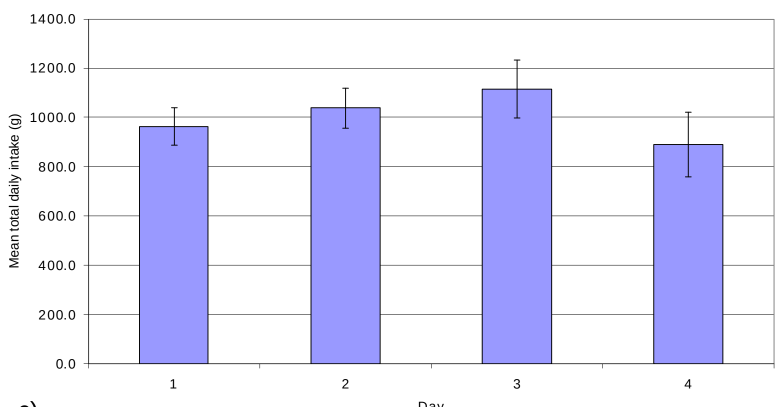


c)

Figure 5.1 The mea**n ± s.e.m. (g) total daily intake of the (a) Young, (b) Middle and (c) Old age group during the training period (n=4 days). Equilibrium in intake was not reached prior to commencement of two-choice preference testing on day 5.**

From the differences in individual left and right intake throughout the training period, a side preference was recorded in five of the 18 horses studied (Young n=2 horses; Middle n=1 horse; Old n=2 horses). All horses exhibiting a significant side preference selected for the left container positioning.

Analysis of the side preference indicated from the duration of attention towards the left or right container found a significant side preference in seven horses (Table 5.5). Inconsistency in side preference identified from intake or behaviour was recorded, with only horses 2, 6, 7, 14 and 16 having a significant intake and behaviour-indicated side preference. The behaviour-indicated side preference recorded in Horse 5 ($F_{(1,6)}$ = 16.4, p<0.01), Horse 13 ($F_{(1,6)}$ = 79.8 p<0.001), and 15 ($F_{(1,6)}$ = 6.89, p<0.05) were not supported by a significant difference in left *versus* right intake.

Table 5.5 The extent of intake-indicated side preference and behaviour-indicated side preference in individuals during training (mean ± s.e.m. (g/s), n=4 days)

	Intake					Behaviour	
	Left	Right				Left	Right
Horse	Mean ± s.e.m. (g)	Mean ± s.e.m. (g)	$F_{(1,6)}$ value	*p* value	Preference*	Mean ± s.e.m. (s)	Mean ± s.e.m. (s)
1	524 ± 140.0	769 ± 232.0	0.82	0.4	NP	240 ± 79.0	361 ± 79.1
2	964 ± 25.9	76 ± 31.4	476.49	<0.001	Left	556 ± 13.4	45 ± 13.4
3	231 ± 85.0	317 ± 94.6	0.45	0.526	NP	278 ± 56.0	275 ± 32.6
4	413 ± 121.0	576 ± 127.0	0.87	0.387	NP	204 ± 68.7	396 ± 68.6
5	772 ± 90.2	406 ± 156.0	4.15	0.88	NP	405 ± 47.9	147 ± 42.1
6	691 ± 91.0	69 ± 45.1	37.5	<0.01	Left	503 ± 42.7	85 ± 37.4
7	1048 ± 223.0	327 ± 107.0	8.49	<0.05	Left	440 ± 53.3	144 ± 38.8
8	303 ± 52.7	255 ± 79.3	0.25	0.636	NP	216 ± 41.8	236 ± 96.1
9	572 ± 156.0	486 ± 143.0	0.16	0.7	NP	315 ± 76.2	286 ± 76.2
10	455 ± 190.0	488 ± 154.0	0.02	0.895	NP	291 ± 98.8	296 ± 94.9
11	687 ± 176.0	299 ± 77.7	4.05	0.091	NP	390 ± 60.8	210 ± 60.8
12	143 ± 48.8	369 ± 154.0	1.95	0.212	NP	117 ± 64.0	230 ± 126.0
13	231 ± 85.0	317 ± 94.6	0.82	0.4	NP	133 ± 26.5	467 ± 26.4
14	964 ± 25.9	76 ± 31.4	476.49	<0.001	Left	510 ± 25.3	101 ± 17.1
15	92 ± 93.6	544 ± 120.0	5.35	0.6	NP	154 ± 78.7	447 ± 78.9
16	841 ± 72.9	228 ± 51.4	47.3	<0.001	Left	472 ± 24.9	128 ± 24.9
17	474 ± 205.0	311 ± 78.4	0.55	0.486	NP	285 ± 107.0	160 ± 45.1
18	518 ± 79.1	574 ± 98.0	0.2	0.671	NP	251 ± 47.7	341 ± 41.3

*NP denotes no preference

5.6.2 Two-choice preference testing

As discussed in Chapter 4, it was necessary to initially determine individual gustatory responses, prior to investigating the effect of age on gustatory function. Consistent with the earlier study on the effect of age on taste response (see Chapter 4), it was also necessary to study the extent of preference, rather than the direction of preference to overcome the issue of individual variation in preference. From these findings the effect of age, if any, on gustatory response could be determined.

No group preference for the control or test feed was recorded within groups (Young $F_{(1,42)}$ = 1.67, p=0.199; Middle ($F_{(1,42)}$ = 0.25, p=0.621; Old $F_{(1,142)}$ = 3.09, p=0.081), however, a significant difference in intake between the control and the test feed was identified in six of the 18 horses studied (Young n=2 horses; Middle n=2 horses; Old n=2 horses) as shown in Table 5.6. Two horses demonstrated a statistically significant preference for the control, with the remaining three horses exhibiting a significant preference, selecting for the test feed.

Inconsistencies between intake-indicated feed preference and behaviour-indicated side preference were recorded in two horses. Horse 8 ($F_{(1,22)}$ = 7.73, $p<0.05$) and Horse 13 ($F_{(1,22)}$ = 9.2, $p<0.01$) were found to have an intake-indicated feed preference not supported by behavioural differences.

No significant preference for the control feed ($F_{(1,426)}$ = 0.30, p=0.587) or for the effect of age on intake ($F_{(1,426)}$ = 0.19, p=0.824) was found. No significant interaction between the factor of feed preference and the factor of age was found ($F_{(2,426)}$ = 2.5, p=0.081).

Table 5.6 Individual horses exhibiting a difference in control or flavour intake during two-choice preference testing (mean ± s.e.m. (g/s), n=12 days).

	Intake					Behaviour	
	Control	Test feed				Control	Test feed
Horse	Mean ± s.e.m. (g)	Mean ± s.e.m. (g)	$F_{(1,22)}$ value	*p* value	Preference*	Mean ± s.e.m. (s)	Mean ± s.e (s)
1	522 ± 170.0	950 ± 161.0	9.2	<0.01	Test feed	186 ± 63.8	415 ± 63.8
2	567 ± 133.0	473 ± 131.0	0.25	0.622	NP	300 ± 73.2	299 ± 72.8
3	382 ± 127.0	527 ± 123.0	0.67	0.42	NP	280 ± 64.0	300 ± 67.5
4	382 ± 130.0	703 ± 116.0	3.39	0.079	NP	231 ± 57.2	369 ± 57.1
5	569 ± 138.0	547 ± 145.0	0.01	0.916	NP	298 ± 70.2	293 ± 70.1
6	450 ± 83.1	106 ± 67.6	9.52	<0.01	Control	383 ± 46.7	81 ± 49.7
7	808 ± 143.0	738 ± 150.0	0.11	0.74	NP	272 ± 51.1	323 ± 52.6
8	364 ± 52.9	599 ± 65.7	7.73	<0.05	Test feed	280 ± 47.5	320 ± 47.5
9	386 ± 105.0	607 ± 115.0	0.11	0.74	NP	259 ± 60.7	342 ± 60.7
10	640 ± 97.7	324 ± 97.4	5.27	<0.05	Control	386 ± 34.1	209 ± 34.2
11	577 ± 116.0	480 ± 132.0	0.31	0.585	NP	335 ± 62.4	264 ± 62.2
12	532 ± 116.0	360 ± 126.0	1	0.327	NP	361 ± 68.4	240 ± 68.4
13	445 ± 135.0	1026 ± 136.0	9.2	<0.01	Test feed	354 ± 52.1	247 ± 52.1
14	870 ± 153.0	405 ± 136.0	5.12	<0.05	Control	455 ± 59.2	146 ± 59.2
15	425 ± 93.0	258 ± 96.7	1.56	0.224	NP	367 ± 74.8	234 ± 75.1
16	450 ± 134.0	489 ± 138.0	0.04	0.842	NP	286 ± 81.8	314 ± 81.9
17	272 ± 78.8	194 ± 64.6	0.58	0.456	NP	250 ± 46.1	180 ± 42.4
18	530 ± 162.0	768 ± 160.0	1.1	0.306	NP	235 ± 70.6	365 ± 70.6

*NP denotes no preference

Analysis of control *versus* test feed intake within age groups found no significant intake-indicated control preference in the Young group ($F_{(1,142)}$ = 1.67, p=0.199) or Middle group ($F_{(1,142)}$ = 0.25, p=0.621), with only a significant difference in feed preference being recorded from behaviour duration in the Old group ($F_{(1,142)}$ = 3.98, p<0.05). This apparent lack of a feed preference was due to variation in the direction of preference found between individuals, with three horses having an intake-indicated control preference and three horses having an intake-indicated test feed preference (see Table 5.9).

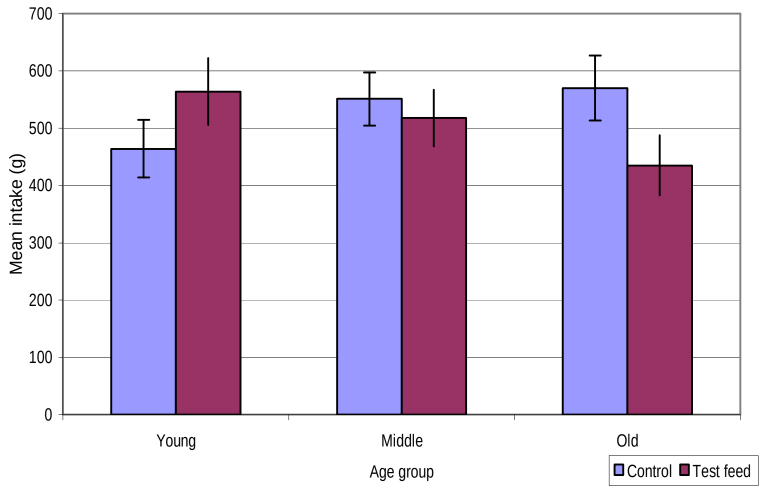
As discussed in chapter 4, this variation in the direction of individual preference resulted in the mean control versus test feed intake for each age group being distorted towards the 0.5 mean (see Table 5.11). In order to overcome this, from hereon in, all results will be discussed in terms of preferred food versus non-preferred food, rather than control versus test feed. In order to study the extent of preference and hence the effect of age on gustatory response rather than the direction of preference, individual control or test feed preference was used to determine the direction of preference. For horses not exhibiting a significant feed preference, preference was calculated as the proportion of intake around 0.5, when calculated from the mean total daily consumed. A proportion of control intake of more than 0.5 supported a greater intake of control feed and a proportion of intake of less than 0.5 supported a greater intake of test feed (see Table 5.7).

Table 5.7 Differences in intake in individuals between the control and test feed and the preferred and non preferred feed (mean ± s.e.m. (g), n=12 days).

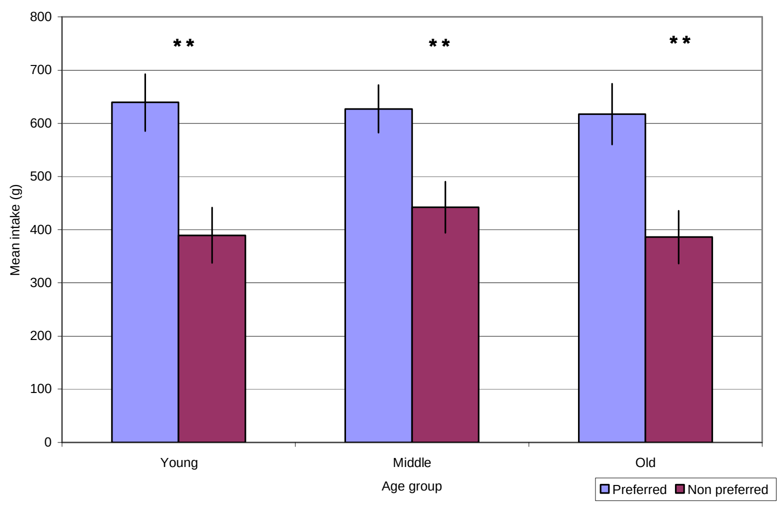
	Intake					
	Control	**Test feed**	**Preferred**	**Non preferred**		
Horse	**Mean ± s.e.m. (g)**	**Mean ± s.e.m. (g)**	**Mean ± s.e.m. (g)**	**Mean ± s.e.m. (g)**	**$F_{(1,22)}$ value**	***p* value**
1	522 ± 170.0	950 ± 161.0	950 ± 161.0	522 ± 170.0	9.2	<0.01
2	567 ± 133.0	473 ± 131.0	567 ± 133.0	473 ± 131.0	0.25	0.622
3	382 ± 127.0	527 ± 123.0	527 ± 123.0	382 ± 127.0	0.67	0.42
4	382 ± 130.0	703 ± 116.0	703 ± 116.0	382 ± 130.0	3.39	0.079
5	569 ± 138.0	547 ± 145.0	569 ± 138.0	547 ± 145.0	0.01	0.916
6	450 ± 83.1	106 ± 67.6	450 ± 83.1	106 ± 67.6	9.52	<0.01
7	808 ± 143.0	738 ± 150.0	808 ± 143.0	738 ± 150.0	0.11	0.74
8	364 ± 52.9	599 ± 65.7	599 ± 65.7	364 ± 52.9	7.73	<0.05
9	386 ± 105.0	607 ± 115.0	607 ± 115.0	386 ± 105.0	0.11	0.74
10	640 ± 97.7	324 ± 97.4	640 ± 97.7	324 ± 97.4	5.27	<0.05
11	577 ± 116.0	480 ± 132.0	577 ± 116.0	480 ± 132.0	0.31	0.585
12	532 ± 116.0	360 ± 126.0	532 ± 116.0	360 ± 126.0	1	0.327
13	445 ± 135.0	1026 ± 136.0	445 ± 135.0	1026 ± 136.0	9.2	<0.01
14	870 ± 153.0	405 ± 136.0	870 ± 153.0	405 ± 136.0	5.12	<0.05
15	425 ± 93.0	258 ± 96.7	425 ± 93.0	258 ± 96.7	1.56	0.224
16	450 ± 134.0	489 ± 138.0	489 ± 138.0	450 ± 134.0	0.04	0.842
17	272 ± 78.8	194 ± 64.6	272 ± 78.8	194 ± 64.6	0.58	0.456
18	530 ± 162.0	768 ± 160.0	768 ± 160.0	530 ± 162.0	1.1	0.306

***NP denotes no preference**

When preferred intake *versus* non-preferred intake was analysed a significant preference was recorded in all three age groups (see Figure 5.2). The duration of behaviour towards the preferred container also recorded a significant preference across the three age groups, with the strongest preference being recorded in the Young group (see Figure 5.3 and Table 5.8).



a) Mean daily control and test feed intake for each age group during two-choice preference testing.

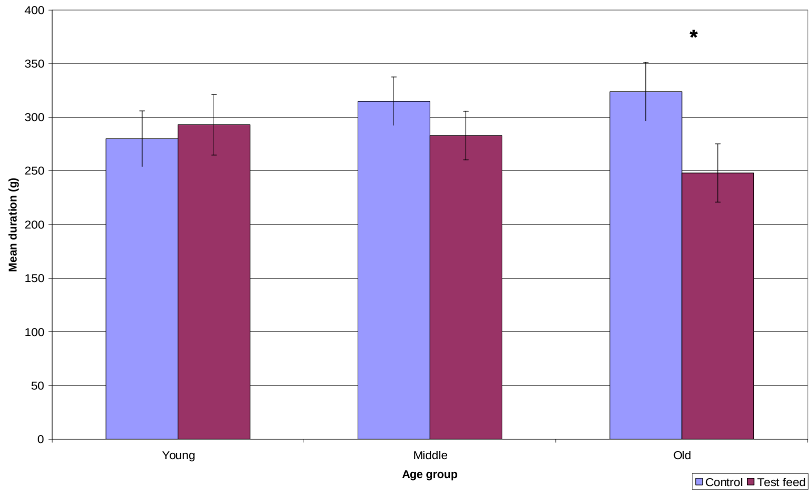


b) Mean daily preferred and non preferred feed intake for each age group during two-choice preference testing.

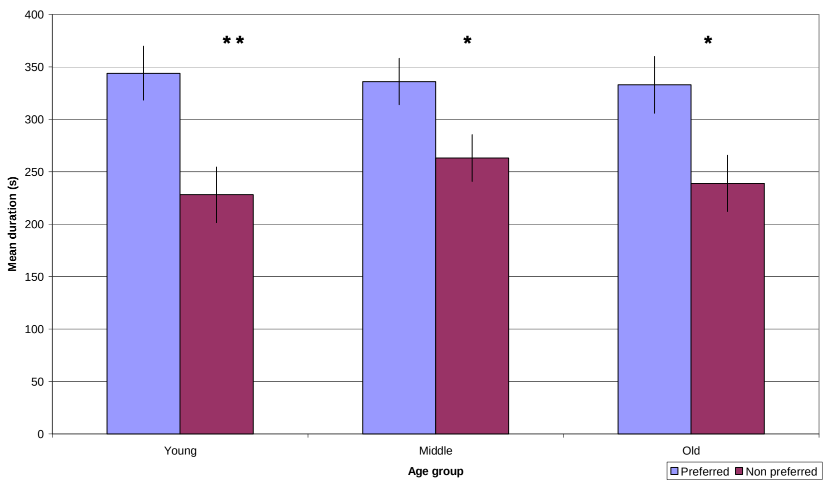
Figure 5.2 The effect of individual variation on the extent of feed preference when comparing control *versus* test feed intake and preferred *versus* non preferred feed intake, indicated by a stronger response to the test feed when preferred *versus* non preferred was analysed.
***denotes $p<0.05$, ** denotes $p<0.01$ and *** denotes $p<0.001$**

Table 5.8 Effect of age on mean control *versus* test feed intake and behaviour compared to preferred *versus* non preferred intake and behaviour (mean ± s.e.m. (g), n=12 days).

		Control	Test feed			Preferred
Age group		**Mean ± s.e.m. (g)**	**Mean ± s.e.m. (g)**	**$F_{(1,142)}$ value**	***p* value**	**Mean ± s.e.m. (g)**
Young	**Intake (g)**	**464 ± 50.4**	**564 ± 58.2**	**1.67**	**0.199**	**639 ± 53.4**
	Behaviour (s)	**280 ± 25.9**	**293 ± 28.1**	**0.11**	**0.735**	**344 ± 25.7**
Middle	**Intake (g)**	**551 ± 46.4**	**518 ± 49.4**	**0.025**	**0.621**	**627 ± 45.1**
	Behaviour (s)	**315 ± 22.5**	**283 ± 22.6**	**1.04**	**0.31**	**336 ± 22.1**
Old	**Intake (g)**	**570 ± 56.6**	**435 ± 52.4**	**3.09**	**0.081**	**617 ± 57.0**
	Behaviour (s)	**324 ± 27.3**	**248 ± 27.1**	**3.98**	**<0.05**	**333 ± 27.1**



a) Mean daily duration of behaviour towards the control and test feed for each age group during two-choice preference testing.



b) Mean daily duration of behaviour towards the preferred and non preferred feed for each age group during two-choice preference testing.

Figure 5.3 The effect of individual variation on the extent of feed preference when comparing control *versus* test duration of behaviour and preferred *versus* non preferred duration of behaviour, indicated by a stronger response to the test feed when preferred *versus* non preferred was analysed.
***denotes p<0.05, ** denotes p<0.01 and *** denotes p<0.001**

Analysis of the effect of inclusion level on preferred *versus* non preferred gustatory response, calculated from the proportion of preferred feed consumed from total eaten, found no significant effect on intake across all age groups (Young $F_{(2,69)} = 1.71$, $p=0.189$; Middle $F_{(2,69)} = 0.23$, $p=0.796$; Old $F_{(2,69)} = 0.08$, $p=0.927$). There was no significant main effect of flavour inclusion level on preferred *versus* non preferred intake, determined from gustatory response, across all age groups ($F_{(2,207)} = 0.11$, $p=0.898$). No significant effect of age on preferred *versus* non preferred gustatory response was found ($F_{(2,207)} = 0.32$, $p=0.728$) with no significant interaction between the factor of inclusion level and the factor of age ($F_{(4,207)} = 0.54$, $p=0.708$).

As shown in Table 5.9 and Figure 5.4, the Young and Middle group showed a significant difference between the preferred and non preferred feed at the highest inclusion level, $20 g.kg^{-1}$(Young $F_{(1,46)} = 6.64$, $p<0.05$; Middle $F_{(1,46)} = 6.76$, $p<0.05$). The Old group exhibited a significant difference between the preferred and non preferred feed at $5 g.kg^{-1}$, the lowest inclusion level, ($F_{(1,46)} = 5.88$, $p<0.05$) and the mid inclusion level ($F_{(1,46)} = 4.71$, $p<0.05$).

Table 5.9 Differences in intake and behaviour for preferred and non preferred feed (mean ± s.e.m. (g/s), n=12 days)

		Preferred	Non preferred			Preferred	Non pr
Age group	Inclusion level (g.kg^{-1})	Mean intake ± s.e.m. (g)	Mean intake ± s.e.m. (g)	$F_{(1,46)}$ value	*p* value	Mean duration ± s.e.m. (g)	Mean d ± s.e.m
Young	2.5	594 ± 88.9	429 ± 93.0	1.64	0.206	452 ± 85.7	88 ± 29
	5	641 ± 93.0	400 ± 90.5	3.45	0.07	477 ± 89.4	90 ± 32
	10	681 ± 98.6	338 ± 89.5	6.64	<0.05	464 ± 90.3	33 ± 24
Middle	2.5	623 ± 74.9	848 ± 84.0	1.53	0.222	563 ± 63.3	237 ± 3
	5	607 ± 89.8	473 ± 86.8	1.16	0.288	701 ± 101.1	206 ± 3
	10	651 ± 71.6	370 ± 81.2	6.76	<0.05	601 ± ± 93.7	205 ± 3
Old	2.5	663 ± 98.6	347 ± 85.1	5.88	<0.05	478 ± 93.7	201 ± 5
	5	642 ± 105.0	352 ± 85.1	4.71	<0.05	390 ± 85.8	195 ± 4
	10	545 ± 94.3	466 ± 92.5	0.36	0.553	476 ± 86.6	98 ± 34

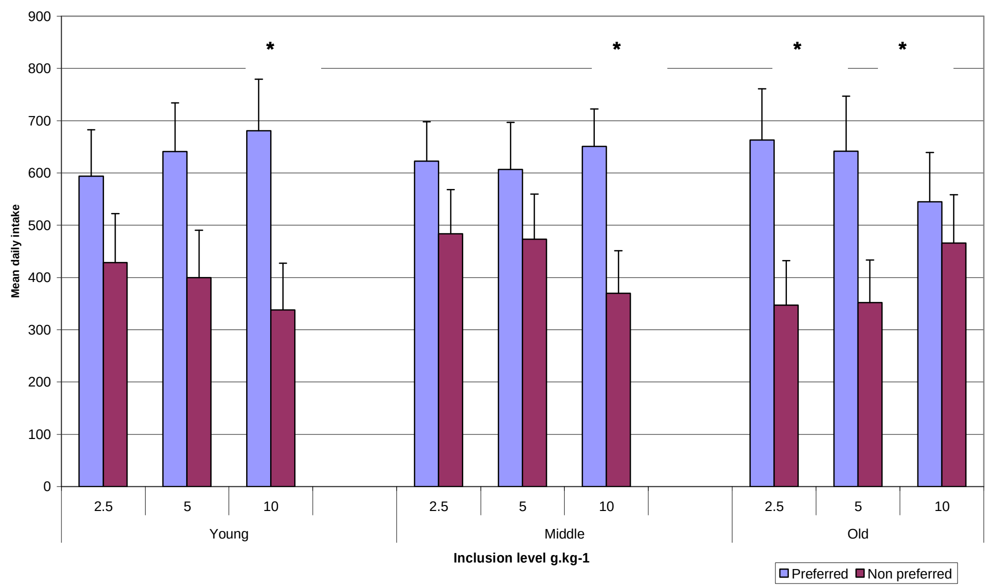


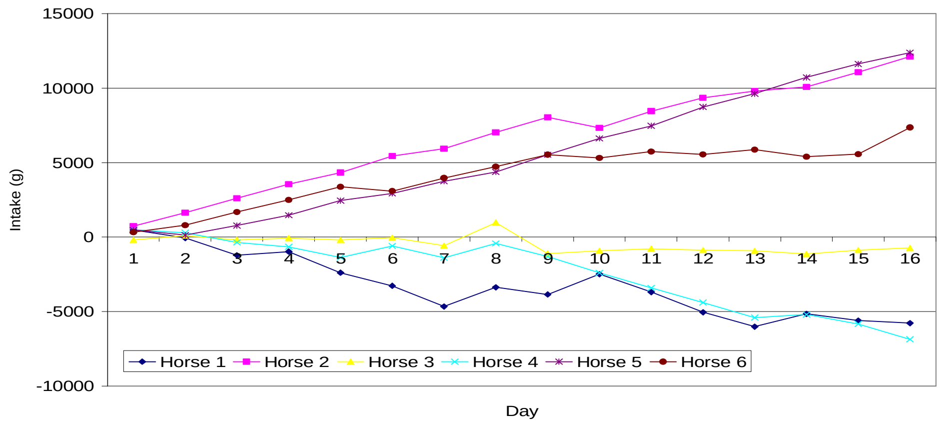
Figure 5.4 The effect of inclusion level on preferred *versus* non preferred feed intake and age.
***denotes p<0.05, **denotes p<0.01 and *** denotes p<0.001**

A significant difference between intake from the left container and right container during two-choice preference testing was recorded in 12 horses (Young n=4 horses; Middle n=5 horses; Old n=3 horses), with an equal distribution of left or right preference (n=6 horses) (see Table 5.10). Consistency in left or right intake between days was evident in individuals (see Figure 5.5). Intake-indicated side preference was supported by the duration of attention towards the left or right container in 11 horses. Horse 10 was found to have an intake-indicated left preference ($F_{(1,22)}$ = 8.17, $p<0.01$) which was not supported by a significant difference in the duration of attention between the left and right container ($F_{(1,22)}$ = 1.72, $p=0.203$). A significant behaviour-indicated right preference ($F_{(1,22)}$ = 11.82, $p<0.01$) was recorded in Horse 13 was not supported by an intake-indicated side preference ($F_{(1,22)}$ = 3.57, $p=0.072$).

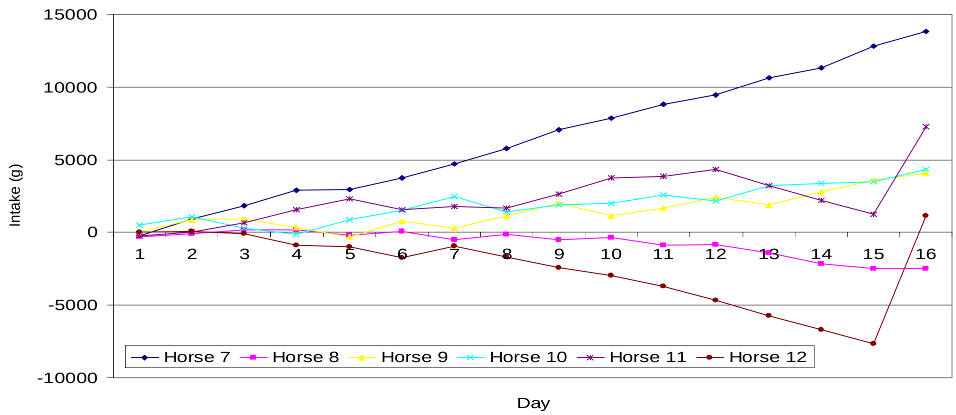
Table 5.10 Individual horses exhibiting a significant difference in left or right intake / behaviour duration during two-choice preference testing (mean ± s.e.m. (g/s), n=12 days).

	Intake					Behaviour	
	Left	Right				Left	Right
Horse	Mean ± s.e.m. (g)	Mean ± s.e.m. (g)	$F_{(1,22)}$ value	*p* value	Preference*	Mean ± s.e.m. (s)	Mean ± s.e.m. (s)
1	489 ± 163.0	983 ± 159.0	3.57	0.072	NP	216 ± 67.9	349 ± 67.9
2	845 ± 78.4	148 ± 71.1	42.05	<0.001	Left	495 ± 42.9	104 ± 43.1
3	157 ± 85.7	753 ± 93.3	22.18	<0.001	Right	97 ± 30.1	483 ± 32.0
4	284 ± 98.7	801 ± 115.0	11.66	<0.01	Right	169 ± 46.3	431 ± 46.2
5	1013 ± 39.3	103 ± 31.8	323.07	<0.001	Left	523 ± 14.8	67 ± 13.3
6	395 ± 102.0	160 ± 62.7	4.15	0.054	NP	305 ± 67.4	159 ± 57.5
7	1226 ± 50.7	317 ± 52.3	152.04	<0.001	Left	460 ± 19.3	135 ± 18.5
8	265 ± 55.3	635 ± 58.5	6.86	<0.05	Right	174 ± 29.1	426 ± 29.1
9	651 ± 105.0	341 ± 106.0	4.34	<0.05	Left	397 ± 54.7	204 ± 54.7
10	669 ± 88.3	295 ± 97.0	8.17	<0.01	Left	336 ± 41.4	259 ± 42.1
11	469 ± 128.0	588 ± 119.0	0.504	0.504	NP	254 ± 61.5	346 ± 61.8
12	127 ± 65.6	765 ± 89.2	33.13	<0.001	Right	116 ± 43.8	485 ± 43.8
13	428 ± 102.0	907 ± 99.7	3.57	0.072	NP	193 ± 44.0	407 ± 44.0
14	376 ± 144.0	852 ± 140.0	42.05	<0.001	Right	189 ± 67.5	411 ± 67.5
15	567 ± 75.3	116 ± 65.8	20.35	<0.001	Left	485 ± 53.9	116 ± 53.9
16	916 ± 27.1	23 ± 14.4	844.37	<0.001	Left	570 ± 11.3	31 ± 11.3
17	185 ± 62.4	281 ± 79.7	0.89	0.356	NP	175 ± 38.9	254 ± 48.4
18	780 ± 173.0	519 ± 146.0	1.34	0.26	NP	340 ± 72.3	261 ± 72.3

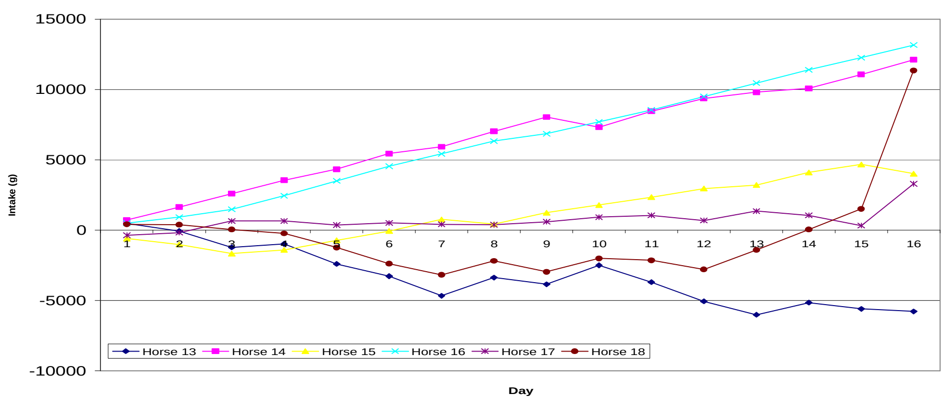
***NP denotes no preference**



a) Young



b) Middle



c) Old

Figure 5.5 Cumulative differences between left and right intake during training (d1-4) and two-choice preference testing (d5-16) in the (a) Young, (b) Middle and (c) Old group.
- values denotes right intake, +values denote left intake

5.6.3 Determination of gustatory response by monadic testing

Alteration in the four inclusion levels failed to elicit a significant change in intake across the three age groups (Young $F_{(3,44)}$ = 0.08, p=0.971; Middle $F_{(3,44)}$ = 0.06, p=0.982; Old $F_{(3,44)}$ = 0.84, p=0.478). Further analysis of the main effect of variation in inclusion levels found no effect on monadic intake ($F_{(3,132)}$ = 0.53, p=0.665). The main effect of age on monadic intake was found to have no significant effect ($F_{(2,132)}$ = 0.90, p=0.411) and no significant interaction between inclusion level and age on intake was recorded ($F_{(6,132)}$ = 0.36, p=0.904).

5.7 Discussion

This present study aimed to investigate gustatory response in the horse, in the absence of additional feed-based olfactory cues. Gustation is intrinsically linked with olfaction and hence, difficulties arise when studying these senses in isolation of each other. This study employed a novel approach to establish gustatory responses in the horse to investigate if, and to what extent, gustatory function is affected by age. In line with previous findings in humans (Chauhan *et al.,* 1987; Murphy and Gilmore, 1989; Stevens and Cain, 1993; Finkelstein and Schiffman, 1999 a; Kaneda *et al*., 2000; Mojet *et al*., 2001; Mojet *et al*., 2005), a decline in gustatory function was indicated with increased age in the horse.

5.7.1 Training

Side preference was recorded from differences between left and right intake in five horses during the four-day training period. Differences between the duration of behaviour directed towards the left and right container identified a significant side preference in eight individuals. These results indicate that whilst attention was being directed towards the least preferred container, ingestion from it was not occurring. The results for the training period need to be interpreted with caution due to limited collection of data, the finding of side preference indicates consistency in intermeal container selection based on positioning cues in individuals.

In contrast to the earlier study of the effect of age on taste response (see Chapter 4), no effect of age on total daily intake was recorded. This may reflect the allocation of subjects previously studied during Chapter 4 and therefore, despite a four month period being left between allocation of horses to trials, familiarity with the feeding environment and basal feed may have reduced the effects of novelty. The older profile of the Young group allocated to Chapter 5 (Chp 4 mean age = 3.5 $\pm$ 0.42; Chp 5 mean age = 4.7 $\pm$ 0.33) meant the horses were undergoing a more advanced stage of training and therefore were more familiar with the stabling environment and presentation of concentrate feed (pers. Comm. Yard Manager, Anna Gregory).

It is proposed that the Young subjects allocated to the present study showed less wariness towards the novel basal feed and presentation method as a result of prior experience. This resulted in mean total daily intake more consistent with those of the Middle and Old groups. Additionally, no significant difference between ingestive behaviour, determined from the duration of 'eat' behaviour, was recorded between age groups. This indicates that the Young group exhibited more consistent behaviour, in line with the Middle and Old group.

5.7.2 Two-choice preference testing

There is an accepted consensus of an age associated development and subsequent decline in taste response in humans. Furthermore, study of gustatory response in isolation of olfactory cues has suggested increased magnitude of loss in the olfactory system over the gustatory system (Rolls, 1999). Whilst studies in humans recognise the difficulties associated with studying gustatory function due to the intrinsic relationship with olfaction, further study is warranted. Whilst no such study of gustatory function has been conducted in the horse, the vital role that gustation plays in diet selection merits further study.

The increased longevity of the domestic horse (Harris, 1999; Hintz, 1999 a; King, 2000), a consequence of improved welfare and its role as a leisure animal, increases the need for the study of any associated decline in gustatory function. As discussed in Section 1.1.1, a decline in taste response has implications for the ability of the horse to make appropriate diet selection and hence has consequences on welfare. This present study aimed to identify if, and to what extent, gustatory function was affected by age in the horse, by investigation of

taste response in isolation from additional feed-based olfactory cues, from hereon in termed the gustatory response.

The study was conducted with 18 horses, allocated to three age groups (n=6 horses), Young, Middle and Old. Gustatory responses in the horse were identified from changes in behaviour and intake in response to the presentation of gustatory cues, in isolation from additional feed-based olfactory cues. Whilst it was recognised that the subjects would still be subject to olfactory cues from the external environment, the test feed aimed to present no additional olfactory cues to the horse on presentation. Two-choice preference testing and monadic presentation were implemented to identify individual gustatory responses for the purpose of age comparison.

Variation in intake-indicated preference between individuals was recorded; with three horses exhibiting a control preference and three horses exhibiting a test feed preference. In contrast to the negative response to the orange test feed presented during the study of the effect of age on taste (Chapter 4), a test feed preference was recorded towards the glycine in three subjects. Glycine has been found to evoke a sweet taste in mammalian species (Glaser *et al*., 2000; Glaser, 2002) and therefore, if detected as sweet by the horse, a positive response would be anticipated, based on the findings of a sucrose preference in the horse (Randall *et al*., 1978; Hawkes *et al*., 1985). A preference for sweet tastes is suggested to be linked to the reinforcement of postingestive consequences following a positive nutritive change (Cabanac, 1971; Drewnowski, 2000).

In order to investigate any effects of age on gustatory response it was necessary to compare the extent of preference, rather than direction of preference. A significant difference between the preferred and the non preferred feed was found across all age groups, supported by both intake and behaviour data. This

indicates that horses were motivated to alter their behaviour in order to select for, or select away from, the test feed. Consistent with the findings of the previous study (Chapter 4), gustatory response was influenced by not only gustatory cues, but also by positioning cues in susceptible individuals.

From analysis of the effect of age on preferred *versus* non preferred feed intake, no significant effect was recorded. Inclusion rate was found to have no significant effect on intake when analysed across the groups, with no significant effect on intake in individuals. Comparison of the response to inclusion levels within age groups found a high significant response to the test feed at the low and mid inclusion level in the Old group, and at the high inclusion level in the Young and Middle group. If an effect of age on inclusion level was found, it would be expected that the Old group would record a stronger response to the test feed at the high inclusion level, demonstrating less discriminatory ability at the lower inclusion levels.

The presentation of a test feed with no additional olfactory cue would have resulted in an increased need for horses to sample, and potentially ingest, from both feeds before identification of the test feed would be possible. This may have contributed to the less significant preference recorded in individuals in this study, in comparison to Chapter 4 where discrimination was possible on olfactory cues alone. This finding is contradictory to a study of changes in discriminatory abilities between asnomic (through medical intervention) and intact dogs through two-choice preference testing (Houpt *et al.*, 1978). Asnomic animals are suggested to be comparable to animals showing a dramatic decline in gustatory function associated with late senescence. Asnomic dogs demonstrated a weaker discriminatory ability, with intake falling nearer the no preference zone. This finding is contradictory to the discriminatory ability identified in older subjects at the high inclusion level.

The lack of additional feed-related olfactory cues would have prevented discrimination between the test feed and control by olfaction alone. Whilst the Old group were suggested to be more reliant on gustatory cues to identify the test feed in the absence of additional feed-related olfactory cues, a mean gustatory response nearer 0.5 may in fact indicate a reduced ability to detect gustatory cues. Studies in humans have identified increased taste thresholds to glycine in human subjects with advancing age (Schiffman *et al*., 1979 a; Schiffman and Clark, 1980) and hence, a similar effect may be applicable to horses.

Although a development in gustatory function immediately after the postnatal period is recognised in humans (Corso, 1981), this study supported no such findings. The horses used in this study were suggested to possess mature gustatory systems. Additionally, the inclusion levels used in this study and the mode of presentation with food as the carrier, reduced the sensitivity of the study beyond the scope to measure gustatory thresholds.

Variation in not only the extent of preference, but also the direction of preference was recorded. Three horses exhibited a control preference, with three horses exhibiting a test feed preference. As glycine is suggested to evoke a sweet response in horses, a preference would be expected (Randall *et al.*, 1978). Moreover, whilst a sweet preference is predicted, intraspecies differences in sweet preference have been recorded (Randall *et al.*, 1978). However, acceptance of the test feed may have been affected by both the novelty of the flavour and prior conditioning to the control during the training period, resulting in a control preference. The Middle group showed the most inconsistency in gustatory response, with an increased response at the middle inclusion rate. This may indicate variation in the extent of preference, as recorded previously in the horse (Randall *et al.*, 1978; Pollack and Burton, 1981).

An apparent lack of discriminatory ability was recorded in twelve horses. This may be the result of indifference towards the test feed as a result of side preference, or an inability to detect glycine as a result of taste blindness. An additional hypothesis is that due to the lack of consequence of glycine ingestion, there was no reason to alter feeding response. This may have been more pronounced in the Old subjects who are suggested to exhibit monotony in the feeding environment, and hence, exhibit limited behaviour change in response to novelty in the environment or when no negative or positive benefit from a change in behaviour arises.

As in Chapter 4, the horses allocated to the Old group were not in the late stages of senescence and therefore a dramatic decline in gustatory function would not be anticipated. The lack of consequence of not selecting for the control or test feed, with the exception of hedyphagic benefits, may have resulted in insufficient motivation to alter behaviour and hence, resulted in a strong side preference across all age groups.

Whilst glycine threshold levels in humans have been found to increase with age in human subjects (Schiffman *et al*., 1979 a; Schiffman and Clark, 1980), no such finding was supported in this study. This is proposed to be due to the allocation of horses not in the late stages of senescence, when a decline in sensitivity would be expected from comparison with other mammalian models. The lack of consequences following ingestion would have resulted in insufficient motivation to select for, or against, the test feed as the cost of a change in container selection would not convey any additional benefit.

5.7.3 Monadic testing

No effect of inclusion level on intake was recorded in individuals or across age groups when glycine was presented monadically. This indicates that any earlier rejection of the test feed was not sufficient to prevent intake, when only one feeding choice was presented.

5.8 Conclusions

No effect of advancing age on gustatory response was recorded in the horses. The lack of additional feed-related olfactory cues in the present study increased the incidence of exploratory behaviour in Young subjects, suggested to be an attempt to discriminate between the test feed and control based on olfactory cues, rather than gustatory cues, resulting in less preference recorded towards the control in this study, compared to the findings of Chapter 4. The Old subjects appeared more reliant on gustatory cues to discriminate between the test feed and control, resulting in lower gustatory responses as a result of increased ingestion of both feeding choices. However, lower gustatory responses in the Old group may result from a lack of gustatory ability resulting in a reduced sensitivity towards the test feed, and hence, gustatory response falling nearer to the no preference zone, though this appears unlikely due to the limitations on reliability imposed by the study design.

Old horses may make appropriate decisions over food selection through gustatory cues; however, increased sampling behaviour increases the risk of ingestion of inappropriate dietary sources, potentially putting the horse at risk of exposure to toxins. Additionally, a reduced ability to identify gustatory cues appropriately has implications for the ability of old subjects to respond to, and form appropriate associations between, the gustatory and olfactory characteristics of a food and postingestive consequences.

Chapter 6:

The effect of age on feeding behaviour in the horse

Changes in diet selection with life stage are accepted in all animals and these are suggested to be attributable to changes in gustatory and olfactory function in humans (Drewnowski, 2000). Diet selection and feeding behaviour are subject to influence from more than just gustatory and olfactory responses. Earlier studies failed to identify an effect of age on taste or gustatory function in the horse, though this is suggested to be due to limitations imposed by the methodology in these studies (see Chapter 4 and Chapter 5). This chapter aims to investigate further the effect of age on feeding behaviour in the horse, with particular emphasis on the exhibition of side preference.

Two-choice preference testing was used to determine the extent of preference or aversion to test feed in order to establish taste and gustatory response. This method is reliant on the selection of equal quantities of food from both containers when no choice is presented. The selection of food based on positional cues was found to have implications on the validity of two-choice preference testing during earlier studies (see Chapter 3, Chapter 4 and Chapter 5).

6.1 Study aims

The aims of this study were three-fold: firstly, to investigate the effect of age on feeding behaviour in the horse; secondly, to investigate if, and to what extent, the exhibition of feeding side preference was affected by age in the horse and thirdly, to consider the implications of feeding side preference for the validity of two-choice preference testing in the horse.

6.2 Materials and methods

The general materials and methods are detailed in Chapter 2. Where appropriate, additional information is provided in Sections 4.2 and Sections 5.2.

To meet the above aims, data generated during Chapter 4, the study of the effect of age on taste response in the horse, was analysed to further study the effect of age on feeding behaviour in the horse. This study comprised of three elements; an initial 9 day training period, a subsequent 12 day two-choice preference testing period, concluding with an eight day period of monadic testing.

6.2.1 Behaviour analysis

Behaviour analysis was conducted using The Observer programme (Noldus, Netherlands). Lag sequential analysis was conducted, in addition to the analysis outlined in section 2.4, in order to research the temporal structure of the sequence of behaviour exhibited during the study. This resulted in the production of transition matrices for the probability of behavioural transitions between two behaviours, namely a criterion event and a target event.

The transition probability, the probability that the criterion event (A) is followed by the target event (B) was calculated as;

P(A->B) = (number of A->B transitions)/(number of A occurrences)

The transition probability was calculated as either the probability of behavioural transitions within each behavioural class, or across behavioural classes.

To record the probability of behavioural transitions within each behavioural class, behaviours were clustered into the three behavioural classes of; area, ingest and locomotory. This enabled the transition probability for behaviours within each behavioural class to be calculated.

During training and two-choice preference testing, the 'area' cluster represented behavioural transitions between behaviours expressed towards the left container (le), right container (ri), the floor (fl) and 'else' area (el), which represented attention away from the feeding area. During two-choice preference testing, the additional transitions between the control (co) and test feed (te) (in addition to left and right transitions), as well as the floor and else areas were recorded. Due to the replacement of the left and right container with a single, monadic container (mo), analysis of behavioural transitions during the monadic period recorded behavioural transitions between the monadic container, the floor and the else area.

A state lag with state order +1 was used to generate transition matrices for each individual 10 minute period. From this, mean probability transitions for individuals and age groups were generated. The state order +1 recorded the probability that the target behaviour would follow immediately after the criterion behaviour, within each behavioural cluster.

From the example transition matrix presented in Table 6.1, a greater probability of the criterion behaviour (A), left, being followed by the target behaviour (B), right, (le->ri = 0.86) was recorded than for the probability of transition from the criterion behaviour, right, to the target behaviour, left, (ri->le = 0.50). Table 6.1 demonstrates that there is an equal probability that following attention directed away from the feeding area (else), the horse returns equally to the left (el->le = 0.50) and right (el->ri = 0.50) container. Additionally other behaviour was always followed by eat behaviour (ot->ea = 1.00) and walk behaviour was always followed by stand (wa->st = 1.00). The Y0 column represents the probability that the criterion behaviour will not be followed by target behaviour; as no target behaviour was recorded prior to the session period ending. Each blank cell within the clustered area represents when no behavioural transition

from the criterion event to the target event has occurred. From the transition matrix in Table 6.1, there was a 0.33% probability that eat behaviour would not be followed by any target behaviours within the ingest cluster (ea->Y0 = 0.33).

Table 6.1 An example state lag +1 transition matrix for Horse 1, training - day 1, showing the probability of behavioural transitions between the criterion event (A) and the target event (B), calculated for the behavioural transitions within behaviour class combinations, clustered into area, ingest and locomotory behaviours.

Criterion event (A) ↓ …followed by target event (B) →

	le	ri	fl	el	ea	al	ot	st	wa	Y0
le		0.86		0.14						
ri	0.50			0.38						0.13
fl										
el	0.50	0.50								
ea							0.67			0.33
al										
ot					1.00					
st									0.83	0.17
wa								1.00		
X0	0.25						0.25	0.25		

Le – left, ri – right, fl – floor, el – else, al – alert, ot – other, st – stand, wa – walk

In order to study whether there was a difference between the temporal structure of events between the first half of each presentation period (0-5 minutes) and the second half of each presentation period (5-10 minutes), time lag was conducted from 0-5 minutes and 5-10 minutes. This enabled the study of the onset, if any, of exclusivity of choice during the 10 minutes period and changes in response to the initial presentation of novel sensory cues.

Time lag recorded the transition probability of a target behaviour occurring at any point during the specified interval (0-5minutes and 5-10 minutes), rather than recording the probability of a target event immediately following the criterion behaviour, as recorded using state +1. Consistent with the state +1

recording, behaviours were clustered into area, ingest and locomotory behaviours (Table 6.2).

Table 6.2 An example transition matrix (Horse 1, training - day 1, 0-5 minutes) showing the probability of all transitions between the criterion event (A) and the target event (B), calculated for the behavioural transitions within behaviour class combinations, clustered into area, ingest and locomotory behaviours.

Criterion event (A) ...followed by target event (B) →

	le	ri	fl	el	Ea	al	ot	st	wa	Y0
le	0.23	0.37	0.05	0.16						0.06
ri	0.31	0.24	0.05	0.16						0.22
fl	0.05	0.05	0.04	0.08						
el	0.18	0.20	0.05	0.13						0.01
ea					0.19	0.04	0.29			0.48
al					0.13	0.01	0.08			
ot					0.32	0.03	0.20			0.02
st								0.21	0.22	0.52
wa								0.34	0.17	0.03
X0	0.12	0.09		0.05	0.12		0.09	0.20	0.02	

Le – left, ri – right, fl – floor, el – else, al – alert, ot – other, st – stand, wa – walk

In contrast to the transition matrix presented in Table 6.1, it can be seen that transitions between identical criterion and target behaviours was recorded, for example left to left (le->le = 0.23) and stand to stand (st->st = 0.21). This is due to the time lag recording the probability of any behaviour within the cluster following the criterion event at any point rather than recording the next behaviour. For state +1, it would not be possible for eat behaviour to follow eat behaviour, whilst during time lag, it is highly probable that left behaviour would be followed by left behaviour at some point during the five minute time interval.

6.3 Results

6.3.1 Training

From analysis of the mean probability of behavioural transitions within behavioural classes for the Young, Middle and Old group (Table 6.3), greater fragmentation of behaviour was recorded in the Young and Middle group, compared to the Old group. The reduced number of behavioural transitions from the criterion behaviours to the target behaviours in the Old group is apparent from the higher number of blank cells recorded in the Old group, compared to the Young and Middle group. The blank cell within the behaviour cluster indicates that there is zero probability of a target behaviour following the respective criterion behaviour. The lack of behavioural transitions within clusters in the Old group was most pronounced in the ingest cluster and locomotory cluster, as discussed later.

A high probability of behaviour transitions between the feed presentation area and non feeding area (else and floor) was recorded from the mean of the Young group. This was indicated by the probability of transition from the left container to the floor (le->fl = 0.25) and else area (le->el = 0.37) and from the probability of behavioural transitions occurring from ingestive behaviour to non ingestive behaviour (ea->ot = 0.61). Additionally, inconsistency in locomotory behaviour was recorded in the Young group, with a high probability that walk would be followed by stand (wa->st = 0.79) and stand would be followed by walk (st->wa = 0.63). The Middle group exhibited a greater transition probability towards the feed containers (le->ri = 0.84; ri->le = 0.79) than away from the feeding area (le->el = 0.02; ri->el = 0.02). A similar finding was recorded in the Old group, with a greater probability of behavioural transition occurring between the left and right containers (le->ri = 0.64; ri->le = 0.74), rather than from or to the non feeding area (le->el = 0.01; ri->el = 0.00).

Table 6.3 Transition matrices for the probability of behaviour transition during the training period for the Young (a), Middle (b) and Old group (c), where the probability of behavioural transition was calculated from the mean total of a criterion event being succeeded by a target event. †

a) Young age group

	le	ri	fl	el	ea	al	ot	st	wa	Y0
Le		0.61	0.25	0.37						0.06
Ri	0.61		0.09	0.22						0.24
Fl	0.10	0.06		0.31						0.05
El	0.43	0.30	0.13							0.13
Ea						0.09	0.61			0.64
Al					0.19		0.22			
Ot					0.6	0.04				0.15
St									0.63	0.46
Wa								0.79		0.07
X0	0.15	0.11		0.08	0.12		0.12	0.22	0.02	

b) Middle age group

	le	ri	fl	el	ea	al	ot	st	wa	Y0
Le		0.84	0.01	0.02						0.13
Ri	0.79		0.02	0.02						0.13
Fl	0.06	0.02		0.04						0.06
El	0.10	0.14	0.04							0.03
Ea						0.02	0.18			0.67
Al					0.02		0.02			
Ot					0.48					0.06
St									0.16	0.84
Wa								0.20		0.02
X0	0.17	0.06		0.04	0.12		0.11	0.23		

c) Old age group

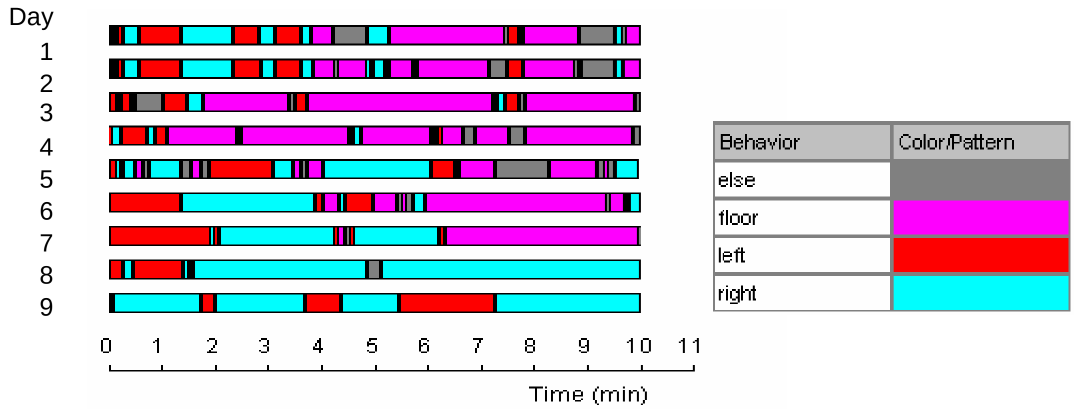
	le	ri	fl	el	ea	al	ot	st	wa	Y0
Le		0.64		0.01						0.33
Ri	0.74									0.13
Fl		0.04								
El	0.15	0.04								
Ea							0.01			0.99
Al					0.04					
Ot					0.17					
St										1.00
Wa										
X0	0.14	0.09		0.03	0.19	0.01	0.04	0.23		

† le – left, ri-right, fl-floor, el-else, ea-eat, al-alert, ot-other, st-stand, wa-walk

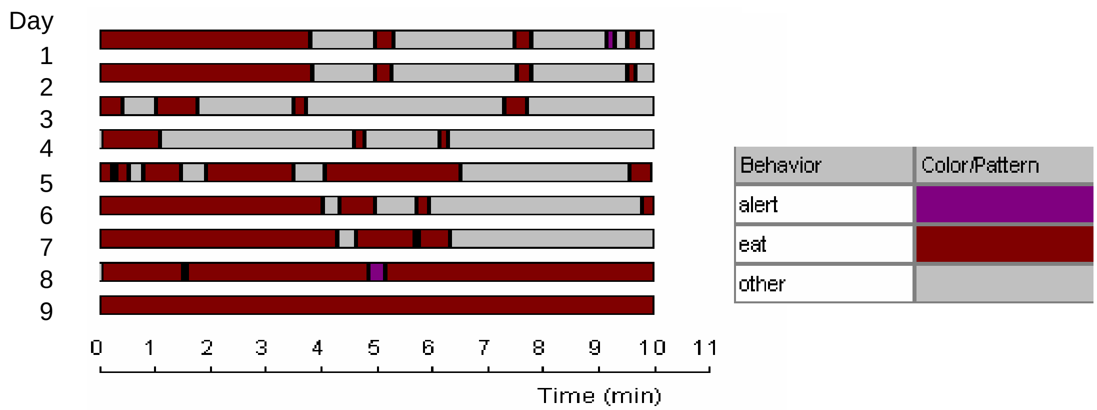
The greater fragmentation in behaviour in the Young group supports the finding of lower total daily intake in the Young group during the training period, when compared to the Middle group ($F_{(1,107)}$ = 37.58, p<0.001) and the Old group ($F_{(1,107)}$ = 56.88, p<0.001). The high probability of behavioural transitions between the feeding area and ingestive and non ingestive behaviour supports exhibition of greater exploratory behaviour and lower ingestive behaviour in the Young group.

In particular, Horse 2 (young) demonstrated fragmentation in behaviour, with a great probability of transfer from eat to other behaviour (ea->ot = 1.00), alert to other (al->ot = 0.11) and other to eat (ot->ea = 0.81). This finding in Horse 2 was also supported by the time event plot for this horse during the training period, as shown in Figure 6.1. This time event plot supports the replacement of non ingestive behaviour with ingestive behaviour with repeated presentation of the control feed (see Figure 6.1). On day 9, prior to the commencement of two-choice preference testing, Horse 2 exhibited ingestive behaviour for the entire 10-minute presentation period.

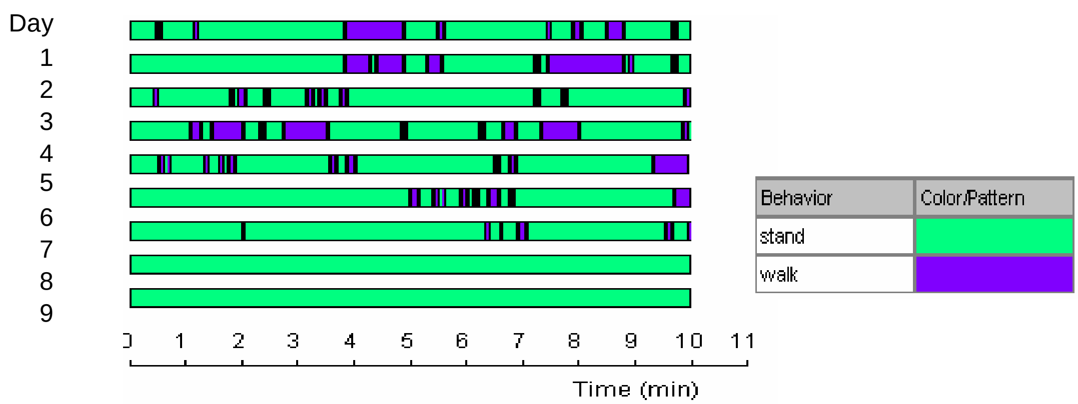
From the transition matrix in Table 6.3, the probability of behaviour transitions within the ingest cluster were minimal in the Middle and Old group. The Middle group exhibited ingest behavioural transitions which fell between the Young and Old group. The Middle group recorded a high probability that other behaviour would be followed by eat (ot->ea = 0.48) and a high probability that eat would not be followed by a target behaviour (ea->Y0 = 0.67). The Old group recorded consistency in ingestive behaviour, with a probability that the criterion behaviour eat would not be followed by a target behaviour (ea->Y0 = 0.99).



a) Horse 2 – time event plot of area cluster behaviour



b) Horse 2 – time event plot of ingest cluster behaviour



c) Horse 2 – time event plot of locomotory cluster behaviour

Figure 6.1 Time event plot of changes in (a) feeding area, (b) ingest and (c) locomotory behaviour during the training period, demonstrating fragmentation in behaviour for Horse 2 (Young).

Analysis of the effect of age on locomotory behaviour found a high probability of transitions from stand to walk and walk to stand being recorded in the Young group (st->wa = 0.63; wa->st = 0.79) compared to the Middle group (st->wa = 0.16; wa->st = 0.20). The Old group exhibited no behavioural transition from stand to walk throughout the training period as indicated by the Y0 value of 1.00 for stand to walk transitions.

The main factor of age was found to have a significant effect on intake ($F_{(2,30)}$ = 3.75, $p<0.05$) during the training period. As discussed in Chapter 4, a significant side preference was recorded in eight of the 18 horses studied during the nine-day training period (Table 6.4). From analysis of the data, the main factor of left and right container position was found to have no significant effect on the proportion of left or right food eaten ($F_{(1,30)}$ = 0.16, $p=0.690$). A significant interaction between age and left / right positioning on age was recorded ($F_{(2,30)}$ = 4.51, $p<0.05$). Horses exhibiting a significant container preference would be expected to have a higher mean transition probability from the least preferred container to the preferred container. Whilst this was supported in Horses 1, 5, 6, 14, 15 and 18, it was not the case in Horses 7 and 16 (Table 6.4). The transition matrices recorded the occurrence of transitions between behaviours and resultant probability of a transition occurring, yet they failed to reflect the duration of behaviours within each transition. Whilst Horse 7 exhibited an equal mean probability of left behaviour being succeeded by right behaviour (le->ri = 0.88; ri->le = 0.88), the transition probability fails to take in to account the difference in mean duration directed towards the two containers.

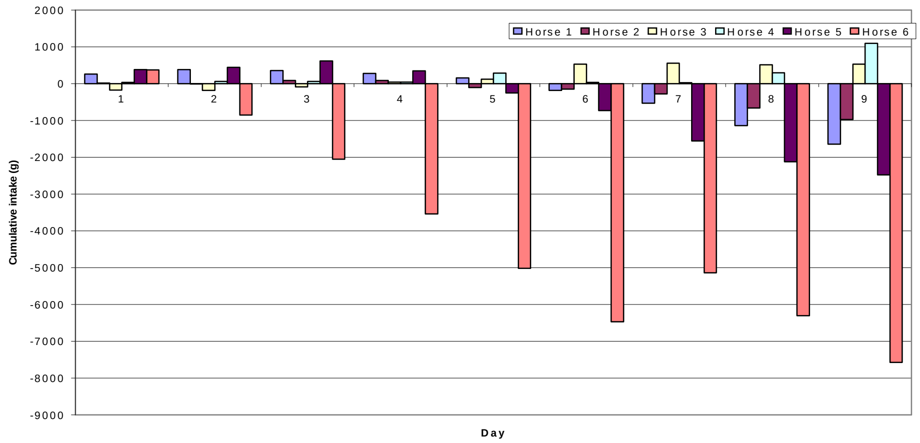
Table 6.4 Individuals exhibiting an intake-indicated and behaviour-indicated side preference during training (mean ± s.e.m. (g/s), n=9 days) (presented as Table 4.5 in Chapter 4)

	Intake					Behaviour		
	Left	**Right**				**Left**	**Right**	
Horse	**Mean ± s.e.m. (g)**	**Mean ± s.e.m. (g)**	**$F_{(1,17)}$ value**	***p* value**	**Pref ***	**Mean ± s.e.m. (g)**	**Mean ± s.e.m. (g)**	**$F_{(1,17)}$ value**
1	337 ± 29.5	521 ± 80.3	4.57	<0.05	RP	221 ± 31.3	370 ± 29.0	12.33
2	137 ±	245 ± 64.3	2.42	0.14	NP	111 ± 12.9	214 ± 54.0	3.45
3	160 ± 55.8	102 ± 22.9	0.93	0.35	NP	176 ± 33.3	101 ± 14.5	4.29
4	247 ± 83.3	126 ± 49.3	1.57	0.229	NP	221 ± 44.1	105 ± 30.0	4.74
5	502 ± 42.2	777 ± 99.2	6.54	<0.05	RP	232 ± 29.2	364 ± 30.8	9.53
6	319 ± 166.0	1161 ± 168.0	12.65	<0.01	RP	110 ± 68.5	490 ± 68.4	15.453
7	944 ± 71.0	681 ± 69.0	7.03	<0.05	LP	328 ± 37.5	272 ± 37.4	1.12
8	493 ± 63.1	611 ± 123.0	0.73	0.406	NP	281 ± 43.1	316 ± 44.2	0.31
9	493 ± 135.0	476 ± 137.0	0.01	0.934	NP	303 ± 83.3	295 ± 84.1	0
10	491 ± 106.0	531 ± 116.0	0.07	0.8	NP	247 ± 49.9	273 ± 38.1	0.18
11	550 ± 172.0	1066 ± 188.0	4.1	0.06	NP	198 ± 68.3	403 ± 68.2	4.51
12	554 ± 125.0	813 ± 123.0	2.17	0.16	NP	228 ± 45.5	321 ± 55.0	1.67
13	568 ± 70.5	576 ± 59.0	0.01	0.93	NP	288 ± 32.1	312 ± 32.0	0.29
14	476 ± 96.3	912 ± 88.2	11.18	<0.01	RP	164 ± 44.4	437 ± 44.4	18.91
15	1254 ± 41.0	222 ± 40.5	321.28	<0.001	LP	543 ± 15.4	58 ± 15.6	487.27
16	982 ± 168.0	319 ± 163.0	8.01	<0.05	LP	425 ± 76.6	175 ± 76.3	5.33
17	820 ± 168.0	589 ± 159.0	1	0.332	NP	340 ± 68.6	254 ± 69.6	0.77
18	1164 ± 87.7	95 ± 55.6	105.96	<0.001	LP	550 ± 22.1	51 ± 22.1	255.77

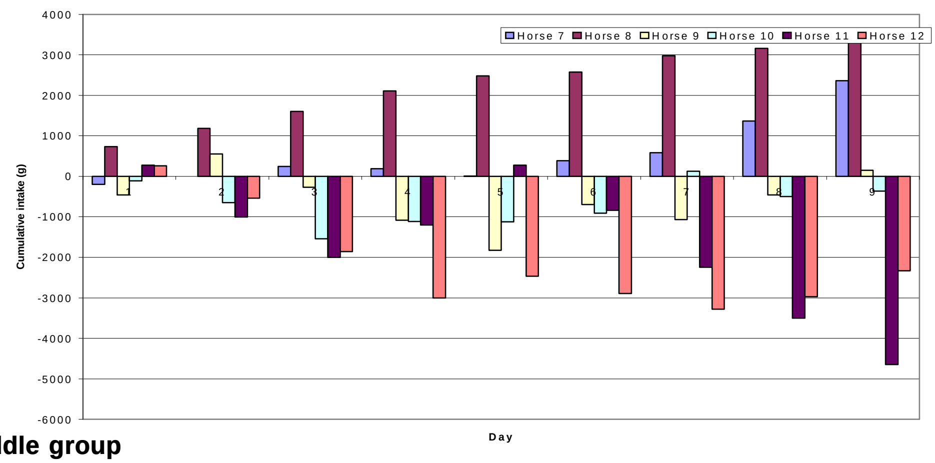
***NP denotes no significant preference recorded, LP denotes a left preference, RP denotes a right preference. [†]TP denotes transition probability.**

Individual variation in the consistency of left container selection or right container selection was evident in the training period, suggesting individual variation in the onset of side preference. This was evident from analysis of cumulative intake, calculated from left and right intake during the training period, whereby left intake was positive and right intake was negative (see Figure 6.2). Horse 14 and Horse 18 demonstrated a consistently higher intake from the left container over the right container during the nine-day training period, whereas Horse 5 consistently selected from the left container from day 5 onwards. The cumulative data indicates that the onset of side preference in Old individuals exhibiting a significant side preference (Horses 14, 15, 16 and 18) was earlier than the onset of side preference in Young individuals exhibiting a significant side preference (Horses 1 and 5). Horse 6 was the only Young individual to have an apparent consistency of left over right intake from day 2, this was followed by inconsistency in selection on day 7. Horse 7 was the only Middle group member to record a significant side preference during the training period, with consistency of left selection from day 5 onwards.

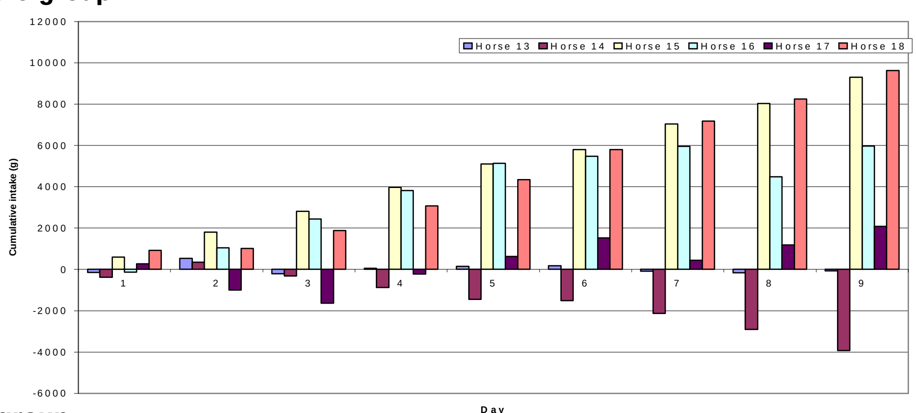
From the time event plots it was evident that horses exhibiting a significant side preference showed consistency of selection within days and between days. Characteristic examples of these are presented in Figure 6.3. Individuals not showing a significant preference for the left or right container exhibited variation within and between days (Figure 6.3).



a) Young group

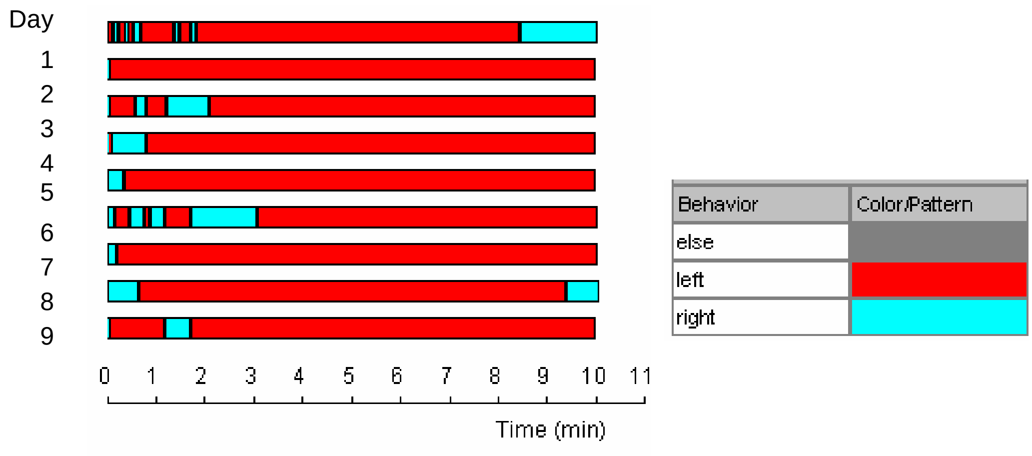


b) Middle group

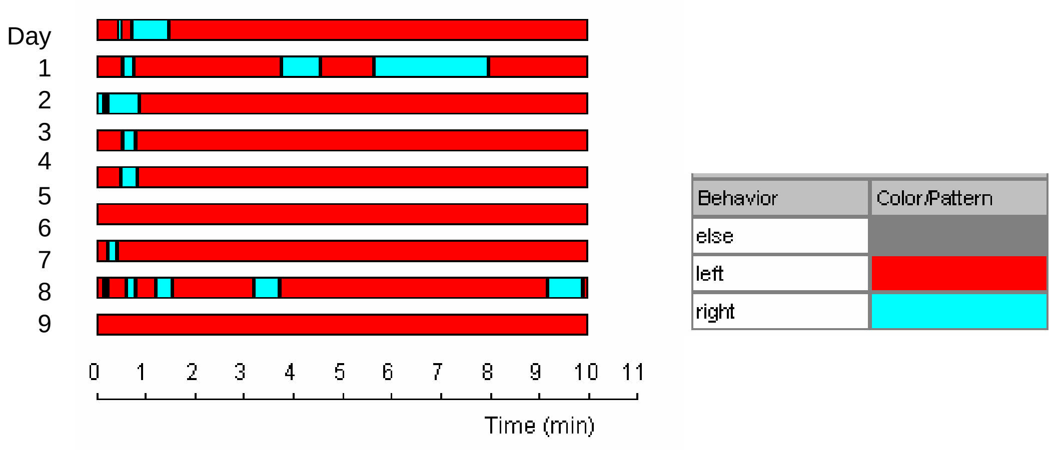


c) Old group

Figure 6.2 Cumulative intake in the (a) Young, (b) Middle and (c) Old group, calculated from the difference between left and right intake during training. + value denotes a left intake, - value denotes a right intake.



a) Time event plot for feeding area behaviour exhibited by Horse 15 during the training period indicating left side preference



b) Time event plot for feeding area behaviour exhibited by Horse 18 during the training period indicating a left side preference

Figure 6.3 Characteristic time event plots for horses exhibiting a significant side preference during training, showing consistent container preference between and within presentation period. a) Horse 15 exhibited a significant left preference (intake $F_{(1,17)}$ = 321.3, p<0.001; behaviour $F_{(1,17)}$ = 482.3, p<0.001). (b) Horse 18 exhibited a significant left preference (intake $F_{(1,17)}$ = 106.0, p<0.001; behaviour $F_{(1,17)}$ = 255.8, p<0.001).

A significant side preference was recorded in individuals showing inconsistency in container selection within and between days. Horse 5 exhibited sampling behaviour between the left and right container over the 9-day training period with a significant intake-indicated right preference ($F_{(1,17)}$ = 6.54, p<0.05) and behavioural-indicated right preference ($F_{(1,17)}$ = 9.53, p<0.001) was recorded in this horse (see Figure 6.4).

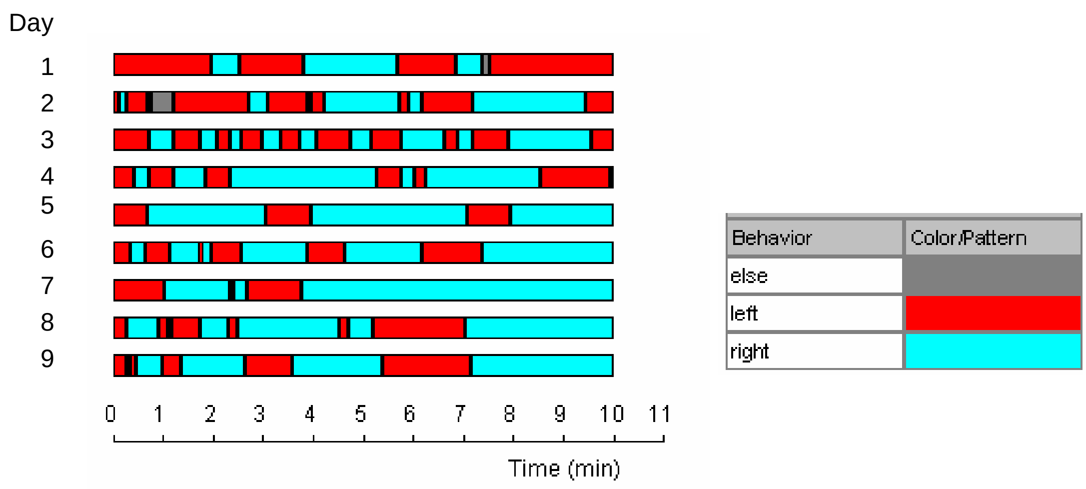


Figure 6.4 Time event plot for feeding area behaviour exhibited by Horse 5 during the training period indicating inconsistency in container selection within, and between, presentation days.

A similar finding of inconsistency of side behaviour between and within days was recorded in Horse 7 (Figure 6.5). A significant left preference was recorded from differences in intake from the left and right container ($F_{(1,17)}$ = 7.03, p<0.05). No preference was recorded from analysis of the difference between left and right behaviour ($F_{(1,17)}$ = 1.12, p=0.305). This inconsistency between the degree of side preference recorded from intake and the degree of side preference recorded from behaviour suggests that while Horse 7 directed attention towards the least preferred right container, less ingestion from the right container was occurring, than suggested for the behavioural duration towards the right container.

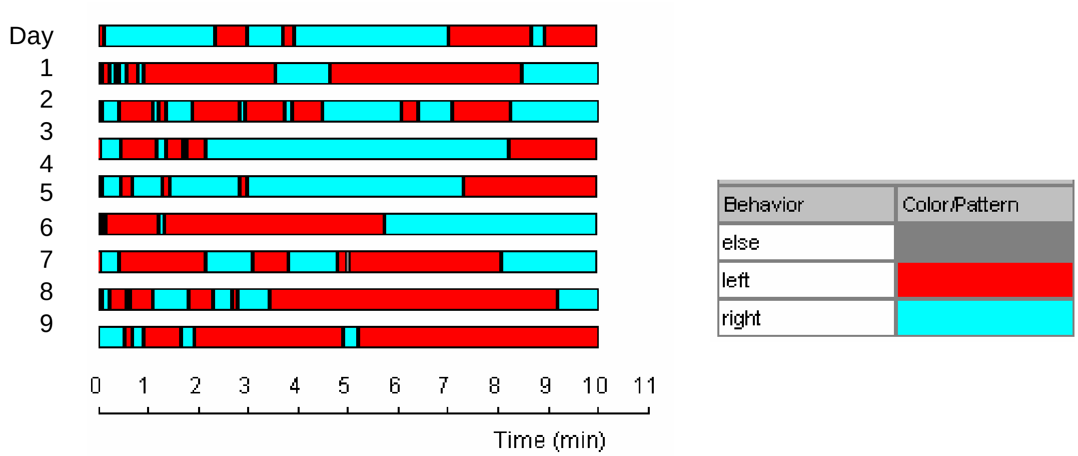


Figure 6.5 Time event plot for feeding area behaviour exhibited by Horse 7 during the training period indicating variation in container selection within and between presentation days.

Analysis of the difference between the mean probability transitions for 0-5 minutes and 5-10 minutes recorded higher behavioural fragmentation and reduced consistency of behavioural states during the 0-5 minute interval (Table 6.5). The 0-5 minute probability transitions within behavioural classes were higher than the probability transitions recorded in the 5-10 minute period. Additionally, the probability of a criterion behaviour not being followed by a target event throughout the entire 5 minute period, indicated by the Y0 value, was higher in the 5-10 interval. This can be illustrated by the probability transition that left criterion would be followed by an area target behaviour during the 5 minute lag period. During the 0-5 minute interval a lower probability that left behaviour would persist throughout the five minute period was recorded (li->Y0 = 0.15), compared to the probability during the 5-10 minute period (li-Y0 = 0.45). This indicates that a more consistent choice in feeding container was more probable during the 5-10 minute period than during the 0-5 minute period.

Table 6.5 Transition matrices for the probability of behaviour transition, calculated from the mean of all age groups, during (a) 0-5 minutes and (b) 5-10 minutes. A lower level of probability transitions is evident during the 5-10 minute interval compared to the 0-5 minute interval.

	le	ri	fl	el	ea	al	ot	st	wa	Y0
le	0.31	0.42	0.02	0.09						0.15
ri	0.44	0.30	0.02	0.09						0.13
fl	0.05	0.05	0.02	0.06						0.02
el	0.14	0.13	0.04	0.08						0.02
ea					0.13	0.02	0.20			0.71
al					0.08	0.01	0.05			
ot					0.32	0.01	0.13			0.03
st								0.14	0.15	0.79
wa								0.24	0.11	0.03
X0	0.16	0.09		0.05	0.14		0.08	0.22	0.02	

a) 0-5 minutes

	le	ri	fl	el	ea	al	ot	st	wa	Y0
le	0.21	0.22	0.03	0.12						0.45
ri	0.23	0.20	0.03	0.12						0.44
fl	0.01	0.04	0.01	0.03						0.10
el	0.09	0.11	0.02	0.08						0.18
ea					0.08	0.02	0.11			0.77
al					0.04		0.04			0.09
ot					0.07	0.02	0.09			0.33
st								0.11	0.13	0.83
wa								0.07	0.08	0.32
X0	0.08	0.07		0.02	0.03		0.02	0.04	0.02	

b) 5-10 minutes

An effect of age on probability transitions was found between age groups during both the 0-5 minute period and the 5-10 minute interval. Greater fragmentation in behaviour was recorded in the Young group in comparison to the Middle and Old group, with the Old group showing the most consistency in behaviour during both intervals, indicated from lower probability transitions and high Y0 values (see Table 6.6).

Table 6.6 Transition matrices for the probability of behaviour transition during 0-5 minutes and 5-10 minutes of the training period for the (a) Young, (b) Middle and (c) Old group.

	le	ri	fl	el	ea	al	ot	st	wa	Y0
le	0.23	0.37	0.05	0.16						0.06
ri	0.31	0.24	0.05	0.16						0.22
fl	0.05	0.05	0.04	0.08						
el	0.18	0.20	0.05	0.13						0.01
ea					0.19	0.04	0.29			0.48
al					0.13	0.01	0.08			
ot					0.32	0.03	0.20			0.02
st								0.21	0.22	0.52
wa								0.34	0.17	0.03
X0	0.12	0.09		0.05	0.12		0.09	0.20	0.02	

a) i Young 0-5 minutes

	le	ri	fl	el	e
le	0.22	0.22	0.05	0.19	
ri	0.22	0.18	0.04	0.21	
fl	0.03	0.04	0.02	0.06	
el	0.16	0.16	0.02	0.15	
ea					0
al					0
ot					0
st					
wa					
X0	0.08	0.07	0.01	0.04	0

ii Young 5-10 minutes

	le	ri	fl	el	ea	al	ot	st	wa	Y0
le	0.33	0.52	0.02	0.03						0.10
ri	0.49	0.34	0.02	0.03						0.09
fl	0.05	0.03	0.01	0.03						0.04
el	0.13	0.13	0.03	0.02						0.02
ea					0.07	0.01	0.10			0.69
al					0.03		0.01			
ot					0.39		0.06			0.03
st								0.07	0.07	0.85
wa								0.14	0.06	0.02
X0	0.18	0.07		0.04	0.12		0.12	0.21	0.02	

b) i Middle 0-5 minutes

	le	ri	fl	el	e
le	0.24	0.24	0.04	0.04	
ri	0.26	0.23	0.04	0.04	
fl		0.01			
el	0.07	0.11	0.01	0.01	
ea					0
al					
ot					0
st					
wa					
X0	0.08	0.07		0.01	0

Ii Middle 5-10 minutes

	le	ri	fl	el	ea	al	ot	st	wa	Y0
le	0.36	0.39	0.01							0.29
ri	0.51	0.32	0.01							0.09
fl	0.05	0.06								
el	0.10	0.08								
ea										0.95
al										
ot					0.25					
st										1.00
wa										
X0	0.17	0.10		0.04	0.19		0.05	0.23		

c) i Old 0-5 minutes

	le	ri	fl	el	e
le	0.19	0.19	0.01		
ri	0.22	0.19			
fl		0.08			
el	0.06	0.07			
ea					0
al					
ot					0
st					
wa					
X0	0.07	0.06			0

ii Old 5-10 minutes

An age effect was most evident in the ingest cluster and the locomotory cluster. The Young group exhibited the highest probability of transitions between ingestive and non ingestive behaviours compared to the Middle and Old group during the 0-5 minute interval (Young ea->Y0 = 0.48; Middle ea->Y0 = 0.69; Old ea->Y0 = 0.95), with greater consistency of ingestive behaviour recorded in the 5-10 minute period across all age groups (Young ea->Y0 = 0.60; Middle ea->Y0 = 0.75; Old ea->Y0 = 0.98). A similar increase in the consistency of stand behaviour was recorded in the Young and Middle from the 0-5 minute period (Young st->Y0 = 0.52, Middle st->Y0 = 0.85) to the 5-10 minute period (Young st->Y0 = 0.63, Middle st->Y0 = 0.88). No behavioural transitions from stand to walk were recorded for the Old group during the 0-5 minute period (wa->Y0 = 1.00) and the 5-10 minute period (wa->Y0 = 1.00).

Analysis of the difference between the mean age group transition probabilities during the first five minutes of the presentation period and the second five minutes of the presentation period, recorded increased consistency of behavioural states during the 5-10 minute interval. This is deduced from the Y0 values which indicated the probability of the criterion behaviour not being succeeded by a target event from within the behavioural class. The lower transition probabilities recorded within the behavioural classes during the 5-10 minute interval indicate reduced behavioural transitions and hence, less fragmented behaviours. This was most pronounced within the Old group.

Increasing consistency in container selection was recorded during the 5-10 minute period in all age groups. In the Young group, the probability of the left behaviour not being followed by a target event, denoted by the value in Y0, was higher during the 5-10 minute interval (le->Y0 = 0.27) than the 0-5 minute interval (le->Y0 = 0.06). The persistence of right behaviour was less strong than that of left behaviour; the probability of right behaviour not being followed by a target event was stronger during the 5-10 minute interval (ri->Y0 = 0.35) compared to the 0-5 minute interval (ri->Y0 = 0.22). A similar trend of consistency of intake was recorded in the Middle group which showed a greater consistency of container selection in the 5-10 minute interval (le->Y0 = 0.45; ri->Y0 = 0.40) than the 0-5 minute interval (le->Y0 = 0.10; ri->Y0 = 0.09). The Old group recorded the greatest consistency of behaviour, with the highest probability that the left container would not be succeeded with another behaviour from the area cluster during the 5-10 minute interval (le->Y0 = 0.63; ri->Y0 = 0.56) than the 0-5 minute interval (le->Y0 = 0.29; ri->Y0 = 0.09).

6.3.2 Two choice preference testing

Analysis of transition probabilities during two-choice preference testing recorded greater fragmentation of behaviour in the Young group compared to the Middle and Old group (see Table 6.7). This was identified in the Young group from a greater probability of transitions within the behavioural classes of area and ingest, indicating greater probability of non ingestive behaviours and attention directed away from the feeding area.

The effect of age on the transition probability between containers was evident, with the Young group having a lower transition probability from the left to right (le->ri = 0.54; ri->le = 0.46) container and from the right to left container due to the transition away from the feeding area (le->el = 0.16; ri->el = 0.20). An example of a horse showing such fragmented behaviour towards the feeding area was recorded in Horse 3 (Figure 6.6). Behaviour was more consistently directed between the feed containers in the Middle (le->ri = 0.76; ri->le = 0.83) and Old group (le->ri = 0.54; ri->le = 0.59).

Table 6.7 Transition matrices for the mean probability of behaviour transitions during two-choice preference testing for the (a) Young, (b) Middle and (c) Old group, where the probability of behavioural transition was calculated from the mean total of a criterion event being succeeded by a target event.

a) Young age group

	le	ri	fl	el	ea	al	ot	st	wa	Y0
le		0.54	0.02	0.16						0.16
ti	0.46			0.20						0.21
fl		0.08		0.04						0.04
el	0.33	0.18	0.02							0.08
ea						0.04	0.45			0.50
al					0.12		0.06			
ot					0.48	0.01				0.09
st									0.34	0.56
wa								0.44		0.02
X0	0.15	0.06		0.07	0.12		0.07	0.19		

b) Middle age group

	le	ri	fl	el	ea	al	ot	st	wa	Y0
le		0.76		0.01						0.23
ti	0.83		0.02							0.15
fl	0.01	0.06		0.01						
el	0.09	0.13								
ea						0.03	0.06			0.74
al					0.06					
ot					0.42					0.01
st									0.22	0.78
wa								0.32		
X0	0.25	0.04		0.04	0.14		0.08	0.22		

c) Old age group

	le	ri	fl	el	ea	al	ot	st	wa	Y0
le		0.54								0.39
ti	0.59									0.16
fl										
el	0.08	0.06								
ea							0.01			0.99
al										
ot					0.15					
st									0.01	0.99
wa								0.03		
X0	0.21	0.06		0.03	0.20		0.03	0.23		

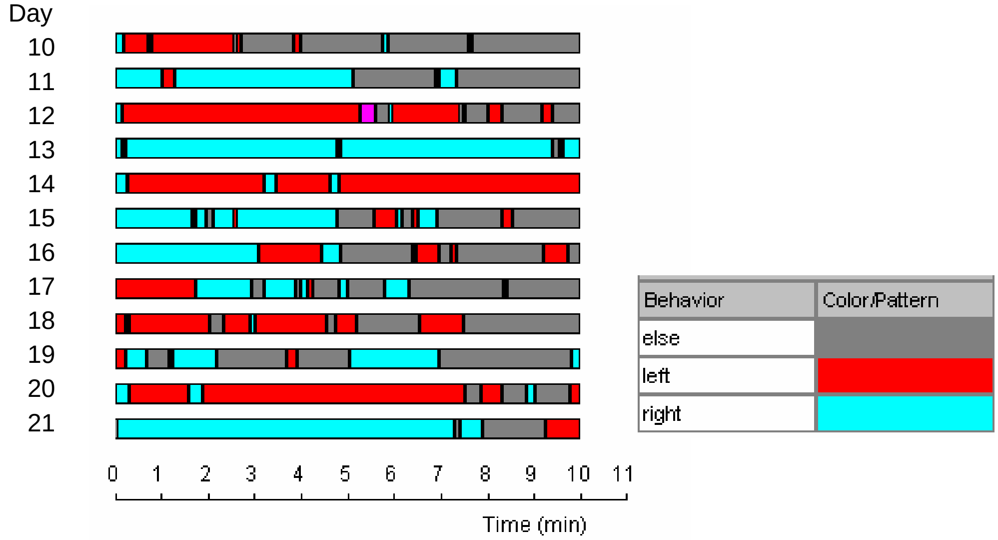


Figure 6.6 Time event chart for Horse 3 during two-choice preference testing illustrating transitions between the area cluster.

As reported in Chapter 4, a significant side preference was recorded in five horses (Table 6.8). Consistency of side preference throughout the training period, persisting into two-choice preference testing is evident in individuals from analysis of individual cumulative intake (see Figure 6.7). This consistency is most evident in the Middle and Old groups. This side preference continued with the presentation of the test feed on alternate days and varying inclusion levels. The effect of control positioning on cumulative intake for the left and right container during two-choice preference testing was evident in horses exhibiting a significant control preference as shown in one individual (Figure 6.8). The daily reversal of the control positioning resulted in horses which showed a control preference having a cumulative difference near to a 0.5 mean.

Table 6.8 Individuals exhibiting an intake-indicated and behaviour-indicated side preference during two-choice preference testing (mean ± s.e.m. (g/s), n=12 days)

	Intake					Behaviour	
	Left	Right				Left	Right
Horse	Mean ± s.e.m. (g)	Mean ± s.e.m. (g)	$F_{(1,22)}$ value	*p* value	Pref*	Mean ± s.e.m. (s)	Mean ± s.e.m. (s)
1	615 ± 154.0	581 ± 150.0	0.03	0.875	NP	267 ± 67.3	294 ± 67
2	447 ± 136.0	456 ± 142.0	0	0.964	NP	302 ± 88.1	298 ± 88.3
3	277 ± 83.7	272 ± 83.0	0	0.961	NP	196 ± 55.8	199 ± 53.9
4	570 ± 157.0	540 ± 153.0	0.02	0.892	NP	293 ± 77.9	287 ± 77.9
5	679 ± 160.0	611 ± 158.0	0.09	0.765	NP	309 ± 71.6	289 ± 71.8
6	374 ± 168.0	583 ± 1131.0	10	<0.01	Right	139 ± 70.9	246 ± 460.2
7	1015 ± 121.0	728 ± 121.0	2.81	0.108	NP	340 ± 39.4	259 ± 39.5
8	950 ± 68.7	238 ± 175.3	64.59	<0.001	Left	500 ± 37.7	131 ± 97.7
9	504 ± 144.0	527 ± 155.0	0.01	0.918	NP	308 ± 85.3	290 ± 85.0
10	924 ± 87.2	302 ± 432.9	20.78	<0.001	Left	396 ± 31.4	109 ± 191.0
11	579 ± 105.0	846 ± 134.0	2.46	0.131	NP	245 ± 52.8	355 ± 52.9
12	741 ± 111.0	801 ± 116.0	0.14	0.713	NP	244 ± 426	352 ± 42.1
13	787 ± 161.0	545 ± 160.0	1.13	0.298	NP	336 ± 63.1	263 ± 63.1
14	726 ± 197.0	674 ± 199.0	0.03	0.855	NP	302 ± 83.4	295 ± 83.9
15	909 ± 195.0	674 ± 569.0	1.56	0.225	NP	371 ± 79.4	275 ± 229.3
16	1242 ± 93.2	323 ± 90.9	81.07	<0.001	Left	531 ± 48.9	169 ± 44.3
17	646 ± 180.0	779 ± 185.0	0.27	0.612	NP	243 ± 69.9	356 ± 69.7
18	1199 ± 118.0	409 ± 215.0	34.3	<0.001	Left	524 ± 48.1	167 ± 75.3

***NP denotes no preference**

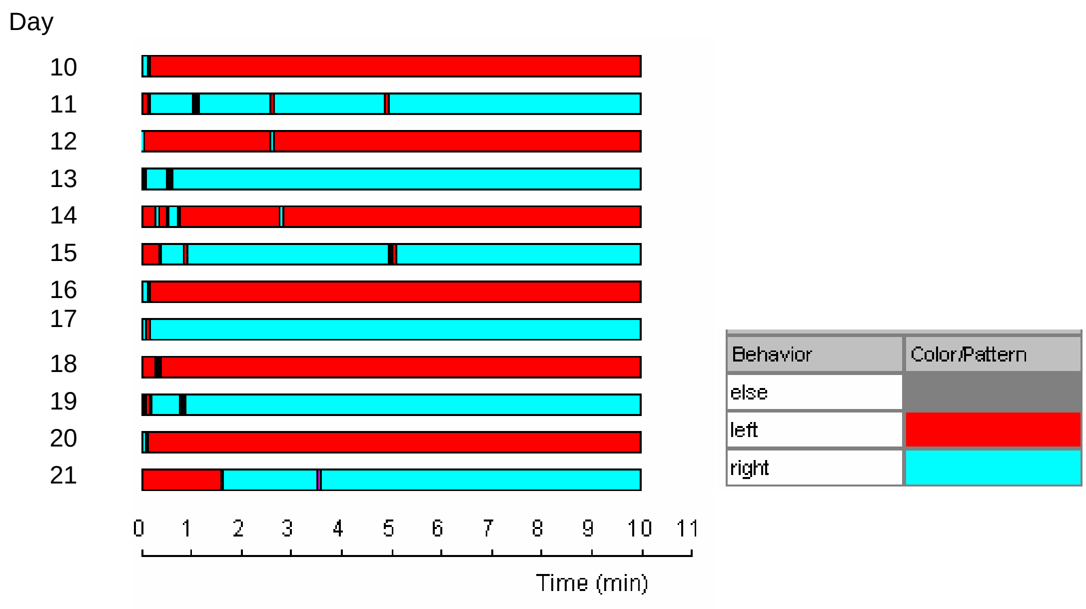
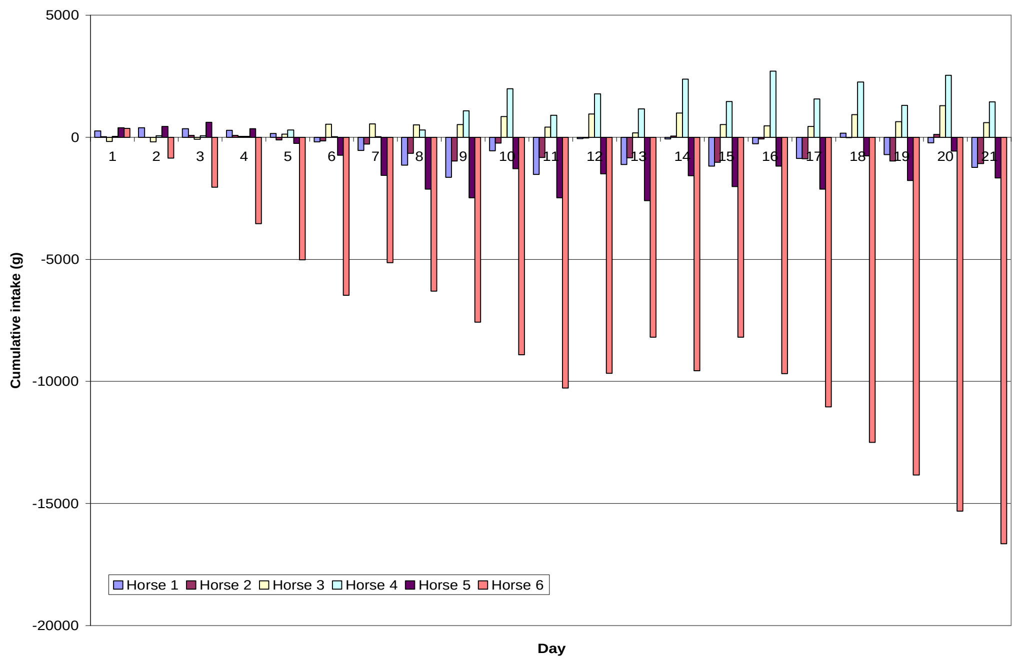
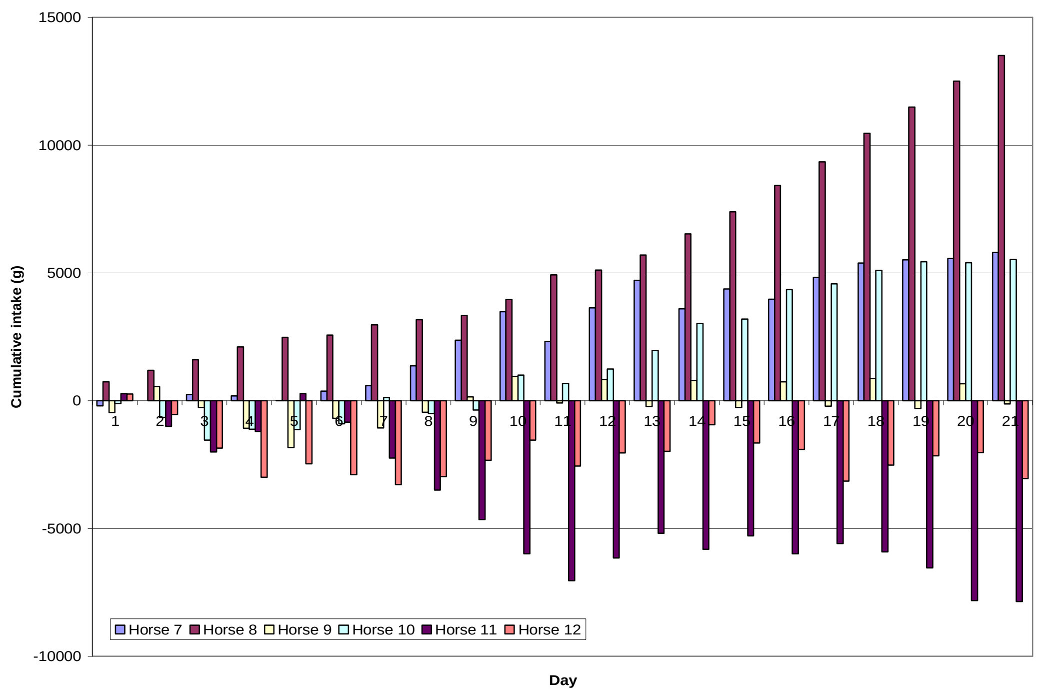


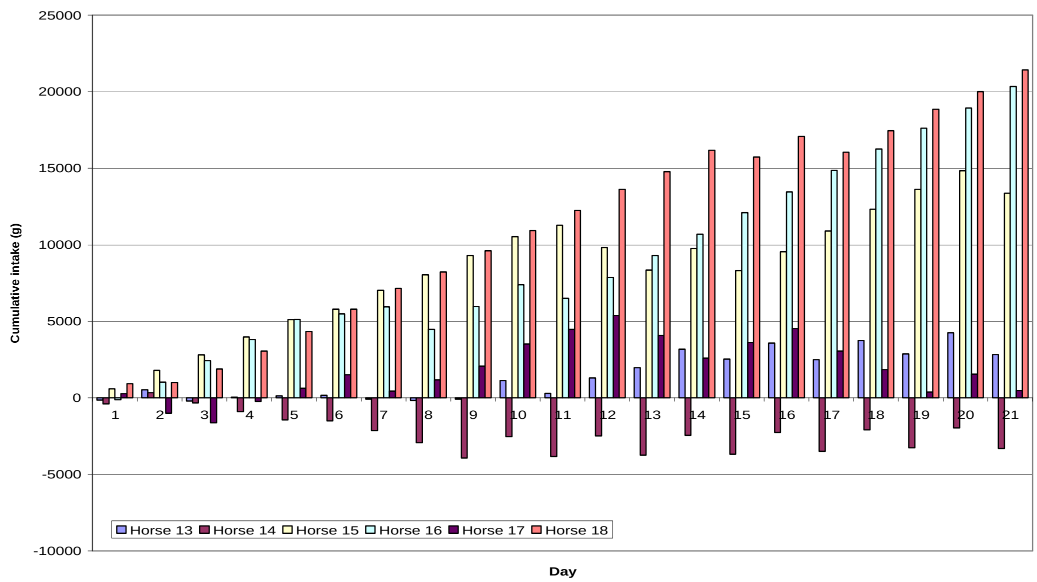
Figure 6.7 Time event chart for Horse 9 illustrating a significant control preference during two-choice preference testing. Initial sampling of both containers is evident within the first minute on all days, with the exception of day 12. The control was positioned on the left on day 9 with daily reversal of the control thereafter.



a) Young



b) Middle



c) Old

Figure 6.8 Individual cumulative intake for the (a) Young, (b) Middle and (c) Old group during the training period (day 1-9) and two-choice preference testing (day 10-21). Negative values denote a left intake, positive values denote right intake

The significant side preference recorded in Horse 6, Horse 16, and Horse 18 during training (Table 6.4) persisted through to a significant side preference during two choice preference testing (Table 6.8). The significant side preference recorded in training in Horse 1, Horse 4, Horse 14 and Horse 15 failed to persist with the commencement of two-choice preference testing. In Horse 1, Horse 4 and Horse 15 this termination of a significant side preference was due to a significant control preference being exhibited. Horse 8 and Horse 10 recorded a significant side preference during two choice preference, having not previously shown a significant side preference during training.

In horses exhibiting a significant side preference, the extent of sampling between the left and right container varied within, and between, individuals. Horse 8 exhibited a significant left preference, yet it can be seen that Horse 8

exhibited more transitions between the left and right container and sampled most when the novel test feed was presented from day 10 onwards (Figure 6.9).

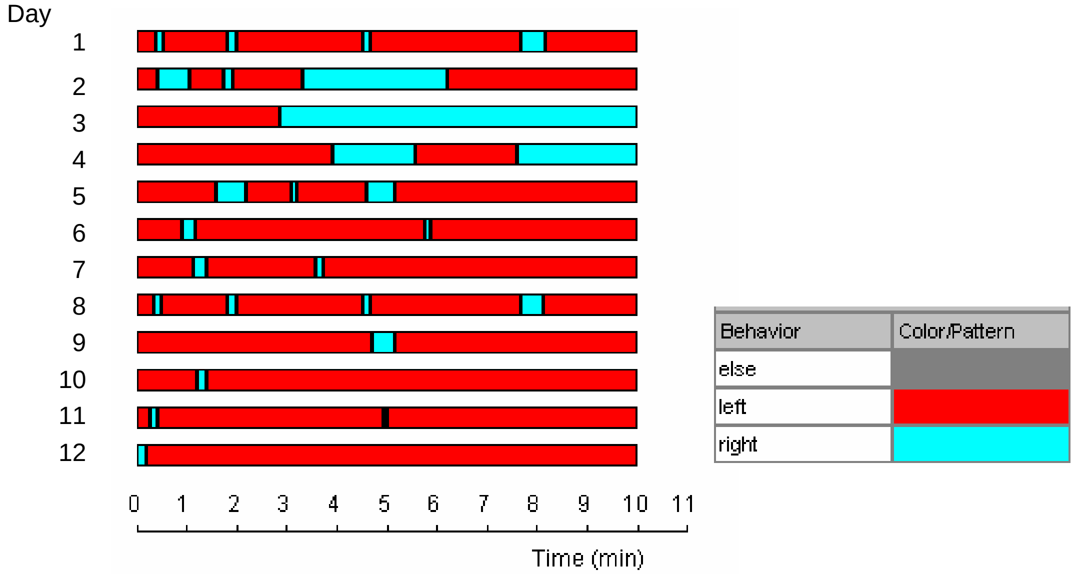


Figure 6.9 Time event chart for Horse 8 demonstrating a significant left preference, with intermittent attention towards the right container more prevalent when the novel test feed was presented on day 10.

Consistent with the findings of the training period, greater fragmentation of behaviour was recorded in the 0-5 minute period, compared to the 5-10 minute period. There was a higher consistency of behaviour, with greater probability that a criterion behaviour would not be followed by a target behaviour, indicated from higher Y0 values for all clusters (see Table 6.9).

Consistent with the effects of the 0-5 minute interval and 5-10 minute interval recorded in the training period, the second time interval recorded a reduction in transitions between behavioural classes (see Table 6.10). This was found in all three age groups, within the three behaviour clusters. Additionally, the consistency of behaviour was more evident during the 5-10 minute period, with greater Y0 values recorded, indicating that the criterion behaviour had not been succeeded by the target behaviour. This was most pronounced in the Old group

who exhibited no behavioural transitions in the ingest and locomotory cluster, as indicated by a Y0 value of 1.

Table 6.9 Transition matrices for the probability of behaviour transition, calculated from the mean of all age groups, during (a) 0-5 minutes and (b) 5-10 minutes for two-choice preference testing. A lower level of probability transitions is evident during the 5-10 minute interval compared to the 0-5 minute interval.

	le	ri	fl	el	ea	al	ot	st	wa	Y0
le	0.25	0.43	0.01	0.04						0.23
ri	0.45	0.24	0.01	0.07						0.16
fl	0.02	0.04		0.01						0.01
el	0.14	0.13	0.01	0.04						0.04
al					0.05		0.02			
ot					0.32		0.08			0.03
st								0.10	0.11	0.78
wa								0.20	0.10	0.01
X0	0.20	0.06		0.05	0.15	0.01	0.07	0.22	0.01	

a) 0-5 minutes

	le	ri	fl	el	ea	al	ot	st	wa	Y0
le	0.13	0.13	0.01	0.05						0.67
ri	0.14	0.11	0.01	0.05						0.64
fl	0.02	0.01		0.01						0.05
el	0.05	0.04	0.01	0.06						0.21
ea					0.08	0.01	0.08			0.83
al					0.01	0.01	0.01			0.05
ot					0.06	0.01	0.06			0.32
st								0.07	0.07	0.92
wa								0.04	0.04	0.23
X0	0.06	0.05		0.01	0.03		0.01	0.04	0.01	

b) 5-10 minutes

Table 6.10 Transition matrices for the probability of behaviour transition for left and right behaviour during 0-5 minutes and 5-10 minutes of two-choice preference testing period for the (a) Young, (b) Middle and (c) Old group.

le	ri	fl	el	ea	al	ot	st	wa	Y0
0.44	0.25	0.01	0.13						0.18
0.44	0.20	0.01	0.14						0.21
0.03	0.01		0.01						0.01
0.27	0.22	0.01	0.09						0.04
				0.20		0.23			0.60
				0.06		0.03			
				0.47		0.17			0.04
							0.20	0.21	0.63
							0.35	0.14	
0.12	0.14		0.07	0.14	0.01	0.10	0.22	0.02	

oung 0-5 minutes

	le	ri	fl	el	ea	al	ot	st	wa
le	0.11	0.10	0.01	0.10					
ri	0.11	0.09	0.01	0.10					
fl	0.01			0.01					
el	0.06	0.06	0.01	0.06					
ea					0.13	0.02	0.14		
al							0.01		
ot					0.10	0.01	0.11		
st								0.09	0.1
wa								0.04	0.0
X0	0.06	0.10		0.02	0.04		0.03	0.04	0.0

ii Young 5-10 minutes

le	ri	fl	el	ea	al	ot	st	wa	Y0
0.43	0.39	0.03	0.01						0.20
0.49	0.38	0.02	0.01						0.14
0.05	0.04								
0.13	0.09	0.01							
				0.03	0.02	0.03			0.79
				0.06	0.01	0.01			0.01
				0.39		0.01			0.01
							0.13	0.13	0.78
							0.25	0.07	
0.16	0.15		0.05	0.14	0.01	0.09	0.22		

iddle 0-5 minutes

	le	ri	fl	el	ea	al	ot	st	wa
le	0.20	0.22	0.01						
ri	0.16	0.20	0.01						
fl	0.01	0.01							
el	0.03	0.07							
ea					0.03	0.01	0.01		
al					0.01	0.01			
ot					0.02		0.01		
st								0.04	0.0
wa								0.03	0.0
X0	0.06	0.07		0.01	0.03		0.01	0.04	0.0

ii Middle 5-10 minutes

le	ri	fl	el	ea	al	ot	st	wa	Y0
0.32	0.24								0.33
0.34	0.24								0.13
	0.01								
0.07	0.05								
									0.95
				0.14					
							0.01	0.01	0.94
							0.03		0.01
0.13	0.14		0.02	0.20		0.03	0.22		

ld 0-5 minutes

	le	ri	fl	el	ea	al	ot	st	wa
le	0.07	0.10							
ri	0.07	0.09							
fl									
el	0.01	0.03							
ea									
al									
ot									
st									
wa									
X0	0.05	0.05			0.02			0.02	

ii Old 5-10 minutes

6.3.3 Monadic testing

From the transition matrices for the mean Young, Middle and Old group during the monadic period (Table 6.11), it is evident that consistency of behaviour was recorded in the old group, with a Y0 value of near 1 for the area, ingest and locomotory cluster. There was a high probability that stand (st->Y0 = 0.11), eat (ea->Y0 = 0.99) and attention towards the monadic (mo->Y0 = 0.98) container would not be followed by a target behaviour during the monadic presentation period. The Young group, consistent with findings in the training period and two-choice preference testing, continued to show the most fragmented behaviour. Of interest was the transition probabilities in the area cluster, indicating attention away from the monadic container (mo->el = 0.35). This reflects the reduced intake recorded in the Young group with presentation of the monadic container, following the previous presentation of a left and right container during two-choice preference testing. The reduction in ingestive behaviour is reflected in the time event chart for Horse 2 during monadic testing where a high level of non ingestive behaviour was recorded following initial presentation of the monadic container on day 22 (see Figure 6.10).

Table 6.11 Transition matrices for the mean probability of behaviour transition for the (a) Young, (b) Middle and (c) Old groups during monadic testing

	fl	el	mo	ea	al	ot	st	wa	Y0
fl		0.02	0.02						
el			0.62						0.01
mo	0.01	0.35							0.62
ea					0.07	0.31			0.58
al				0.18		0.02			0.04
ot				0.58					0.03
st								0.33	0.65
wa							0.48		
X0		0.10	0.15	0.12		0.12	0.24		

a) Young group

	fl	el	mo	ea	al	ot	st	wa	Y0
fl			0.06						
el			0.20						
mo	0.03	0.03							0.93
ea					0.07	0.10			0.65
al				0.10					0.02
ot				0.38					
st								0.12	0.86
wa							0.19		
X0			0.04	0.08	0.16		0.08	0.24	

b) Middle group

	fl	el	mo	ea	al	ot	st	wa	Y0
fl									
el									
mo									0.98
ea					0.01				0.99
al				0.02					
ot				0.08					
st									1.00
wa									
X0			0.24	0.23		0.02	0.25		

c) Old group

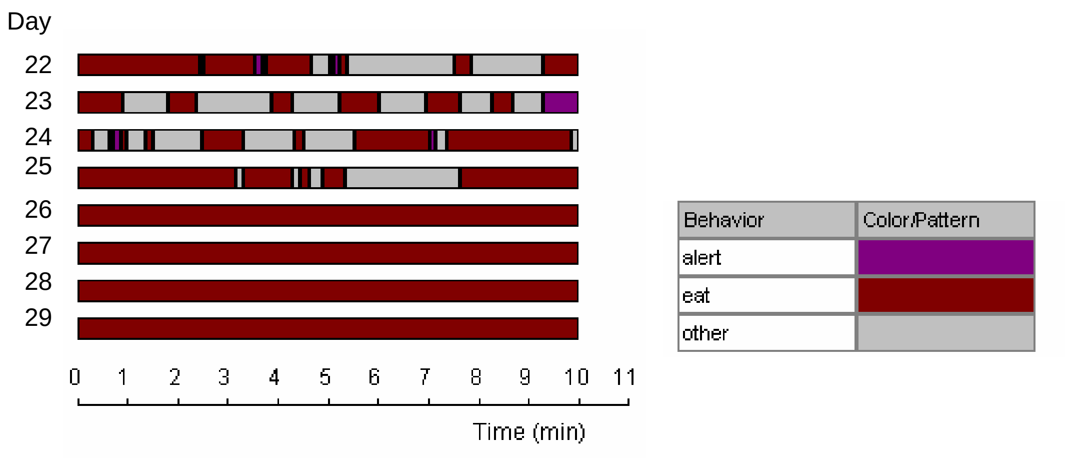


Figure 6.10 Time event plot for Horse 2 during monadic testing showing a high level of non ingestive behaviour with presentation of a single container following two-choice preference testing.

Consistent with earlier findings, the Young were characterised by more fragmented behaviour, most pronounced during the 0-5 minute period (see Table 6.12). The Old group exhibited the greatest consistency of behaviour between the 0-5 minute period and the 5-10 minute period, with a Y0 of 1 recorded for walk, eat and monadic, indicating exclusivity of choice.

Table 6.12 Transition matrices for the probability of behaviour transition for monadic presentation during 0-5 minutes and 5-10 minutes of two-choice preference testing period for the (a) Young, (b) Middle and (c) Old group.

	fl	el	mo	ea	al	ot	st	wa	Y0
fl		0.01	0.03						
el	0.01	0.14	0.49						
mo	0.01	0.21	0.19						0.60
ea				0.20	0.05	0.18			0.54
al				0.18		0.03			0.04
ot				0.44	0.03	0.13			0.02
st							0.20	0.20	0.60
wa							0.39	0.11	
X0		0.10	0.15	0.12		0.12	0.23	0.02	

a) i Young 0-5 minutes

	fl	el	mo	ea	al	o
fl						
el		0.08	0.08			
mo		0.09	0.09			
ea				0.10	0.02	0
al				0.04		0
ot				0.09	0.01	0
st						
wa						
X0		0.03	0.05	0.05		0

ii Young 5-10 minutes

	fl	el	mo	ea	al	ot	st	wa	Y0
fl			0.06						
el	0.01	0.01	0.20						
mo	0.01	0.01	0.02						0.95
ea				0.06	0.01	0.05			0.70
al				0.10					0.02
ot				0.34		0.04			
st							0.07	0.07	0.86
wa							0.16	0.03	
X0		0.04	0.20	0.16	0.02	0.08	0.24		

b) i Middle 0-5 minutes

	fl	el	mo	ea	al	o
fl						
el		0.01	0.01			
mo	0.01	0.01	0.02			
ea				0.06	0.04	0
al						
ot				0.04	0.02	0
st						
wa						
X0		0.01	0.03	0.04		0

ii Middle 5-10 minutes

	fl	el	mo	ea	al	ot	st	wa	Y0
fl									
el									
mo									1.00
ea				0.01	0.01				1.00
al				0.02	0.01				
ot				0.08					
st									1.02
wa									
X0			0.25	0.23		0.02	0.25		

c) i Old 0-5 minutes

	fl	el	mo	ea	al	o
fl						
el						
mo						
ea				0.01		
al						
ot						
st						
wa						
X0			0.03	0.03		

ii Old 5-10 minutes

6.4 Discussion

The assumption that animals select around a theoretical mean of 0.5 when two identical choices are presented simultaneously fails to take into account the effect that positioning cues may have on choice. From this study it was observed that horses may choose to select based on positional cues when no additional cues are provided and in some individuals, may persist even with the presentation of additional cues.

Training, or acclimatisation, is commonly used in animals prior to two-choice preference testing. This period, normally immediately prior to two-choice preference testing, aims to ensure acceptance of the basal feed, awareness of two-feeding choices and replacement of ingestive behaviours with exploratory behaviours. The findings of this study support the replacement of exploratory and non ingestive behaviour with ingestive behaviour, following repeated presentation of the novel basal feed. Individual variation in the Young group in response to the presentation of the novel test feed during two-choice preference testing and single container positioning during monadic testing resulted in an increase in non ingestive behaviour similar to that observed during the initial training period.

Whilst this study satisfies these aims, it was observed in some animals that lack of variety between choices presented resulted in selection based on positional cues. Furthermore, once established in individuals, side preference, (also termed lateralisation (Rogers and Andrew, 2002; Murphy *et al*., 2004; McGreevy and Rogers, 2005)), was observed to persist and continue to be influential on feeding choice, even when additional cues were presented (see Section 4.6.2 and Section 5.6.2).

Side preference has been previously recorded in one horse during two-choice preference testing (Hawkes *et al*., 1985), with the horse selecting 92% of intake from the right container, regardless of contents. Hawkes *et al.* (1985) rejected the horse from further study. Due to the high incidence of side preference recorded in this present study, such rejection of individuals exhibiting side preference would not be feasible.

The susceptibility of a horse to exhibit side preference may be influenced by both genetic and environmental factors. Side preference is suggested to occur at population level in the horse, determined from motor positional bias relating to foreleg and nostril advance (McGreevy and Rogers, 2005). An increased preference for left foreleg advance and right nostril advance was found. Additionally, left preference in the horse was found to be more apparent in males, with right preference more common in females (Murphy *et al*., 2004). Further study of feeding side preference, with specific reference to gender, may support these findings as allocation of horses failed to consider gender allocation in the current study. The findings of this study do not support side preference being present at population level, with a significant difference between left and right container preference not being recorded in all individuals. It is suggested that the exhibition of side preference in individuals is the result of the lack of consequence of selecting for side due to limitations in the study design, rather than side preference being an inherent inevitability as argued by Rogers and Andrew (2002) with regard to Thoroughbred and Standard bred horses.

Rogers and Andrew (2002) suggested side preference to be the exhibition of differences in functional activity in the cerebral hemispheres. Studies of side preference in neonatal lambs failed to identify a preference and this may suggest that side preference is not exhibited in the perinatal period (Lane and Phillips, 2004). Environmental cues may be more influential in the expression and

development of a genotypic predisposition for a side preference. Rogers and Andrew (2002) suggest that emotionality may increase the susceptibility of horses to exhibit side preference, supporting earlier suggestions of a link by Grandin and Deesing (1999).

The motivation to exhibit side preference in individuals can be seen to be strong, with cows exhibiting increased levels of the stress hormone cortisol and reduced milk production as a result of frustration in response to being forced to be milked on their least preferred parlour side (Hopster *et al.*, 2000). This current study found side preference to be more influential than taste response on intake in some individuals, with side preference persisting over a dislike for the test chemical which was rejected by conspecifics.

An effect of age on the extent of side preference was exhibited. Old subjects showed a higher degree of side preference over the Middle and Young subjects. Several reasons are proposed to account for this. An increase in lateralised motor bias was recorded in older horses by McGreevy and Rogers (2005) and was suggested to be the result of maturation and training. This is not relevant to feeding side preference due to the lack of prior experience to two-choice preference testing in these horses prior to the commencement of the study.

For side preference to be recorded, the horse needs to exhibit not only intrameal consistency, but also intermeal consistency. The following discussion deals with proposed reasons for consistency of selection based on positional cues in the horse.

An effect of age was observed on intrameal behaviour, with the Young group exhibiting more fragmented behaviour, exemplified by a higher occurrence of transitions between ingestive and non ingestive behaviour and stand to walk.

The higher levels of non ingestive behaviour rather than ingestive behaviour reflect the altered motivations in the Young group, as compared to the Middle and Old group. The study was based on the assumption that individuals would be subject to the same motivational state. It was apparent that this was not the case with the Young group exhibiting a higher wariness towards the basal food and test food at the onset of the training period and two-choice preference testing respectively as well as the change to single container positioning during monadic testing. Inspective exploration towards the novel basal feed and container presentation was found, alongside an increase in non ingestive behaviour. This was supported by a lower total daily intake in the Young group and increased walk behaviour, reducing the likelihood of intrameal consistency.

Spontaneous reversal, whereby the previous choice is reversed, is suggested to have been evident in the Young group. Spontaneous reversal is normally restricted to reversal in choice between different presentation periods (Toates, 1980). Due to the duration of each presentation period, characterised by several feeding bouts in Young animals following periods of intermittent non ingestive behaviour, it is suggested that spontaneous reversal occurs within the presentation period in some individuals. Following behaviour directed away from the feeding area there is an equal chance of either container being selected on resumption of ingestive behaviour. Spontaneous reversal suggests that the selection of the alternative container is through choice, rather than chance (Toates, 1980). The Young group may have been selecting for novelty in feeding choice on resumption of ingestive behaviour. If this was exhibited throughout the presentation period following each exhibition of non ingestive behaviour, there would be no opportunity for the selection of a container based on positioning or taste response. This would result in no significant preference recorded between the container options in these individuals. It is suggested that spontaneous reversal is most likely to be exhibited in the initial stages of the

presentation period, due to an intrasession decline being recorded. An intrasession decline would account for reduced behavioural fragmentation in the second half of each presentation period, resulting in an increased probability that a container choice would be made. Spontaneous reversal may be expressed alongside inspective exploration in the Young group. Toates (1980) suggests that spontaneous exploration occurs due to motivation for novelty, with an inter trial decline following increased presentation. This combined with the presentation of variation in inclusion level and positioning of the test feed may result in increased exhibition of exploratory or non ingestive behaviour in Young subjects. Difficulties arose in distinguishing between non ingestive behaviour and exploratory behaviour as, in this study, the two appeared intrinsically linked. Inspective exploration was most commonly observed in the Young group following presentation of the novel test feed at the onset of two-choice preference testing and the presentation of one container choice at the onset of monadic testing.

It has been observed in young pigs that feed deprivation increases exploratory motivation (Day *et al*., 1995). Whilst feed was available to the horses in this study, the wariness to novelty and initial avoidance of the test feed may have resulted in increased exhibition of non ingestive behaviour, in order to attempt to satisfy feeding motivation. The frustration of not being able to satisfy motivations has been found to result in increased levels of the stress hormone, cortisol and redirection of behaviours (Mason and Cooper, 2001).

Variation in intrameal choice may be the result of increased attention away from the presentation area and feeding container, indicated by the duration of non-ingestive behaviour and walk behaviour, most notably in Young and Middle subjects. Termination of intrameal feeding bouts increases the probability of the horse altering its feeding choice, as observed in the Young group. The subjects

that made an exclusive choice and ate to completion had less opportunity to select a different container and hence, were more likely to exhibit side preference if associated with intermeal consistency of container selection. The Old group exhibited the lowest probability of behavioural transitions, within the behavioural classes of ingest and locomotory. Increased consistency of intrameal selection in the Old group may be the result of monotony as a result of a lifetime of limited choice in the feeding environment. The win-stay concept put forward by Hosoi *et al.* (1995) appears relevant to the Old group. Initial sampling from both containers, followed by an intrasession decline, may have resulted in consistency of choice being recorded. Rapidity of exclusive choice was more apparent in Old subjects, exemplified by a limited probability that a change in behaviour would occur, prior to termination of the presentation period. Consistency of this choice between presentation days would result in a significant preference being recorded. Increased awareness of the external environment in the Young group is proposed to increase intrameal variation. The subjects used in the training period during Chapter 4 were unfamiliar with the stabling environment. Although introduced to the stables several days prior to the trial commencing, they may still have been subject to novelty in response to changes in their environment. Additionally, changes in the animals' external environment may not be discernible to humans but might affect short term feeding behaviour. The fragmentation of behaviour in the Young group supported an increased response to changes in the environment through a high probability of non ingestive behaviours being recorded.

The lower probability of behavioural transitions in the old group and persistence of ingestive behaviour suggest monotony in Old subjects and suggests more rapid acceptance of change and consequently a lower response to novelty. The lack of consequence for selecting for a feed based on container positioning of behaviour is suggested to result in persistence of container choice in all

individuals exhibiting side preference. A reduced neophobic response towards novel foods has been found in humans to be attributable to a loss of olfactory function (Pelchat, 2000) and hence, may be applicable to the horse. Such monotony, or reduced selectiveness, may account for the persistence of side preference with test feed presentation during two-choice preference testing. It may not be that the Old group are less sensitive to the test feed, it may be that they are more tolerant and more rapidly aware of no negative or positive nutritive consequences of test feed ingestion.

The reduction in transition probabilities between the 0-5 minute period and the 5-10 minute period indicates changes in the motivational state of the horses during presentation period. In the Young group there were fewer exhibitions of non ingestive behaviour in the 5-10 minute period, suggesting acceptance of the feeding environment and an increase likelihood of a feeding choice being made. In the Middle and Old group the reduction in behavioural transitions represented a feeding choice had been made, with a higher probability of consistency in this choice to completion in the Old group, supporting win-stay in horses exhibiting a significant side preference.

A decline in transition probabilities between the initial five minute period and the second five minute period was recorded across all age groups. This is suggested to be the result of intrasession decline where a lower exhibition of non ingestive behaviour was recorded in previous time period, compared to the start of the next period (Toates, 1980). A characteristic increase in sampling behaviour, recorded between the two containers during two-choice preference testing or within the monadic presentation is suggested to be a mechanism to ensure consistency of cues between intervals (Toates, 1980). The methodology employed in this study may have confounded that ability of horses to determine consistency of cues between presentation periods due to variation in inclusion levels and daily reversal of control positioning during two-choice preference testing. In the short term it could not be concluded whether horses were moving towards one container as a result of inspective exploration, or whether they were exhibiting an avoidance of cues in the other container, indicating lose-shift (Hosoi *et al*., 1995). The consistent selection for the control feed recorded in some individuals during two-choice preference testing is most likely to represent avoidance of the test feed, rather than inspective exploration. The short term sampling of the test feed within presentation periods is suggested to be the result of inspective exploration as a result of variation in inclusion levels and daily reversal of positioning.

The consistency of intrameal choice increases the likelihood of side preference being recorded subjects, if this was accompanied by intermeal consistency in container preference. During two-choice preference testing, the consistency of container selection during the 0-5 minute period and 5-10 minute period was influenced by taste cues in some individuals, rather than positioning cues. In individuals selecting for the test feed, intermeal consistency resulted in intake falling around the 0.5 mean due to the daily reversal in test feed positioning.

In addition to intrameal consistency, the second necessity for side preference to be recorded is intermeal consistency. Whilst intrameal variation may be explained by increased non ingestive behaviours and initial spontaneous reversal recorded in the Young group, the consistency of selection between meals will be subject to other factors.

It has been recorded in deer that a memory of a successful pathway to food enables them to maximise their feeding environment through spatial memory at patch level (Gillingham and Brunel, 1989). If subjects were aware of no difference between the feed presented in the two containers from prior experience this may influence selection. Memory of the feeding environment, and hence awareness of the homogenous properties of the two containers' contents, would benefit the horse, enabling maximisation of the feeding environment through reduced exploratory behaviour. It has been demonstrated in cows that whilst young subjects learn more rapidly, memory is retained for longer in older subjects (Kovalcik and Kovalcik, 1986). The study of mental capabilities in horses demonstrated a lack of ability to retain short term spatial memory (McLean, 2004) and, hence, would bring into question the ability of the horse to select for side, based on a memory of a successful pathway to food (Gillingham and Brunel, 1989). The McLean (2004) study was conducted over 3 days, in comparison to a total of 29 days in the present study. The retention of memory in the present study may have been aided by consistent presentation of containers over a longer period.

The persistence of a positioning choice in Old subjects would have resulted in improved associations between the consequences of ingestion from either the left or right container or control or test feed being formed. Exclusivity of choice followed an inter trial decline in sampling in Old subjects. The fragmented behaviour exhibited in the Young group may have caused learning based on taste cues to be confused due to sampling from both feed options and the exhibition of non ingestive behaviour. Monotony and consistent behaviour in the Old subjects may account for intermeal consistency, influenced by the win-stay strategy (Hosoi *et al.*, 1995), with the Old subjects exhibiting an earlier onset of side preference during the training period in comparison to the Middle and Old groups and hence, a greater degree of side preference was recorded in the Old group. Cognitive function has been shown to decline with age in humans, and, if applicable to all mammalian systems, this may result in reduced learning ability to form associations with age in the horse. The horses allocated to this study

were not believed to be in the late stages of senescence and were presumed not to have been subject to such losses.

The lower incidence of behavioural transitions recorded in the Old group, in comparison to the Young and Middle group, across the study is suggested to be the result of several factors. Sampling, evident between the two containers during the initial stages of each presentation period, was suggested to be the result of an inter trial decline, was followed by consistency of container intake for the remainder of the presentation period. This suggests that the Old group were exhibiting the 'win-stay' strategy (Hosoi *et al.*, 1995) where consistency for a choice was the result of reduced costs as a result of changes in behaviour. The consistency of container intake resulted in a higher total daily intake in the Old group, providing a possible benefit for the exhibition of side preference.

The validity of two-choice preference testing is reliant on the selection around a mean of 0.5, when no preference is shown. It is assumed that when two identical feeding choices are positioned simultaneously, due to a lack of choice, selection will be equal from each container. This study brings into question the validity of this assumption in relation to this study and hence, the adoption of two-choice preference testing to determine taste response using the test chemicals presented in this study.

Based on the findings of this study, it is perhaps necessary to consider the age of horses allocated to two-choice preference testing. Variation in motivational state was apparent between age groups. Motivational state was also recorded to alter within presentation periods, with an increase in ingestive behaviour with time. It is necessary to consider the implications of changes in motivational state on behaviour during presentation period. An approach to overcoming side preference or preventing its influence on the results of two-choice preference

testing would be to present test foods which animals select based on consequence, rather than hedyphagia alone. This would enable animals to satisfy the goal of selecting for, or avoiding a consequence, hence, over-riding side preference.

Whilst the results of these studies were affected by side preference, the importance of an initial training period to enable ingestive behaviours to replace exploratory behaviours cannot be ignored. Two-choice preference testing remains a suitable method for determining preference in the horse, provided cues are presented which are discernable to the horse and result in a change in behaviour.

6.5 Conclusions

Side preference is proposed to be the result of consistency in intrameal and intermeal positioning choice, influenced by the win-stay strategy (Hosoi *et al.*, 1995) and learning. Such consistency is reliant on persistence of ingestive behaviour rather than exploratory behaviours within presentation periods and consistency of choice between presentation periods. The higher degree of side preference in the Old group is suggested to be the result of consistency of container selection based on learned associations of no consequence of selection based on position, enabling them to maximise their feeding environment. The cost of selecting for side, such as ingestion of a least preferred food option, must convey a benefit for the behaviour to persist. Increased probability of behaviour transitions and lower ingestive motivation in the Young group increased the probability of a consistent container choice not being made, resulting in a lower incidence of side preference. The Middle group demonstrated less probability of behavioural transitions in comparison to the Young group, yet less consistency of choice was recorded, suggesting the Middle group are selecting for choice.

The conclusion from this study is that the effect of age on the incidence of side preference is the result differences in motivational state, and hence behaviour, between age groups. The Young subjects show greater non ingestive behaviours and behavioural transitions, increasing the likelihood of intrameal container variation. The Middle group demonstrate a preference for choice, rather than exclusive choice and may be attempting to satisfy a patch feeding motivation (Goodwin *et al.*, 2005). The Old group demonstrated rapidity of exclusive choice and appear to exhibit monotony in the feeding environment and as such, exhibit more ingestive behaviour.

With increasing time during the presentation period, fragmentation in behaviour reduced, indicating a choice was being made, even if only transient in the Young

group. It is concluded that while side preference may appear to have no apparent evolutionary benefit, it may enable the maximisation of the feeding environment in Old subjects.

Chapter 7:

Discussion and future work

Changes in the olfactory and gustatory system with age are recognised in humans (Chauhan *et al*., 1987; Murphy and Gilmore, 1989; Stevens and Cain, 1993; Finkelstein and Schiffman, 1999; Kaneda *et al.*, 2000; Mojet *et al*., 2001; Mojet *et al*., 2005) and dogs (McCune, 2001). Such changes are characterised by an initial development, followed by maturity then senescence. Whilst the gustatory and olfactory systems of the horse are stated to be similar to those of other mammalian species (Pfeiffer *et al.*, 2000), changes in the functioning of the gustatory and olfactory systems with life stage are not yet established in the horse.

The horse is reliant on the olfactory system for the identification of airborne chemicals and on the gustatory and olfactory systems for identification of solution based chemicals (Scott, 2001). The gustatory and olfactory systems are therefore intrinsically involved in the perception of flavour. Appropriate functioning of the taste system is essential for nutrient acquisition and toxin avoidance in the horse, and as such, is fundamental to survival. Any age associated decline in gustatory or olfactory function, as identified in humans, has consequences for diet selection and consequently intake. Compromised functioning of the gustatory and olfactory response has implications on welfare and performance of the elderly horse, of increasing relevance due to increased longevity in the horse (Harris, 1999; Hintz, 1999 b; King, 2000). Reduced intake, and consequent weight loss, is a recognised problem in older horses, commonly attributed to poor dental condition. Reduced appetite as a consequence of reduced gustatory and olfactory function, as recognised in elderly humans, may also be involved in reduced feed intake in older subjects, in addition to poor dental condition. Changes in the human and rat gustatory and

olfactory systems with age have been attributed to changes at the receptor, neural and cognitive levels as a result of chronological and physiological age.

Initial preliminary studies were conducted to develop the techniques to identify taste and gustatory response in the horse. The materials and methods employed in Chapter 4 and Chapter 5 were developed in these initial studies. The pilot trials identified side preference in individuals and suitable inclusion levels were identified as those that were appropriate to identify taste and gustatory response and sufficiently strong enough to reduce the incidence of side preference in individuals. Following the pilot trials, two-choice preference testing and monadic testing were deemed appropriate to establish individual taste responses for the purposes of age comparison. The test chemicals used to evoke taste response in Chapter 4 (artificial orange) and Chapter 5 (glycine) were selected due to their non-nutritive value in order to prevent taste response based on nutritive consequence

No significant effect of age on taste response was recorded in Chapter 4. Individual ability to discriminate between the orange test feed and control was identified in individuals, recorded as avoidance, but inclusion level had no significant effect on intake. No significant effect of age on gustatory response was recorded in Chapter 5. As in Chapter 4, individual discriminatory ability was recorded towards glycine, with both individual preference and avoidance recorded, but change in inclusion level had no significant effect on intake. Significant side preference was recorded in individuals in both studies.

The control preference recorded during two-choice preference testing may be influenced by a conditioned preference during training and avoidance of the novel test feed. Preference exhibited for the novel orange test feed by one individual may reflect phenotypic variation in preference, suggested to convey an

ecological advantage due to distribution of resource demand. A preference for the glycine supports findings of a sweet preference in the horse (Randall *et al.*, 1978), whilst an aversion to glycine supports intraspecies variation in preference. Therefore, individual variation in preference may result from genetic variation or conditioning from prior exposure and as such needs to be considered when developing flavours for inclusion in horse feeds.

The findings of these studies suggest that due to the lack of positive or negative nutritive consequences of ingestion of the test feed, there was no motivation for a behavioural change to occur. This was coupled with the presentation of differing levels of inclusion rate and with daily reversal of container position, believed to reduce the ability of horses to make consistent choices over time.

A study by Cairns *et al.* (2001) has previously established the association between flavour and postingestive consequences in horses. It is therefore proposed that if the taste cue was associated with a consequence, the horses would be motivated to change their behaviour. This would potentially diminish or remove the occurrence of side preference. It is also recommended that one inclusion level be employed to determine taste response, enabling consistency in the presentation of the test feed. Alternatively, test chemicals to determine taste response could be presented with water as the carrier, suggested by Randall *et al*. (1978) to increase sensitivity in the horses towards the test feed due to less confounding factors from the orosensory characteristics associated with the basal feed.

Whilst electrophysiological and morphological studies would enable the study of olfactory and gustatory response in isolation of confounding factors, this would fail to adequately represent behavioural response. Additionally, the influence of prior experience or learning on taste or gustatory response would not be

achieved using these methods. A longitudinal study, though impractical here due to time and cost implications, would enable the age related changes to be studied throughout the development, maturation and senescence periods, with the individuals acting as their own controls. In order to accurately determine whether an age related decline in gustation and olfaction occurs in the horse, morphological and physiological studies of the gustatory and olfactory systems are necessary. Additionally, morphological and physiological studies would enable investigation of the development in the gustatory and olfactory systems during the postnatal period in the horse.

A limitation of this study was the lack of availability of study horses in the late stages of senescence. It is suggested that the horses allocated to the Old age group were not undergoing a dramatic decline in gustatory and olfactory function as is characteristic of late senescence in other mammalian systems. The use of older subjects would enable a predicted gustatory and olfactory decline to be studied. A confounding limitation associated with this would be the concurrent decline in cognitive function with age, potentially compromising validity of results.

Differences between the exhibition of ingestive behaviour and non ingestive behaviour were recorded from analysis of behavioural data collected in Chapter 4. The Young group exhibited greater fragmentation in behaviour transitions compared to the Middle and Old group. Evidence of an intra session decline in behavioural transitions was recorded from 0-5 minutes to 5-10 minutes. The difference in motivational state between age groups resulted in differences in behaviour responses being recorded.

The Old group demonstrated the most consistency of behaviour throughout the study. It is suggested that for the Old group to alter their behaviour in response to a test feed, the consequences would need to be sufficient to alter their motivational state from monotony to sampling behaviour. The motivation to change feeding behaviour is reliant on the cue strength, derived from external factors such as sight and taste of the food, and the energy state signals to be sufficient to cause an alteration in motivation (Toates, 1980). The reduced behavioural transitions in the Old group provide evidence of a lack of motivation to alter behaviour in response to the gustatory or olfactory cues presented in the test feeds. Costs are involved in changing behaviour and it is suggested that the benefit of less behavioural transitions recorded in the Old group resulted in an increase in total daily intake as a result of consistency of ingestive behaviour, rather than non ingestive behaviour and fragmented locomotory behaviour. This consistency and rapidity of choice is suggested to increase the incidence of side preference in Old subjects.

The Young subjects exhibited the greatest probability of change in behaviour in the presentation period. A higher incidence of non ingestive behaviours was followed by an increase in ingestive behaviour with repeated days. Initial sampling between containers was recorded in all age groups, but was most pronounced in the Young group. The presentation of the novel test feed and alterations in container presentation evoked inspective exploration, this may have been coupled with spontaneous reversal following the introduction of novelty. The Middle group exhibited behaviour most similar to the Old group, with more consistency in behaviour compared to the Young group recorded. This age group may therefore represent the stage between high exploratory behaviour in young horses and monotony in older horses.

This study was based on all subjects having the same motivational state, though it was evident from behavioural analysis that this was not the case. Difficulties may arise when such a study is conducted in Young subjects due to their increased response to novelty in comparison to the Old subjects. The training period attempted to equalise motivational state prior to the onset of two-choice preference testing. The nine-day training period did meet the aims of replacing exploratory behaviour with ingestive behaviour. It was evident that with presentation of the novel test feed and monadic presentations, a decrease in ingestive behaviours were most pronounced in the Young group. These findings suggest a conflict between feeding motivation and exploratory motivation due to the unacceptable nature of the feeding choices (Kyriazakis, 1997), suggesting increased avoidance of the test feed.

Two-choice preference testing is reliant on intake around a theoretical mean of 0.5 when no choice is presented. However, it was found that in some individuals the positioning cues, left and right, represented a choice. A high incidence of side preference was recorded during the training in Chapter 4, which persisted even with the presentation of additional taste cues.

The high incidence of side preference has implications for the validity of employing two-choice preference testing when determining preference in the horse, in particular the identification of taste thresholds in this study. Further study is necessary to explore alternative methodologies or revisions to two-choice preference testing to determine taste response in the horse in the absence of side preference. As stated earlier, it is proposed that the presentation of flavours associated with postingestive consequences may result in taste response over-riding side preference. Alternatively the presentation of short term two-choice preference testing, studying latency to approach, may enable the response to orosensory cues to be determined, without the fixing of positional choice identified during this study.

The study found no effect of age on gustatory and taste function in the horse, as might have been predicted from other mammalian systems. The absence of an identified effect of age may be attributable to the limitations of the study design, namely: the lack of positive or negative consequence following ingestion of the test chemical; the use of inappropriate and varying inclusion levels of the test chemical; the differences in motivation between age groups; and the allocation of Old horses suggested not to be subject to the late stages of senescence. Whilst the validity of two-choice preference testing has been questioned in relation to this study, if the test chemical presented provided a reason for change in behaviour, such as a nutritive consequence, then it is predicted that the limitations associated with selection of feed based on container position may not arise.

Further study is therefore necessary to identify if, as characterised in other mammalian systems, gustatory and olfactory function are affected by age in the horse. A loss of gustatory and olfactory response in the horse has profound implications for the ability of the ageing horse to make appropriate diet selection, and consequently for welfare. Although the need for appropriate diet selection is less relevant to the stabled horse due to human determination of diet, hypophagia is a common observation in the horse. Reduced food intake is of concern, not only in terms of potential weight loss, but also the increased risk of nutrient deficiency that may exacerbate an already reduced taste function.

Taste is involved in both short and long term control of food intake, influencing appetite (Goatcher and Church, 1970b; Rolls, 1999; Schiffman, 2000). It is suggested that reduced food intake in elderly horses, commonly attributed to poor dental condition (Ralston, 2001), may be additionally affected by reduced appetite as a result of a reduction in taste sensitivity. Amplification of flavour presentation in elderly humans subjects identified to have a reduced taste

response significantly increased their food intake (Schiffman, 2000). Similar findings have been demonstrated in dogs (McCune, 2001). It is suggested, on the basis of the findings of this present study, that further research is necessary to identify if such changes in gustatory and olfactory function occur in the horse. If this work were to identify a decline in taste and gustatory function with age, application of such findings would support the amplification of flavour inclusion levels in older horses, alongside the presentation of more liquid feeds to counteract the effects of reduced dental condition.

Increased flavour inclusion may have an appetitive effect on hypophagic horses of all ages and again, warrants further study. The greater behavioural fragmentation recorded in the Young group, in comparison to the Middle and Old groups, suggests that further study of flavours to encourage consistency of intake in the Young group may be appropriate, particularly to counteract novelty in the feeding environment.

A further development from this present study would be an investigation of the effects of the removal of additional feed-related gustatory cues on intake and behaviour in the horse, in order to study olfaction in isolation of gustatory cues. The addition of olfactory cues to the feeding environment has previously been employed in cattle (Corley *et al*., 1999) and therefore may represent a suitable method to determine olfactory response in the horse and hence identify any age effect on function. As with the study of taste response, the odour would need to evoke a change in behaviour to prevent selection based on positioning cues.

Whilst assumed to be similar to those of other species, further investigation of the anatomy and physiology of the gustatory and olfactory systems in the horse is necessary. Moreover, investigation of morphology and physiology of the

gustatory and olfactory systems during the development, maturation and senescence stages would enable more valid conclusions to be drawn.

With the average life span of the domestic horse increasing due to improved awareness of welfare and the role of the horse as a leisure animal there is an increasing justification for the study of the effects of advancing age on body systems. Moreover, alongside the need to study age-related decline in gustatory and olfactory function, further research of the equine gustatory and olfactory systems is necessary to ensure that assumptions about anatomy and function, based on comparative studies, are valid.

The importance of the role of gustation and olfaction to the horse in terms of short and long term controls of feeding behaviour results in any decline in function having severe implications on appropriate diet selection, and consequently welfare.

Chapter 8:

Conclusions

There is a paucity of research relating to the gustatory and olfactory systems in the horse. However, their effective interaction is fundamental to appropriate diet selection in the horse, both in the short and long term. An age-related decline in taste and gustatory systems in the horse is suggested from findings in other mammalian systems, and if proven, would have potential implications for appropriate diet selection, and consequently welfare, in the ageing horse. This study failed to identify an effect of on taste response or gustatory response in the horse.

Whilst a significant response was recorded in individuals towards the test feeds in both the study of the effect of age on taste and the study of the effect of age on gustation, no significant effect of inclusion levels on intake was recorded. This is proposed to be the result of limitations in the study design, rather than a conclusive finding that no effect of age on gustatory and olfactory function in the horse occurs in the horse. Further study using techniques suitable to meet the aim are recommended, alongside morphological and physiological study to determine whether changes in the gustatory and olfactory system of the horse occur with age.

The domestic horse is subject to increasing longevity and is increasingly exposed to the negative consequences associated with senescence. Reduced intake with age is a common problem in the older horse; however, a potential application of the findings of this study would be the amplification of flavour to increase intake, not only in older horses, but also those subject to hypophagia.

A difference in behaviour was recorded between age groups, with the Young group exhibiting greater behavioural fragmentation compared to the Middle and Old group. This finding supported greater exploratory behaviour in the Young group, compared to greater ingestive behaviour in the Old group. The incidence of side preference is suggested to be the result the methodology employed, rather than two-choice preference testing in general.

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