The role of learning in the selection for dietary protein by sheep

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Dedication

This work is dedicated to my parents Evagelia and Ioannis for encouraging me to set my goals high.

"Man is the measure of all things"

Protagoras (480-411 BC)

Abstract

The purpose of this thesis was to investigate in a series of five experiments the role of learning in the selection for dietary protein by sheep

Experiment 1 investigated whether an 'unlearned' appetite for dietary protein exists in sheep, and whether such an appetite depends on the previous protein feeding of the animal and the source of dietary nitrogen. No evidence was found for such an unlearned appetite; selection for protein was a learned response, which depended on the source of dietary protein of the foods offered as a choice. Subsequently, emphasis was given to the dietary factors that affect learning to select for dietary protein by sheep. This was done by the use of a classical conditioning methodology.

Experiment 2 tested the hypothesis that a continuum links the development of learned preferences and aversions towards a food flavour associated with the post-ingestive consequences (PIC) induced by the administration of a rumen degradable protein (RDP) source. The results showed that sheep are able to distinguish between two flavoured foods associated with increasing doses of a RDP; whereas small doses led to the conditioned flavour preferences (CFPs), higher doses led to conditioned flavour aversions (CFAs). It was concluded that the diet selection for protein by ruminants could be influenced quantitatively by RDP provision. A model to account for the conditioned response of an individual sheep towards administration of increasing doses of the same nutrient was put forward

Experiments 3 and 4, tested whether a delayed type of learning could account for the development of CFPs and CFAs by sheep towards food flavours associated with PIC induced by the administration, at different points in time, of either a low or a high dose of RDP, respectively. The results showed that the temporal contiguity between the consumption of the flavoured food and the administration of the low or the high dose of RDP did not affect the development of either CFPs or CFAs. It was concluded that a process of delay learning applied to both the development of CFPs and CFAs by sheep.

The final experiment tested whether sheep are able to develop conditioned responses for food flavours when they are associated with a protein source that is undegradable in the rumen, but readily digestible (DUP) within the post-ruminal digestive tract. In particular, the relative importance of either RDP or DUP as well as their concurrent administration (RDP+DUP) in the development of conditioned responses were investigated. Sheep developed CFPs towards food flavours associated with the administration of both RDP and DUP. In addition sheep were able to distinguish between food flavours associated with the administration of either RDP or DUP; they preferred flavours associated with DUP over flavours associated with RDP. However, such preferences did not develop when DUP was administered concurrently with RDP.

The results of the above experiments were characterised by a considerable variation in the feeding responses between individual sheep. To see clearly the effects of the variation between individuals on the diet selected by groups, a model of group diet selection was developed. Based on simple assumptions about the characteristics of the individuals in a population, including the variation between them, the model confirms the view that their feeding responses could be masked by averaging the data from animals which are treated the same. Possible extensions of the model to allow such variation to be accurately estimated were proposed.

The role of conditioned feeding responses in the control of food intake and diet selection of ruminants for a diet, which meets their requirements at a particular point in time, is considered in the General Discussion.

Declaration

I, hereby declare that this thesis is of my own composition, and that all assistance from other people has been duly acknowledged. The results presented herein have not previously been submitted for any other degree or qualification.

Georgios Ioanni Arsenos

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Chapter 1

General Introduction

"Their food depends chiefly on the substance of which they are severally constituted; for the source of their growth in all cases will be this substance. And whatsoever is in conformity with nature is pleasant, and all animals pursue pleasure in keeping with their nature"

Aristotle, (History of animals, 350 BC).

1.1 General context

1.1.1 The overall problem: The feeding behaviour of farm animals

Farm animals (and animals in general) require many nutrients, in various amounts and proportions, to fulfil their needs for maintenance, growth and reproduction. Of the many materials, which serve as food for animals, few are capable of supplying all the required nutrients. Most consist of several, often numerous, different chemical compounds, the relative levels of which can vary substantially even among different items of the same food type. Similarly many materials are toxic or harmful if eaten, or eaten in large quantities. If the animal is to meet its nutrient requirements, and avoid toxicity, it has to make a choice or choices from the available foods (Emmans, 1991; Forbes, 1995; Kyriazakis, 1997).

The nutritional choices available to farm animals relate to one of the following two questions: (i) how much to eat and (ii) what to eat over a particular time period (see review by Kyriazakis, 1997). The first question applies mainly to the simple feeding situation where animals are given free and continuous access to a single homogeneous food. In this system the only way available to the animal to regulate its diet is to eat more or less of the food. Both questions apply to situations where animals are given access to two or more foods as a choice or in extensive systems (Kyriazakis, 1997). In such cases farm animals are faced, to a greater or lesser extent, with foods that are more likely to differ in their nutritional or other characteristics. For example, foods may differ in their nutrient and possible toxin contents, (Provenza, 1996), their organoleptic or sensory properties (Forbes and Kyriazakis, 1995), or even in their availability, which might change over temporal and spatial scales (Provenza, 1995a,b; Illius and Hodgson, 1996).

Irrespective of whether farm animals have a limited degree of choice or unlimited choices, their feeding behaviour is then a description of what an animal does when it encounters food. The prevailing view in the literature, regarding their feeding behaviour, is a functional one based on the evolutionary idea of fitness, i.e. rapid growth and production of viable offspring, whilst minimising any disadvantages which may be incurred (Rozin and Schulkin, 1990; Forbes, 1995; Emmans and Kyriazakis, 2000). For example, grazing ruminants will select a diet that is higher in nutrients and lower in toxins than would be ingested if selection was random (see Provenza and Balph, 1990; Provenza, 1995a,b). Also, animals will select a diet that meets their requirements, even when the foods available as a choice have not been previously encountered by them in their natural feeding environment e.g. a choice between a high and a low protein concentrate food offered to sheep (Kyriazakis and Oldham, 1993).

The desire to achieve a better understanding of the control and to predict the feeding behaviour of farm animals has been a long-standing one. It has been suggested that the successful predictions of feeding behaviour of animals require detailed information about the system to be described (Emmans and Fisher, 1986). Recently, the problem of predicting diet selection of farm animals in a given set of circumstances, has undergone considerable revision. This has occurred in the light of new scientific knowledge and due to the ecological and the agricultural importance of farm animals (see reviews by Leng, 1993; Faverdin and Bareille, 1999; Forbes, 1999; Steffens and Benthem, 1999; Tolkamp, 1999).

Studies of feeding behaviour in natural environments concentrate on describing what the animal chooses to eat when it forages and has access to foods that are part of its natural feeding environment (Kyriazakis et al., 1999). The difficulties in describing adequately the

feeding, social and thermal environments in nature (Emmans, 1991; Perry and Pianka, 1997; Raubenheimer and Simpson, 1997 and 1999) have led workers in the field to study the diet selection of animals that have a very narrow feeding repertoire (i.e. specialist animals) or to collapse the problem of diet selection to that of a single resource, almost invariably energy (Westoby, 1974; Krebs et al., 1981; Stephens and Krebs, 1986). However, this is not considered sufficient now and the understanding and prediction of diet selection in natural environments still remains a challenge. As Illius and Hodgson (1996, p. 438) stated:
"Investigations of diet choice and foraging behaviour must therefore seek explanations, and not be content with description".

The fact that more controlled environmental conditions can be achieved in the laboratory, where animals are given access to few foods whose composition can be adequately described, enables a detailed study of diet selection (Emmans, 1991; Kyriazakis et al., 1999). This strategy was followed also in the early studies of diet selection suggesting that animals exhibit a degree of selection amongst available foodstuffs and somehow control their intake of nutrients (Dove, 1935; Richter, 1943; Lat, 1967; Overmann, 1976).

The common, but not universal (e.g. Galef, 1991) assumption made in such studies is that, even under such partly artificial conditions, the diets selected by the animal will represent the general adaptive nature of feeding behaviour (Siegel, 1993). The behaviour under such conditions can be called 'preferred diet selection', since it is the feeding behaviour shown by the animal when 'constraints' arising from the (feeding) environment are removed (Nielsen, 1999). This behaviour forms the starting point from which various controlled modifications of the environment, including the feeding environment, can be imposed in order to investigate which features and variables matter, and how they influence diet selection (Kyriazakis et al., 1999). This approach has been applied with an appreciable degree of success (e.g. Kyriazakis, 1997; Kyriazakis et al., 1998a; Day et al., 1998; Hutchings et al., 1999) to develop frameworks of feeding behaviour, which allow feeding behaviour (in terms of food intake and diet selection) to be both explained and predicted. Any 'rules of diet selection' which emerge from such work are expected to be general, to be characteristic of the animal in the same way as its physiology is characteristic, and to apply in more complex natural environments (Kyriazakis et al., 1999).

Good theories seek to be general. For example the idea of 'eating to meet requirement subject to constraints' that goes back at least to Adolph (1947) has been criticised especially for ruminants (see Grovum, 1987; Tolkamp and Ketelaars, 1992), whereas others have progressively diluted it (see Forbes, 1996; Illius and Jessop, 1996; Ellis et al., 1999; Illius and Gordon, 1999). While large amount of research effort over many years has been aimed at identifying the nutrient requirements of farm animals and investigating whether they are able to select a diet that meets their requirements, there has been less effort put into understanding how farm animals reach decisions related to their feeding behaviour both in terms of food intake and diet selection.

The idea behind this thesis is that the factors, which have an influence on the animal's ability to reach at such decisions, must be identified. This approach should give us insights on how various factors influence and modify feeding behaviour, within the goal of arriving at more robust predictions of both food intake and diet selection. The thesis focuses on the particular question of how ruminant animals, in particular sheep, select for the protein content in their diet. The reason for choosing to deal with this particular question will be explained in the following sections of this Chapter.

The aim of this chapter is to present an overview of the theoretical and experimental evidence on the ability of animals to control their food intake and diet selection. The emphasis will be on studies with ruminant animals, but, the results from studies with other farm or laboratory animals will be used where necessary. A more detailed description of the relative literature on ruminant animals will be given in each of the experimental chapters. This chapter is, thus, intended both as an introductory presentation of the literature and as an attempt to set the ground for the themes to be dealt with in the experimental Chapters.

1.1.2 What are the underlying hypotheses?

Currently, there are two hypotheses to account for how animals control their feeding behaviour both in terms of food intake and diet selection. The debate concerns the relative importance of unlearned (instinctive) and learned responses towards food and is often referred to as nature (genes) versus nurture (environment) since the factor influencing learned behaviours is the environment in which the animal lives (Staddon, 1983; Owen, 1990; Rozin and Schulkin, 1990).

The first hypothesis stems from the idea that there are evolutionary advantages for some behaviours to be genetically pre-programmed because there is unlikely to be a second chance to learn them (Staddon, 1983). Studies, in which animals are deprived of particular nutrients, have typically been used to help distinguish between unlearned and learned feeding responses. A common paradigm for these studies is to induce a dietary need for a particular nutrient through the feeding of a diet deficient in the nutrient and then to allow the animal to select between the deficient food and a food supplemented with the needed nutrient. The fact that nutrient deficiency leads to an increased preference for foods that are rich in the required nutrient led to the idea of 'unlearned appetites' which are defined as an inherited ability of animals to readily identify a food rich in a particular nutrient in demand almost instantaneously and without any previous experience on it (Harris et al., 1933; Richter, et al., 1938; Richter, 1943; Scott et al., 1950; Rozin, 1966 and 1976; Garcia et al., 1974). The results of these studies led Hughes, (1979) to further suggest that animals possess a series of unlearned appetites for many specific nutrients. The appetite for sodium (Richter, 1943; Denton, 1982; Schulkin, 1991) and calcium are the most characteristic examples of unlearned appetites (Wood-Gush and Kare, 1966; Hughes and Wood-Gush, 1971).

The second hypothesis assumes that unlearned appetites should not be the normal means by which an animal selects its diet given that animals have a requirement for a large number of nutrients in their diet. The idea is that animals need to have some knowledge of the consequences of eating a food in order either to include or exclude it from their diet. Animals acquire such knowledge through behavioural mechanisms they have developed. These mechanisms allow them to recognise foods on the basis of their nutritional as well as other properties and to include them in their diet according to the post-ingestive consequences (PIC) they have (Provenza and Balph, 1990; Forbes, 1995; Provenza, 1995c; Kyriazakis, 1997; Kyriazakis et al., 1999). In particular for ruminant animals, Provenza (1995c) suggested that they learn which food to prefer or avoid by developing learned associations between food flavours and the PIC induced by the ingestion of foods. Such learning provide the opportunity for ruminants to predict what will be the consequences of eating a food and there is strong evidence of its role in diet selection (Kyriazakis et al., 1999). Some more detailed accounting of the literature dealing with the relevance and influence of learning on the feeding behaviour of animals is given in Section 1.3. However, the exact boundaries between unlearned and learned appetites and their role in diet selection of farm animals are not clear.

1.2 Unlearned or learned appetite for protein?

In the preceding sections it was indicated that our knowledge about how animals select for the nutrient content in their diet is limited. There is no solid evidence to back either ideas of unlearned or learned appetites (Le Magnen, 1985). Apart from the evidence of unlearned appetites for sodium and for calcium, as mentioned before, there is remarkably little firm evidence of unlearned appetites for other nutrients. In the particular case of diet selection for protein the evidence remains inconclusive. For example, experiments with rats support both the ideas of a learned appetite (Baker et al., 1987; Gibson and Booth, 1986) and of an unlearned appetite (Deutsh et al., 1989) for protein.

The evidence from non-ruminant farm animals is consistent with the idea that they are able to select a diet which is a reflection of their protein needs, whilst at the same time avoid an excess of protein intake and therefore, their feeding responses towards foods with different protein content are predictable to a great extent (Forbes, 1995 and Kyriazakis, 1997). For example, strong support, to back the latter view, comes from experiments with pigs (Kyriazakis et al., 1991; Kyriazakis and Emmans, 1992a,b; Kyriazakis and Emmans, 1991 and 1993).

The evidence, however, from ruminant animals does not always seem to be consistent with the above suggestion (for a debate see Kyriazakis et al., 1999). Previous research has shown that ruminants in general, and sheep in particular, are able somehow to regulate their food intake and select a diet that reflects their protein needs according to their physiological state (e.g. Cropper, 1987; Kyriazakis and Oldham, 1993; Cooper et al., 1994; Kyriazakis et al., 1994; Hutchings et al., 1999). However, one unresolved issue is how do they come to select such a diet? For example, there is no evidence in the literature that an unlearned appetite for protein exists in sheep or other ruminants. The issue becomes more complicated because there are a number of striking peculiarities which make the process of nutrient digestion and metabolism, and protein in particular, distinctive in ruminant animals when compared with non-ruminants (McDonald et al., 1988; Van Soest, 1994).

1.2.1 The digestive peculiarities of ruminant animals

The ability of an animal to derive nutrients from the food it eats is determined by the chemical composition of the food and the digestive system of the animal that eats it (Mc Donald et al., 1988). It is the peculiarities of their digestive tract that enable ruminants to take advantage of the symbiotic micro-organisms in the rumen that can ferment fibrous materials to derive energy and can synthesise microbial protein from non-protein nitrogen sources that can be used to supplement low protein foods (Ørskov, 1988; Van Soest, 1994). Ruminants can also use the endogenous N, that is returned into the rumen as urea; such urea is further hydrolysed and used as a N source for microbial protein synthesis (Firkins et al., 1992; BrunBellut, 1997; Sarraseca et al., 1998). There is no doubt that such peculiarities have not only an ecological importance but also major economic consequences.

The structure and function of the digestive tract and its associated organs for the metabolism of N in ruminants results in two interrelated sets of N requirements: those of the rumen microbes and those of the host animal (Van Soest, 1994). Despite these peculiarities, N needs of the tissues of ruminants, as in most other animals, are met by amino acids, which are absorbed from the small intestine (see reviews by Oldham, 1993; Oldham et al., 1997). Ruminants have evolved to rely substantially on rumen synthesised microbial protein as a source of essential amino acids (Van Soest, 1994). The rumen microbes further modify the composition of the dietary protein sources *en route* to the post-ruminal digestive tract. They can degrade dietary protein yielding peptides, amino acids and ammonia (NH_3). The achievement of a maximal utilisation of dietary protein and its conversion into microbial protein is correlated with its supply of nitrogenous substrate to microbes in the rumen (Hume et al., 1970; Lewis and Hill, 1983). It has been suggested that this might vary between different sources of dietary protein or because of other components of the diet or the level of feeding (Ørskov, 1988). These processes affect both the quantity and the quality of the amino acids available for absorption in the small intestine (Wallace et al., 1999). This has led to the protein sources of food eaten by ruminants being divided into two types: (i) rumen degradable protein (RDP), which mainly provides the N for microbial protein synthesis, and (ii) digestible undegradable protein (DUP) which directly supplies amino acids for digestion and absorption in the small intestine. The sum of DUP and that fraction of RDP converted into digestible microbial protein is known as metabolisable protein (MP) which is considered to be the best estimate of the supply of amino acids to the ruminant animal (AFRC, 1993). Therefore, amino acids are supplied in part by the microbial protein synthesised in the rumen, and in part by dietary protein which escaped the 'attack' by the microbes in the rumen and remained intact. The extent of microbial protein synthesis depends mainly on the availability of N and energy to the rumen micro-organisms (Leng and Nolan, 1984; Kyriazakis and Oldham, 1997; Oddy et al., 1997; Wallace et al., 1999).

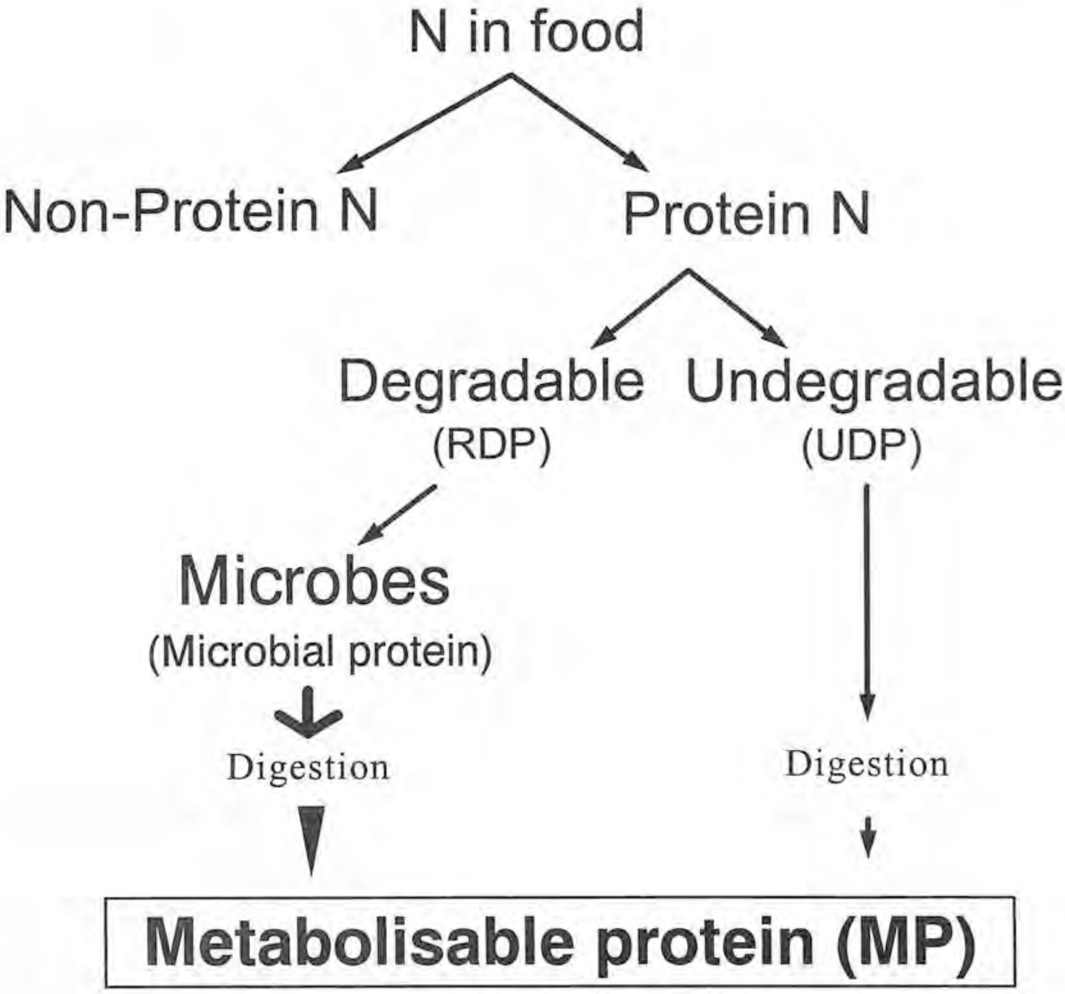


Figure 1.1 Schematic diagram of nitrogen metabolism in ruminant animals (AFRC, 1993).

The above definitions of dietary protein for ruminants, shown in Figure 1.1, will be widely used in this thesis because it is believed that they provide a useful basis on which to discuss how the peculiarities of their digestive physiology modify their feeding behaviour. This will help us to understand how ruminants reach decisions related to their feeding behaviour both in terms of food intake and diet selection for protein. For example, as mentioned earlier, the answer to the question of whether sheep, or other ruminants, have an inherited ability to select for protein in their diet or whether they have to learn to do so, is still unclear. The following sections will briefly introduce some of the major factors, which are though to affect learning about foods.

1.3 Learning about foods

Learning is the modification of behaviour in response to specific experiences and there are several types of learning that may occur in an animal's life (Staddon, 1983). Once we shift the focus on the animal's ability to learn about foods, we must first consider the different types of information that the animal accumulates throughout its life. Several classification schemes (for example, Mathews and Kilgour, 1980; Arnold, 1981; Chapple and Lynch, 1986; Provenza and Balph, 1987; Provenza, 1994; Forbes, 1995) have been proposed to categorise the different types of learning about foods in ruminant animals. It is clear however, that learning about foods may be seen as a result of both social factors and learned associations.

1.3.1 Social factors

Galef (1996) stated that an understanding of the feeding behaviour of animals requires not only knowledge of the physiology of the individual, but also of the interaction between the individual and the ecological and social environments in which it feeds.

Sheep and other ruminants have evolved as a flock species and this is a key factor in terms of social transmission of information about what foods to consume or avoid. It has been suggested that learning through social interactions is markedly affected by factors such as dominance hierarchies, family bonds, age, and sex of the interacting individuals (Provenza and Balph, 1987; Provenza, 1994 and 1995a,b). A critical time for the development of an animal's feeding behaviour is during the transition from maternal-food-dependence to independent feeding. For example, young lambs can extract information about the mother's diet as early as *in utero* (Nolte et al., 1992) and subsequently, from the taste of the mother's milk (Nolte and Provenza, 1992). The consequences of these experiences shape an animal's preferences and aversions for foods and create an interplay between the familiar and the novel. Animals typically prefer the familiar to the novel and they generally regard anything novel with caution (Zahoric and Houpt, 1981). The conservative approach of ruminants towards novel foods, termed food neophobia, is considered to be a survival mechanism that protects them from the over-consumption of toxic compounds (Chapple and Lynch, 1986; Forbes, 1995; Provenza, 1996; Launchbaugh, 1996).

As animals grow they can obtain dietary information by following the mother or other adults to feeding sites (Galef and Stein, 1985; Provenza and Balph, 1987 and 1990; Provenza, 1994). Both timing of feeding and choice of food are partly affected in this way. Several studies suggest that animals may gain a large portion of their dietary information from animals within their social group (related or unrelated). Such learning is acquired when an individual's behaviour is influenced by observation of, or interaction with, another animal or its actions and it has been extensively documented in a broad variety of animal species (Provenza, 1994; Forbes, 1995; Galef, 1996; Nicol, 1996; Shettleworth, 1998). Shettleworth (1998) proposed that such learning allow individual animals to *"exploit the expertise of others"*. At the same time it has been suggested (see reviews by Matthews and Kilgour, 1980; Provenza and Balph, 1987 and 1990 Provenza, 1995a,b) that the interaction of an animal with its feeding environment generates a wealth of information about cause and effect, about the consequences of feeding, and such learning is characteristic for each individual animal.

Heyes (1994) stated that persistence of a socially learned feeding behaviour, in an individual animal, depends on whether the alternative, which is acquired through experience is equally rewarding. On the other hand, it has been suggested that individual and social learning are not independent processes but that interaction between these two learning processes leads to maintenance of adaptive feeding repertoires in individuals and diffusion of such feeding behaviour through populations (Slater, 1981; Sutherland, 1996).

1.3.2 Learning by association

Learning by association takes place whenever an animal is able to associate an external event with a change in its own internal state, or a change in its behaviour. An animal can also learn to associate an act that it performs with some kind of consequences. Theories of learning by association date back to Thorndike's (1911) 'law of effect', which states that learning depends on the consequences of a behaviour performed by the animal, and Pavlov (1927) who demonstrated that the phenomenon of learning includes inherited stimulus-response associations, which may become replaced by an otherwise neutral ('conditioned') stimulus. There are two major types of associative learning, the first is named operant or instrumental conditioning and the second one is named Pavlovian or classical conditioning. Thorndike (1911) and Pavlov (1927) respectively, are credited with initiating the experimental analysis of associative learning in animals and the notion is that the establishment of learned associations involves feedback and reinforcement (see Mackintosh, 1994). Feedback refers to information about the consequences, e.g. the PIC of foods, whereas reinforcement affects the tendency to demonstrate a specific response again. Feedback can be positive, negative or neutral; reinforcement is either positive (increases the response) or negative (decreases the response), (see reviews by Rescorla and Wagner, 1972; Rozin, 1977; Revusky, 1977; Mackintosh, 1994; Hall, 1994). The methodology used in this thesis makes it necessary for the rest of this section to describe Pavlovian of classical conditioning in more detail.

Pavlov (1927) was the first who studied learning in animals in an orderly and systematic way, using a standard series of techniques and a standard terminology to describe his experiments and their results. His experiments with dogs are classical. He found that salivary secretion was elicited not only by placing food in the dog's mouth but also by the sight and smell of food and even by the sound of a buzzer previously associated with the arrival of food. For Pavlov, at first, these 'psychic secretions' merely interfered with the planned study of the digestive system. But he then saw that he had a tool for the objective study of something even more interesting: how animals learn.

Pavlov's experiments on conditioning employed a standard, simple procedure. It was established that experience results in an animal being able to associate a stimulus having no specific meaning with some meaningful stimulus producing either positive or negative effects. As a result, on a following encounter, the response elicited previously only by the meaningful stimulus was then elicited by the neutral stimulus. In the literature, the meaningful stimulus is termed the unconditioned stimulus, whereas the neutral one that comes to be associated with it is termed the conditioned stimulus. The elicitation of a specific response by the conditioned stimulus is termed a conditioned response, the occurrence of which is reinforced by the presentation of the unconditional stimulus. The verb 'to condition' was soon introduced to describe the experimental activity (Rescorla and Wagner, 1972; Mackintosh, 1994; Hall, 1994).

Classical conditioning methodologies have dominated the research on animal learning mainly by using laboratory animals. It was revealed that they can encode and remember detailed information about the events they encounter (Rozin and Zellner, 1985; Mackintosh, 1994; Hall, 1994). Moreover, it was suggested that through classical conditioning, an animal not only learns to perform a behaviour in response to a stimulus that previously had no effect, but can store information about predictive relationships between different events and about specific conditions under which those predictive relationships might be modified. Thus, classical conditioning serves to prepare an organism for a forthcoming event (Mackintosh, 1994; Hall, 1994). One view is that conditioned responses provide the mechanisms underlying the formation of all behaviours, including feeding behaviour (Skinner, 1931).

1.3.2.1 What are the important cues?

Using a series of experimental manipulations, scientists tried to classify the important cues for farm animals (e.g. Kendrick, 1994; Keverne, 1995) and how they use their senses to exploit their feeding environment (e.g. Arnold, 1966; Orians, 1981; Provenza and Balph, 1987 and 1990; Provenza, 1994; Forbes, 1995). Kendrick (1994) made a detailed description of the ability of farm animals for visual perception of their environment. He suggested that animals could make use of this ability to discriminate between food and non-food items (see also reviews by Lomas et al., 1998; Piggins and Phillips, 1998).

Reliance on visual cues of food, amongst farm animals, is more pronounced in birds (Forbes, 1998). It has been demonstrated that chicks have a well-developed visual perception (colour or shape) of foods and can learn to prefer (Kutlu and Forbes, 1993) or avoid (Martin et al., 1977) specific foods on that basis. Similarly, ruminants can also use visual cues to locate and discriminate between food resources as shown in experiments with sheep (e.g. Illius et al., 1992; Illius and Gordon, 1993; Edwards et al., 1997; Dumont and Petit, 1998; Dumont et al., 2000) and cows (e.g. Laca, 1998; Howery et al., 2000). There is also evidence that farm animals might also use the position of a particular foods as a cue in their diet selection when offered a choice between two foods (e.g. pigs, Kyriazakis et al., 1990; chickens, Forbes and Shariatmadari, 1996; sheep, Kyriazakis and Oldham, 1993; and cows, Tolkamp and Kyriazakis, 1997).

According to Rozin (1976 and 1977) the most helpful cue in the 'omnivores dilemma' on which foods to choose is the flavour. Garcia (1989) stated that the flavour (taste and odour) of a food is the dominant cue for mammals in terms of encoding, storage and retrieval of information for food selection. There is some confusion about the exact terminology for the use of the word flavour for animal foods and how its attributes should be defined, mainly because such terminology originated from studies with humans (Bradley, 1978; Rozin and Schulkin, 1990).

Ruminants like other animals seem to rely heavily on food flavours to control their feeding behaviour. Provenza (1995c) stated that ruminants, like other animals, learn to distinguish between foods using flavours by either choosing or ignoring food flavours that anticipate nutrients or toxins respectively. On the other hand, there are also cases where ruminants generalise between different food flavours because of their resemblance, although these are associated with different foods (Early and Provenza, 1998) and cases where ruminants avoid foods simply because of their flavour (Augner et al., 1998). Augner et al. (1998) proposed that: *"the avoidance of foods with strong flavours may be an expression of a rule of thumb of the type given a choice, avoid food with strong flavours. Such a rule could be part of a risk-averse foraging strategy displayed by mammalian herbivores, and which could be of particular importance when they encounter unfamiliar foods"*.

It has been suggested that a range of genetic, physiological and metabolic variables can influence the responses of animals to the flavour of foods (Le Magnen, 1985; Provenza, 1995c). However, the prevailing view is that the nutritional consequences of food go beyond, i.e. they overcome any predisposition towards specific food flavour (Forbes and Kyriazakis, 1995; Wang and Provenza, 1996).

1.3.2.2 The importance of the post-ingestive consequences of foods

Provenza and Balph (1990) have proposed that animal's food choices are the result of the positive and negative PIC of feeding. In their model, preferences for foods would be acquired when their ingestion cause satiety or ameliorates needs and aversions would be developed towards foods that cause malaise as a result of excesses of nutrients or toxins, or a deficit of nutrients (Provenza, 1995c). This learning model requires that the animal is able to distinguish between the foods it eats and that is able to form associations between cues related to food and the PIC induced by the ingestion of these foods (Provenza and Cincotta, 1993; Provenza, 1995c).

1.3.3 Learned food aversions

Food aversion learning is a type of classical conditioning in which an animal learns to associate a food flavour (conditioned stimulus) with the administration of a toxin (unconditioned stimulus) which produces illness or nausea. The animal subsequently, exhibits a learned aversion by avoiding the consumption of that flavoured food. The conditioned flavour aversions (CFAs) paradigm is the most common procedure used in the experimental study of how animals learn about foods (Capaldi, 1992; Sclafani, 1995 and 1997). This is because CFAs have a number of striking properties, which have attracted the attention of those interested in nutrient and food selection. It was Garcia et al. (1966) who challenged the traditional idea that learning cannot occur if there are delays of more than a few seconds between two events and many repetitions are often necessary in order for learning to occur (Seligman, 1970). The temporal contiguity that was presumed as necessary in classical conditioning was violated in a series of experiments that was begun in 1966 by Garcia and his colleagues. They exploited Pavlov's discovery in taste aversion learning and uncovered some important methodological issues. Garcia and Koelling (1966) and Garcia et al. (1966) found that the ability of rats to learn to avoid a poison (lithium chloride, LiCl) has some special features. They demonstrated that strong flavour aversions could be learned within just one conditioning ('flavour-illness pairing') period and such learning occurs even when illness occurs after long delays of food consumption. Such flavour aversions were developed with delays of up to 24 hours between consumption of the food and illness (Garcia et al., 1966; Kalat and Rozin, 1973). The latter discovery made a strong case for its generality. Its popularity was also stimulated by another characteristic, the cue-consequence specificity. The term cue-consequence specificity was used to emphasise that the flavour of a food is the most important cue for the formation of learned associations compared with other cues (e.g. auditory, Domjan, 1985).

Although food aversion learning was studied mainly under controlled environmental conditions, the results led to suggestions that it is a form of learning with a clear function in natural environments. Its unusual properties led to the idea that such learning has principles that may be adapted to a species specific ecological niche (Rozin, 1976; Garcia et al., 1977 and 1985; Revusky, 1977; Logue, 1979; Provenza and Balph, 1987 and 1990).

Westoby (1974 and 1978) was the first to raise the issue of associative learning in ruminant animals, emphasising the limitations that might be imposed by their ingestive physiology. Considering Garcia's experiments (1966 and later), Westoby (1974) suggested that the existence of a learning mechanism in ruminants would explain *"how they manage to optimise a nutrient mix in the diet"*. Moreover, the cue-specificity and the long delays over which associations could be formed were just what would be expected of a ruminant animal for associating the qualities of food with the consequences of ingesting it because such learning would have survival value in toxin avoidance. The particular characteristics of food aversion learning were therefore ideal for assisting ruminants to avoid foods containing potential toxic compounds and to learn about spatial and temporal changes in their food supply (Westoby, 1974 and 1978)

Applying the same methodology as used with the food aversion studies in rats, Provenza and his colleagues investigated how ruminants learn to avoid toxic foods. They conducted a series of experiments, which demonstrated that ruminants are able to develop CFAs towards food flavours that are associated with gastrointestinal malaise (activation of the emetic system), primarily induced by the administration of LiCl. Some examples are the studies by Provenza et al., 1990; du Toit et al., 1991; Ralphs, 1992; Provenza et al., 1994a,b; Burritt and Provenza, 1996; Ralphs and Provenza, 1999. The special characteristics of all these studies of CFAs by ruminants, (irrespective of whether a delay between the 'food flavour-illness pairing' was imposed), is that learned associations were established very quickly (in most cases only a single conditioning period was necessary), were very robust and persisted for a very long time. The adaptive significance of this fact led Provenza and colleagues to put forward a theory of the mechanisms that govern the feeding behaviour of ruminants. Their view is that feeding behaviour and diet selection of ruminant animals arises basically through the processes of learned aversions (e.g. see Ralphs, 1992; Provenza, et al., 1990 and 1994a,b; Provenza, 1996; Ralphs and Provenza, 1999)

Kyriazakis et al. (1997) suggested that the use of the LiCl paradigm and its consequent CFAs was probably an extreme case and may not account for how ruminants might learn what foods to eat or avoid in nature, where they face a wide variety of potential foods (plant species) that vary in both nutritive value and toxicity. For this reason they tested the hypothesis by using natural toxins. Kyriazakis et al. (1997) demonstrated that sheep are able to develop CFAs towards food flavours associated with the administration of mild toxic substances (secondary plant compounds) that occur naturally in plants (e.g. oxalic acid), and whose physiological consequences do not involve the activation of the emetic system. Adding to the existing theory of learned aversions, Kyriazakis et al. (1997) suggested that CFAs are a wider phenomenon and mechanism, than previously thought, and could perhaps be invoked more generally to account for how ruminants learn to select a diet that minimises the ingestion of harmful compounds.

The studies on CFAs by both Provenza and Kyriazakis and their colleagues respectively, were critical in opening a new approach to research on feeding behaviour and diet selection by ruminant animals. However, Duncan and Gordon (1999) suggested that *"further experimentation is required before invoking conditioned food aversions as an influence on toxin avoidance in the field"*. They also stated that *"there is a substantial gulf between this scenario and the free-grazing situation where animals select a wide range of plants within a single bout and where the aversive stimuli do not necessarily stimulate the emetic system"*.

1.3.4 Learned food preferences

Rozin (1976) was the first to underline the inadequacy of the learned food aversion model to account adequately for the feeding behaviour of animals by proposing that: *"although the major phenomenon appears to be learning from the aversive consequences of feeds, animals* *must be able to learn (as well) about the positive effects of feeds"*. However, the evidence in the literature from studies with laboratory animals suggests that the development of conditioned flavour preferences (CFPs) has been much more difficult to establish compared with CFAs (e.g. Booth, 1985; Rozin and Zellner, 1985; Capaldi, 1992; Sclafani, 1995 and 1997). The questions that arise are, does it apply and how difficult it is to demonstrate in ruminant animals?

It has been proposed (Provenza and Balph, 1990; Provenza, 1995c) that ruminants should also be able to form learned associations for food flavours associated with positive and negative PIC. By postulating a theory which states that ruminants are able to form learned associations between food flavours and the positive PIC of foods, it is necessary that CFPs are added as a second dimension in the control of feeding behaviour. It implies that ruminants should be also able to learn which foods lead to positive PIC (e.g. increased supply of nutrients). This is an important phenomenon, considering that foods rich in nutrients are not easy to find in environments where ruminants have evolved (Provenza and Balph, 1990).

The experimental evidence to justify the latter theoretical approach is limited (Hills et al., 1999). For example, contrary to the plethora of information that ruminants are able to form CFAs, the evidence that they can also develop CFPs for food flavours associated with nutrients is rather limited. Although there have been investigations towards this effect their outcomes have been inconclusive (Ralphs et al., 1995; Villalba and Provenza, 1997a,b,c). In subsequent chapters I will provide further support for this and detail the experimental shortcomings that have led to the inconclusive outcomes.

1.3.5 What are the problems relative to learning of ruminants?

The extent to which the structure and the function of the digestive system in ruminants for nutrient digestion and metabolism might affect their ability to form learned associations is unclear. To investigate whether ruminants are able to form CFPs for food flavours associated with PIC of nutrients it is important to consider some of the findings from the studies on the development of CFAs in ruminants. For example, when a food flavour was associated with the intraruminal administration of a strong artificial toxin such as LiCl, the degree of development of CFAs was dependent on the strength of the induced negative PIC (Launchbaugh and Provenza, 1994). Moreover, the degree of persistence of the developed CFAs was dependent on the strength of the negative PIC (e.g. Ralphs and Cheney, 1993). Similar findings regarding the rate and the strength of development of CFAs were reported in the case of experiments of Kyriazakis et al. (1997) and Kyriazakis et al. (1998b). In the latter experiments the CFAs depended both on the dose and the type of secondary plant compounds and there was a considerable variation in terms of the persistence of the developed CFAs in the absence of exposure to secondary compound; CFAs extinguished rather quickly (Kyriazakis et al., 1997).

The above studies although they provide support to the idea that ruminants can form CFAs despite the delays imposed by their digestive system, they provide limited conclusion on how learning for food nutrient composition is modified by the ruminant digestive tract. This is because they have dealt with toxins rather than nutrients.

1.3.5.1 The continuum hypothesis

Booth (1985) stated that preference conditioning must have an optimum strength beyond which the animal is receiving 'too much of a good thing' and hence sufficient excess of even an essential nutrient can be toxic. Faced with an excess of a nutrient an animal has three options: it can refrain from absorbing it, it can excrete it, or it can change it to a less harmful form (Emmans, 1981). Irrespective of the underlying physiological and biochemical mechanisms the presumption (Figure 1.2) is that both low and excessive release of nutrients cause preference to decrease, while intermediate concentration of nutrients cause preference to increase (Provenza, 1995c). In the case of protein, an important feedback signal to a ruminant that a food is good for microbial protein synthesis is recognition (by some means) of ammonia generation in the rumen. It has been suggested that the food which generates ammonia (NH_3) in the rumen signals that essential amino acids will follow (Faverdin, 1999). However, protein degradation within the rumen results in increasing level of NH_3, which is partly used by microbes to form microbial protein while the rest, is absorbed through the rumen wall. In large doses NH_3 may become toxic when the detoxification capacity of the liver is exceeded (Russell et al., 1982; Rooke et al., 1987; Urbaniak, 1995). For example, Tolkamp et al. (1998) showed that a change in RDP content of foods on offer had a strong effect on diet choice of dairy cows. The question that arises is whether ruminants are able to learn to distinguish between two foods associated with a rumen degradable protein (RDP) source and whether such learned responses are influenced quantitatively by RDP provision.

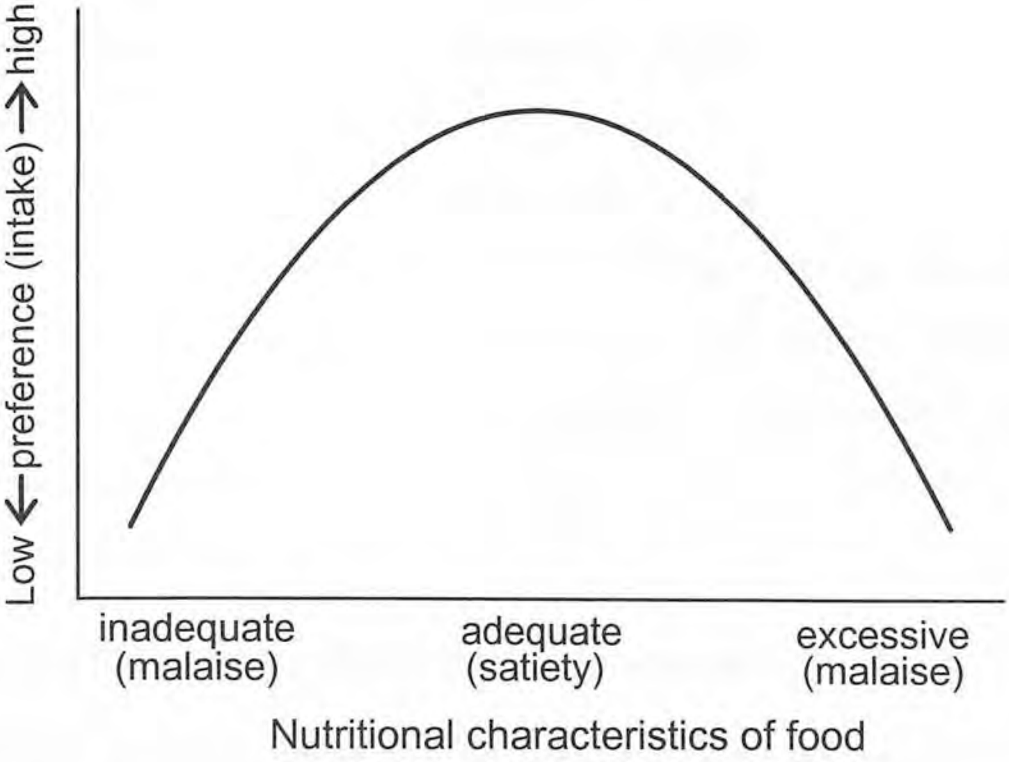


Figure 1.2 Schematic diagram of the continuum hypothesis (Provenza, 1995c).

1.3.5.2 PIC arising with a considerable delay from food consumption

The idea that animals need to have some knowledge about the PIC of food before they select a diet (Provenza, 1995c), implies that an animal will need some time before developing an association between the food flavour and its PIC (Capaldi, 1992). It should be noted that the feedback signals might take a long time to arise in the ruminant digestive tract compared with those that arise in the digestive tract of non-ruminant animals (VanSoest, 1994). The development of CFPs over delays has been very difficult to demonstrate even in experiments with laboratory animals (Booth, 1985; Capaldi, 1992; Sclafani, 1995 and 1997).

The evidence from the studies on CFAs in ruminants suggest that the time interval which an animal takes to form associations is important and, in the case where the toxin dose is not very high, the longer the time interval the weaker the strength of the developed CFAs (Ralphs and Provenza, 1999). In ruminants feeding is generally of very long duration and there might be substantial time intervals between the consumption of a food and the onset of its PIC (Provenza, 1995c). Therefore, the existence of long delay learning seems necessary. The question that arises is whether the time interval between the consumption of a food by sheep and the onset of PIC induced by its protein content could affect the formation of learned associations.

1.3.5.3 PIC arising from both rumen and post-ruminal digestive tract

Recently the basis of diet selection for protein by ruminants has been questioned by Tolkamp et al. (1998). They suggested that diet selection by ruminants is based on the RDP content of foods whereas the MP yield has no effect, when the foods offered as a choice contain adequate RDP. Following Tolkamp et al.'s (1998) suggestion it seems that selection for protein would be dictated by the PIC attributed to the RDP rather than to the DUP content of foods. However, the rumen micro-organisms in some cases cannot supply enough MP to meet the amino acid needs of the ruminant animal. For example, the net requirements for amino acids are high in rapidly growing ruminants and their potential for growth cannot be met by diets formulated to satisfy only the needs for RDP (AFRC, 1993). Moreover, the portion of DUP in dietary protein may supplement the microbial protein in providing a source of one or more limiting amino acids for absorption in the small intestine of the host animal (Merchen and Titgemeyer, 1992; Froetschel, 1996; Sloan, 1997). In this case, if the animal has a demand for essential amino acids in a particular circumstance, which is more than rumen microbial synthesis can provide, it is possible then that the supply of DUP sources could lead to positive PIC induced by provision of such limiting amino acids. The questions that arise are whether sheep can learn to select only for foods that contain RDP or also for foods that yield DUP when this is beneficial. The fact that protein digestion in ruminants has two steps that are separate but also interdependent implies that there might be PIC arising both from the rumen and the post-ruminal digestive tract. This calls for a detailed investigation of what is the relative importance of RDP and DUP in the ability of sheep to form learned associations. The issue has not been addressed systematically before.

1.4 Variation amongst individual animals

Diet selection measurements in most experiments are at the level of individual animals. However, it is usual to report results that are averaged across individuals of particular treatment groups. The existence of individual differences in behaviour is an almost universal phenomenon and feeding behaviour is no exception (Matthews and Kilgour, 1980; Slater, 1981; Staddon, 1983; Emmans and Fisher, 1986; Sutherland, 1996). Shettleworth, (1998) proposed that the variation both between and within animals of a group in their feeding behaviour may be useful in quantifying the effect of the individual on the population. She stated that variation is presumably due either to differences in individuals decision-making capacities, or their physiological state, or to differences in individuals feeding strategies.

Farm animals, in general, are herd species and in practice one does not deal with individuals but with populations. Emmans and Fisher (1986) suggested that the existence of individual differences in diet selection of animals should not be considered as 'noise' in the system. Over the last few years there is a growing interest in the variability in the feeding behaviour among individuals of a group or population especially in grazing systems. For example, the existence of significant variation in feeding behaviour and diet selection between individuals in a group, has been demonstrated in recent studies with ruminant animals (e.g. Illius et al., 1995; Howery et al., 1996 and 2000; Gordon et al., 1996; Scott and Provenza, 1998 and 1999; Ingrand et al., 1999; Duncan et al., 2000). The consequences of such differences for the group seemed to have great practical value for effective management (e.g. Illius et al., 1995; Howery et al., 1996). In other cases the existence of individual differences in diet selection between animals of a group (e.g. dairy cows, Lawson et al., 2000) affected significantly the interpretation of the results of such studies. Therefore, when one is dealing with the specific problem of predicting the diet selection of a group of animals we should consider the variation in feeding responses between individual animals and not treat a population as one 'large individual', unless this can be otherwise justified. In this thesis I shall return to the issue of individual versus group diet selection, after the issues that underlie diet selection of ruminants in relation to protein contents of the foods have been considered in detail.

1.5 Aims and objectives of the thesis

The experiments and ideas presented in this thesis are directed towards obtaining an answer to the question of how sheep come to select for protein in their diet. Within this aim the specific objectives were:

(i) To investigate whether an 'unlearned appetite' for dietary protein exists in sheep.

(ii) To test whether sheep are able to form learned associations towards a food flavour paired with the intra-ruminal administration of an RDP source. A further objective was to see whether the rate of development, magnitude and the persistence such responses would be affected by the dose level of RDP

(iii) To test whether a delayed type of learning could account for the development of learned associations, especially conditioned preferences, towards food flavours associated with either a low or a high dose of RDP administered intra-ruminally, at different points in time.

(iv) To investigate whether sheep are able to form conditioned responses towards a food flavour associated with the intra-ruminal administration of DUP. A further objective was to investigate the relative importance of RDP or DUP in the formations of learned associations.

(v) To develop a model that would account for diet selection of a population of animals by making simple assumptions about the characteristics of the individuals that comprise the population, including the variation between them.

Chapter 2

Does previous protein feeding affect the response of sheep towards foods that differ in their form, but not content of nitrogen?

"The Creator who made man such that he must eat to live, incites him to eat by means of appetite and rewards him by pleasure"

Brillat-Savarin, (1755 - 1826)

2.1 Abstract

The objective of the experiment was threefold: (i) to test whether an 'unlearned appetite' for dietary protein exists in sheep, (ii) whether such an appetite and subsequent diet selection depend on the degree of protein deprivation and, (iii) whether the N-source in the foods offered as a choice influences diet selection. Differences in protein deprivation were achieved by feeding sheep on a food with either high (H) or low (L) protein; sheep were fed on food L either for the same period of time as sheep on H or until they reached the same live weight as sheep on H. Following the feeding regimes, which induced differences in animal state, sheep were given a choice between a novel, low protein food (T) or food T supplemented with isonitrogenous amounts with one of three nitrogen sources: urea, casein or formaldehyde treated casein. Diet selection measured in the sort term, or over the first few days did not provide any evidence in support of an unlearned appetite for protein by sheep of different states. In fact diet selection for all animals was characterised by a strong avoidance of the food supplemented with one of the protein sources used. Selection of considerable amounts of supplemented foods was gradual and consistent only after animals gained experience of them, i.e. were allowed to consume a single supplemented food for a period of 7 days. Following this period, animals that had previously consumed food L for the same period of time as animals on food H, selected a higher proportion of the supplemented food than the other sheep. The results support the view that there is no unlearned appetite for protein in sheep and that control over diet selection is learned.

2.2 Introduction

Animals previously deprived of a nutrient and subsequently given a choice between a food supplemented with this nutrient and an unsupplemented one, are able to select for the former food and hence replenish the deficiency (Hughes and Wood-Gush, 1971; Kyriazakis and Emmans, 1991; DiBattista and Holder, 1998). Currently there are two hypotheses to account for the ability of animals to achieve this. They can be seen as differing mainly in respect of the time scale over which deprived animals are expected to select for supplemented food. The first hypothesis stems from the view that animals need to learn the properties of the foods, before they select an appropriate diet (Provenza, 1995b,c). Thus, a deprived animal would be expected to take some time to learn to select for the food that allows it to return to an appropriate state. However, the duration of learning can vary, as the greater the nutrient deprivation, the greater will be the reinforcing properties of the supplemented food and hence faster the learning (Kyriazakis, 1997; Kyriazakis et al., 1999). The second hypothesis suggests that animals possess a number of 'unlearned' appetites for many, if not most, specific nutrients. This allows them to select the supplemented food almost instantaneously, and without any previous experience of it. Such 'unlearned appetites' have been shown to exist for nutrients such as calcium (Hughes, 1979; Leshem et al., 1999), phosphorus (Blair-West et al., 1992) and sodium (Denton, 1982; Leshem, 1999).

Here we first investigate the possible role of an unlearned appetite for protein in the selection of a diet by sheep, previously fed on a food low in protein. In ruminant animals the issue has an added complexity due to the digestion and metabolism of protein in the gastrointestinal tract, (Van Soest, 1994) and the potential of this to influence diet selection (Tolkamp et al., 1998). For this reason we also investigate the existence of an unlearned appetite for protein in foods supplemented with protein/nitrogen sources that have the potential to be degraded, or are undegradable, in the rumen. Our null hypothesis is that the existence of the unlearned appetite for protein in a supplemented food should be independent of the protein/nitrogen source used to supplement the food. Deprived sheep would be expected to benefit from the metabolisable protein that would eventually reach their small intestine either as microbial protein or as undegraded protein (Van Soest, 1994). The existence of an unlearned appetite for protein has previously been investigated in a non-ruminant animal (i.e. rats, Deutsch et al., 1989), but the outcome of investigations to date have been far from conclusive (Galef, 2000). Additional objectives are to see (i) whether such an appetite and subsequent diet selection depend on the degree of protein deprivation and, (iii) whether the N-source of the foods offered as a choice influences diet selection.

2.3 Materials and methods

2.3.1 Animals and management

Seventy-two Texel x Greyface female sheep were used. They had been reared indoors with their dams until weaning at approximately 8 weeks of age. The sheep had a mean live-weight (LW) of 28.3 ± 3.0 kg (mean ± SD). They were moved immediately after weaning to individual pens. Each individual pen measured 1.29 x 1.53 m and had a sawdust bedding. It was equipped with two adjustable feed troughs and a water bucket. An initial acclimatisation period lasted for 10 days, and was considered long enough to allow sheep to become accustomed to the new environment and procedures. During this period a high quality pelleted concentrate feed was offered to all sheep. Its quantity was gradually increased during this period from 500g per day to allowances that were well above the voluntary food intake of all sheep. Natural lighting and ventilation were used throughout the experiment.

2.3.2 Experimental foods

Three iso-energetic foods, two basal (HP and LP) and one test (T), with different protein contents were formulated (Table 2.1). Foods HP and LP were made as pellets, whereas food T was made in a meal form, using similar ingredients to those of LP. Food HP was intended to supply sufficient protein (metabolisable protein, MP) to support the requirements for potential growth, of these relatively fast growing sheep when offered ad lib (AFRC, 1993). On the other hand, both LP and T foods were formulated to yield similar but low MP, and hence to be inadequate to support the requirements of the sheep. All foods were similar, and high, in energy, mineral and vitamin contents (AFRC, 1993).

Three more foods were made after supplementing food T with one of three different N-sources. The N-sources were either urea (U), or casein (C, sodium caseinate, Bacarel and Co Ltd, Asford, Kent) or casein that was protected from microbial degradation in the rumen by formaldehyde treatment (FC) using a dry method as described by Ketelaars and Tolkamp (1991). The basis of our calculations for supplementation of food T was U, added at a rate of 20.5 g U/kg food T. The expectation was that the contribution of N from U would correct the protein deficit in food T (AFRC, 1993). The amount of the other two N–sources was calculated such that it would be isonitrogenous to U (9.4 g N/kg food T). The resulting supplemented foods were TU, TC and TFC respectively. The N concentration in the supplemented food was further verified by chemical analysis of a composite sample for each food. The effectiveness of the formaldehyde treatment for the preparation of FC was also tested with the method of Ørskov, (1988); it had an apparent degradability of proportionately 0.14. The *in vitro* digestibility was tested with the method of Tilley and Terry, (1963); it was found to be 0.92.

The above N-sources were chosen after considering the manner of their contribution to protein metabolism in ruminant animals (Van Soest, 1994; Ørskov, 1988). For example, it was expected that supplementing food T with either U or C, would lead to the enhancement of microbial protein synthesis in the rumen and thus indirectly contribute to MP yield within the post-ruminal digestive tract (Leng and Nolan, 1984; Wallace et al., 1999). Conversely, supplementing food T with FC was expected to increase substantially the MP yield 'directly' within the post-ruminal digestive tract (Ketelaars and Tolkamp, 1991; Froetschel, 1996).

***Table 2.1** The composition and the chemical analyses of the three foods used*

	Food		
	LP	HP	T
Ingredients (g/kg fresh matter)			
Barley	165	200	200
Oatfeed	285	298	303
Citrus pulp	476	227	472
High-protein Soya	-	200	-
Molasses (CMS 20) [†]	50	50	-
Salt	8	10	8
Dicalcium phosphate	11	4.6	10
Limestone flour	-	8.9	1.4
Scotmin ewe/lamb [‡]	2.2	2.2	2.2
Scotmin dairy/beef [‡]	0.35	0.13	0.38
Sodium sulphate [‡]	1.9	-	2.5
Analysed chemical composition (g/kg DM)			
Dry matter (g/kg fresh food)	879	854	887
Crude protein	82	170	82
Gross energy (MJ/kg DM)	17.5	18.6	17.5
Ash	71.8	78.2	71.1
ADF (Acid Detergent Fibre)	234	164	251
NDF (Neutral Detergent Fibre)	290	237	313
Calcium	12.4	11.2	13.5
Phosphorus	3.7	3.7	4.0
Sulphur	4.5	4.5	4.5
Calculated protein and energy components (g/kg DM)[¶]			
Effective rumen degradable protein (eRDP)	62	121	61
Digestible undegradable protein (DUP)	11	36	10
Metabolisable protein (MP)	50	104	49
Metabolisable energy ME (MJ/kg DM)	10	10	10
Effective metabolisable energy fME (MJ/kg DM)	9.7	9.7	9.5

[†] CMS 20 condensed molasses soluble (Intermol, Cobham, Surrey)

[‡] Vitamin and mineral premix, supplied by Scotmin Ltd, Ayr, Scotland.

[¶] Calculated according to AFRC (1993).

2.3.3 Experimental design

The experiment consisted of two main periods. The objective of the first period, hence called *'state period'*, was to manipulate the state of the sheep through protein feeding (Kyriazakis and Emmans, 1991; Kyriazakis et al., 1999). Sheep were randomly assigned to one of three groups (n=24), designated as H, L1 and L2 according to the basal food and the length of time it was offered. Food HP was offered ad lib to group H and food LP to groups L1 and L2 respectively. The duration of this period was set to 28 days for both groups H and L1, whereas for L2 group it ended four weeks later; this was the extra time taken for L2 sheep to reach an average LW similar to that of group H. Therefore any comparisons of the consequences of the protein feeding to sheep during the state period could be made either on equal time (groups H vs. L1) or at equal LW (groups H vs. L2).

Immediately after the completion of the state period, sheep within each group were assigned to one of four sub-groups that were defined by the feeding treatment offered (n=6 per treatment) for a period of 21 days. The allocation was such that mean LW of sheep did not differ significantly between feeding treatments. This period, hence called 'test period', consisted of three consecutive sub-periods (TP1, TP2 and TP3) that lasted for 7 days each. During these sub-periods the following four feeding treatments were offered to sheep:

2.3.3.1 TP1: a choice between two foods

Sheep within each feeding treatment were offered ad lib a choice between two foods. The foods used were T, TU, TC and TFC and four choice-feeding treatments were considered: T vs. TU, T vs. TC, T vs. TFC and TC vs. TFC respectively. For the first three the expectation was that sheep would select for the supplemented food. The fourth one was designed to assess the relative importance of the source of dietary protein in the feeding responses of sheep when given a choice between two isonitrogenous foods. The position of the two food troughs was initially randomised within pens and then remained in the same position throughout the experiment (Kyriazakis and Oldham, 1993).

2.3.3.2 TP2: access to one food only

We decided to include this intermediate sub-period because the diet selection of sheep in groups H and L1 during the 7 days of TP1 was characterised largely by avoidance of the supplemented foods (see Results for details). The purpose of this intermediate sub-period was therefore to give sheep experience on the supplemented foods. Sheep previously given a choice between food T and T supplemented with a N-source, were then offered singly the supplemented food, i.e. TU, TC and TFC respectively. Those given previously the TC vs. TFC choice treatment were then offered singly food TFC. This 'experience period' was a modification of the method described by Kyriazakis et al. (1990). In all cases the food offered during TP2 was placed in the same trough as it would have been placed when offered as a choice; the other trough remained empty. This was done to maintain continuity throughout the test period.

2.3.3.3 TP3: a choice between two foods

All sheep were offered again the same choice-feeding treatments as in TP1.

2.3.3.4 Measurements

The sheep were weighed in the morning of the day prior to the start of the state and the test periods, and subsequently on the same day, each week, throughout the experiment. At the end of the state period, the body composition of sheep was assessed by measuring the muscle and fat depth at the 13th vertebra by ultrasound scanning (Glasbey et al., 1996).

Food refusals were collected daily, in the morning, before fresh food was offered; the refusals were weighed and then discarded. In addition, food refusals were weighed after 30-min, 1 h and then in the morning of the next day, at the start of the choice-feeding regime for sheep H and L1 (first day of TP1). These particular measurements were expected to provide information on the existence of an 'unlearned appetite' for protein in sheep. Our experimental design did not allow us to obtain measurements on a shorter time scale. Groups H and L1 were considered as the most appropriate for this test. The diet selection of sheep offered one of the choice-feeding treatments T vs. TU, T vs. TC and T vs. TFC (during TP1 and TP3), was expressed as a proportion of food T selected over total food intake (g of food T/g TFI) in each choice- feeding treatment respectively. For the TC vs. TFC choice-feeding treatment, the diet selection of sheep was expressed as a proportion of food TC selected over total food intake (g of food TC/g TFI).

2.3.4 Statistical analysis

All statistical analyses were performed using GENSTAT, version 5.3 (Lawes Agricultural Trust, 1993). A one-way analysis of variance (ANOVA) was used to test for differences between the three groups of sheep (H, L1 and L2) in their total food intake and their body composition (in terms of muscle and fat depths) at the end of the state period. Comparisons either on equal time (groups H vs. L1) or at equal LW (groups H vs. L2) were made through the use of *t* tests.

The average daily food intakes of sheep during the two sup-periods of choice-feeding (TP1 and TP3) were analysed using a split-plot analysis of variance model. These data were tested for the effects of, and interactions between, animal state (S), choice-feeding treatment (FT) and sub-period (TP). The nesting structure was sub-periods within individual sheep (Horgan and Sword, 1995). The data regarding daily food intakes of sheep during TP2 were tested for the effects of, and interactions between, animal state (S) and food offered, by a two-way analysis of variance. The LW of sheep at the beginning of the test period was used as a covariate in all the above analyses of daily food intake. The daily rates of LW gain (DLWG, g/d) of sheep during the whole test period, were estimated by linear regression, and analysed using a two-way analysis of variance.

The data for diet selection of sheep during the two sub-periods of choice-feeding (TP1 and TP3) were analysed first for treatments: T vs. TU, T vs. TC and T vs. TFC (diet selection was calculated as g of food T/g TFI in each choice). The analysis was carried out using a split plot analysis of variance model and tested for the effects of, and interactions between, animal state (S), choice-feeding treatment (FT) and sub-period (TP). The nesting structure was days within sub-period and sub-period nested within individual sheep. A similar split plot analysis of variance model was used to analyse the data regarding the diet selection of sheep of TC vs. TFC choice-feeding treatment (diet selection was calculated as g of food TC/g TFI). These data were tested for the effects of, and interactions between, animal state (S) and sub-period (TP). The LW of sheep at the beginning of the test period was used as a covariate in all the above analyses of the diet selection of sheep. Prior to analysis, diet selection data (proportions) were tested for meeting the criteria of normality.

To test whether the diet selection of sheep, measured at 30-min, 1 h and at the first day of TP1, was different from random selection, a *t* test was used with a null hypothesis of mean = 0.5 and an alternative hypothesis of mean $\neq 0.5$.

2.4 Results

Data from two sheep in group L1, for choices T vs. TC and T vs. TFC, were omitted because of their very poor performance and irregular intakes during the test period of the experiment. Data from these animals were treated as missing values in all the statistical analyses.

2.4.1 State period

2.4.1.1 Comparisons made on equal time (groups H vs. L1)

Sheep given ad lib access to food HP consumed significantly more food ($p<0.001$, $t = 8.12$, d.f. = 45), reached a higher LW ($p<0.001$, $t = 6.36$, d.f. = 45) and had greater muscle ($p<0.001$, $t = 5.48$, d.f. = 45) and fat depths ($p<0.01$, $t = 3.44$, d.f. = 45) than sheep that were given ad lib access to food LP for the same period of time (28 days) (Table 2.2).

Table 2.2 *The performance of sheep given ad lib access to single foods yielding either high (group H) or low (groups L1 and L2) metabolisable protein. Sheep in groups H and L1 received the foods for 28 d, whereas sheep in L2 group had access to the food for 56 d. This allowed comparisons to be made either on equal time (H vs. L1) or at equal live-weight basis (H vs. L2).*

	Group				
Performance	H	L1	L2	s.e.d.	Significance
Initial Live-weight (kg)	28.4	28.1	28.4	0.88	NS
Final Live-weight (kg)	38.2	31.5	38.0	1.15	***
Time taken (days)	28	28	56		
Food consumed (kg)	45.7	33.9	75.6	2.4	***
Muscle depth (mm) †	26.1	22.1	25.6	0.74	***
Fat depth (mm) †	4.72	3.48	5.67	0.408	***

†measured by ultrasound scanning at the 13th thoracic vertebra of sheep at the end of the state period.

2.4.1.2 Comparisons made at equal LW (groups H vs. L2)

Sheep given ad lib access to food HP took half the time to reach the same LW, consumed significantly less food ($p<0.001$, $t = -10.99$, d.f. = 30) and had a lower fat depth ($p<0.05$, $t = -2.23$, d.f. = 46) than sheep that were given ad lib access to food LP over the same LW range (28-38 Kg LW). However, sheep fed on either food had very similar muscle depths ($p>0.05$, $t = 0.63$, d.f. = 46) (Table 2.2).

2.4.2 Test period

2.4.2.1 Food intake, sub-periods TP1 and TP3

The average values of daily food intake of the three groups of sheep on each of the four choice-feeding treatments are shown in Table 2.3 for both sub-periods. Daily food intake was significantly affected (at most $p = 0.01$) by sub-period, animal state (group) and choice-feeding treatment. The food intake of sheep was significantly higher in sub-period TP3 rather than in TP1 (1885 vs. 1602, s.e.d. 24.43; $F(1, 57) = 133.98$, $p<0.001$). Sheep on group L2, which were delayed in their growth, had a lower food intake than sheep on the other two groups (L1 and H), ($F(2, 57) = 6.70$, $p<0.01$), and sheep on choice-feeding treatments T vs. TU and TC vs. TFC consumed more than animals on T vs. TC and T vs. TF, ($F(3, 57) = 7.74$, $p<0.001$). There was also a significant interaction on the daily food intake between sub-period and animal state, ($F(2, 57) = 5.86$, $p<0.01$); this was due to the fact that sheep in group L2 did not increase their food intake to the same extent as those in groups H and L1 during TP3. Live-weight at the start of TP1 significantly affected the daily food intake of sheep, ($F(2, 57) = 35.71$, $p<0.001$).

Table 2.3 *The effects of animal state (previous feeding on a high (H) or Low (L1 and L2) protein food) on the average daily food intake (g/d) of sheep given ad lib a choice between two foods over two sub-periods (TP1 and TP3). The choice was between a food low in protein (T) and food T supplemented with either urea (U), casein (C) or formaldehyde treated casein (TFC) (n=6 per choice); there was also a choice between foods TC vs. TFC (n=6).*

		Food intake (g/d)								
	Sub-period	TP1				TP3				
	Animal state	H	L1	L2		H	L1	L2		
Food 1	Food 2				Means				Means	
T	TU	1671	1835	1587	**1699**	2018	2311	1854	**2061**	
T	TC	1658	1511	1420	**1534**	1977	1830	1613	**1802**	
T	TFC	1446	1513	1521	**1494**	1824	1777	1594	**1731**	
TC	TFC	1635	1782	1617	**1679**	1988	2097	1770	**1951**	s.e.d. 72.1[†]
Means		**1604**	**1661**	**1540**		**1951**	**2003**	**1704**		s.e.d. 66.3[‡]

Significance of:

Sub-period (TP)	***
Animal State (S)	**
Choice-feeding treatment (CFT)[¶]	***
TP x S	**
TP x CFT	NS
S x CFT	NS
TP x S x CFT	NS

[†] For TP x CFT interaction; except when comparisons are made within the same level of choice-feeding treatments where s.e.d. 48.9.

[‡] For TP x S interaction; except when comparisons are made within the same level of animal state where s.e.d. 44.9.

[¶] Choice-feeding treatment applies to the pairs of food 1 and food 2.

*** $p < 0.001$; ** $p < 0.01$; NS, non-significant.

2.4.2.2 Food intake, sub-period TP2

When a supplemented food was offered singly, daily food intake was not affected by animal state, (*F*(2, 58) = 1.83, $p>0.05$), and type of food offered, (*F*(3, 58) = 0.92, $p>0.05$). Average daily food intake was 1879, 1776, 1752 and 1848 (s.e.d. 87.4) g/d on foods TU, TC, TFC and TFC respectively.

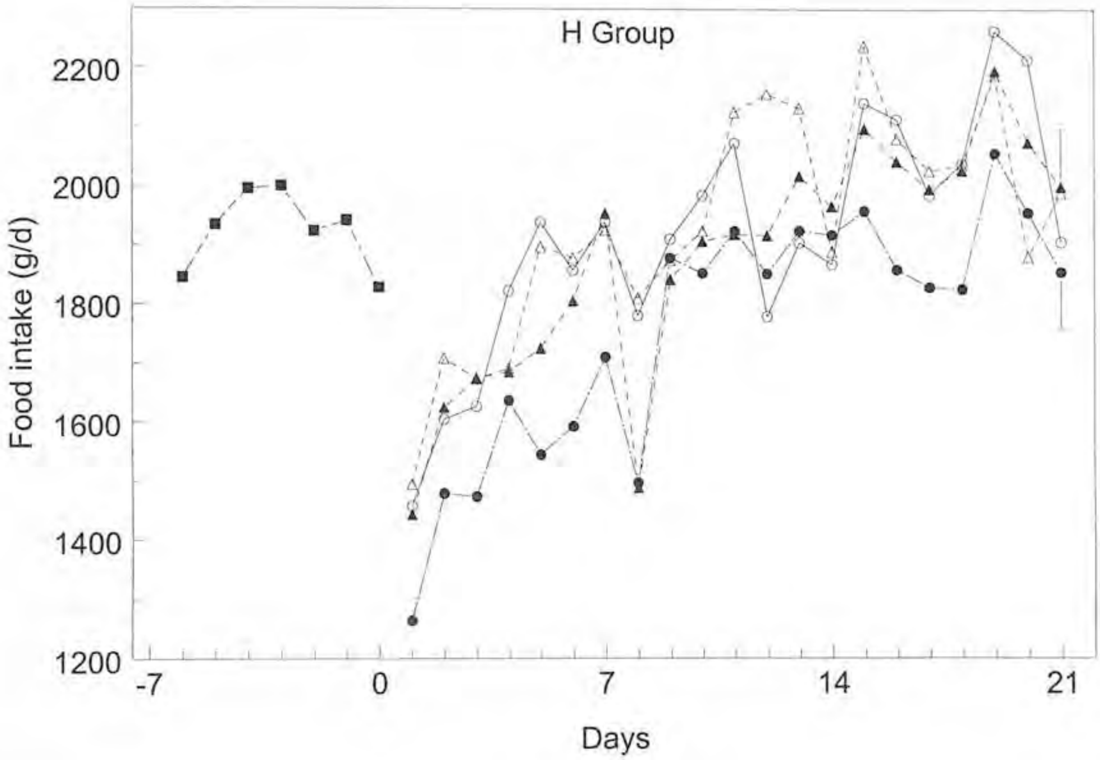


Figure 2.1 Average daily rates of food intake (g/d) of sheep from group H over a period of 4 weeks. During days –7 to -1 sheep (n=24) were given ad lib one food yielding high protein (HP). From day 1 to 7, sheep were given ad lib a pair of foods as a choice. The choice treatments were between: (i) a food low in protein, T and T supplemented with either urea (T vs. TU, ○), casein, (T vs. TU, Δ) or formaldehyde treated casein (T vs. TFC, ●) and, (ii) food T supplemented with either casein or formaldehyde treated casein (TC vs. TFC, ▲) (n=6 per choice). For days 8 to 15 supplemented foods TU, TC, TFC and TFC were offered singly respectively for each of the above treatments During days 15 to 21 sheep were treated identically as in days 1-7.

2.4.2.3 Evolution of food intake

The evolution of daily food intake was similar for the three groups of sheep (H, L1 and L2). Food intakes of sheep from group H over the final 7 days of state period and the whole test period are given here as an example (Figure 2.1). On introduction to the choice-feeding treatments (sub-period TP1) food intake was immediately reduced, but recovered to 'state period' levels by the end of the first choice-feeding sub-period (TP1). In general, food intake continued to increase during both sub-periods TP2 and TP3, but the introduction of a single supplemented food (TP2) was seen to induce different sheep responses. On the first day of TP2 food intake was reduced on food TFC, but not on foods TU and TC; thereafter, food intake continued to increase at/or above TP1 rates.

2.4.2.4 Diet selection during day 1

There was no difference in the diets selected over the first 30-min and 1h after the introduction of the choice-feeding treatments; for this reason we report here only the diet selection of sheep over the former interval. There were no differences in diet selection between sheep of the two animal states (0.687 vs. 0.696, s.e.d. 0.070; $F(1, 47) = 0.02$, $p>0.05$, for H and L1 respectively). However, diet selection over the first 30-min was significantly affected by choice-feeding treatment ($F(3, 47) = 4.27$, $p<0.05$). The proportion of food T selected was (mean ± SE) 0.491 ± 0.090, 0.686 ± 0.067 and 0.820 ± 0.065 g/g TFI for treatments T vs. TU, T vs. TC and T vs. TFC, respectively. Only for the first treatment was diet selection indistinguishable from random selection. In treatment TC vs. TFC sheep selected clearly for TC (mean ± SE: 0.769 ± 0.051 g of food TC/g TFI). Animal state and choice-feeding treatment did not significantly affect the total food intake of sheep over the 30-min (overall mean ± SE: 72.2 ± 4.17 g).

By the end of day 1 diet selection had changed to preference for the non-supplemented food, but to a different degree for each choice feeding treatment (mean ± SE: 0.660 ± 0.051, 0.673 ± 0.066 and 0.943 ± 0.024 g of food T/g TFI for treatments T vs. TU, T vs. TC and T vs. TFC respectively). The diet selection of sheep in treatment TC vs. TFC was similar to that observed during the first 30-min (mean ± SE: 0.887 ± 0.024 g of food TC/g TFI). The total food intake of sheep over the first day was not significantly affected by animal state and choice-feeding treatment (overall mean ± SE: 1352 ± 47.80 g/d).

2.4.2.5 Diet selection during sub-period TP1 and TP2

Table 2.4 shows the average values of diet selection of sheep in different states, given access to choice-feeding treatments where one of the foods was supplemented with a different N-source; the diet selection is given separately for the two sub-periods. Diet selection (g of food T/g TFI) was significantly affected (at most $p = 0.05$) by sub-period, animal state and choice-feeding treatment. The proportion of food T selected declined significantly from sub-period TP1 to TP3, (0.676 and 0.575, s.e.d. 0.023; $F(1, 42) = 22.71$, $p<0.001$). Animal state significantly affected diet selection, with sheep in group L2 selecting a lower proportion of food T than those in groups L1 and H respectively, ($F(2, 43) = 5.12$, $p<0.05$). Sheep on the choice-feeding treatments T vs. TC and T vs. TFC selected the lowest and highest proportion of T in their diets respectively; however, this difference was most evident in TP1 rather than TP3, and contributed to the formally significant interaction between sub-period and choice-feeding treatment, ($F(2, 43) = 3.85$, $p<0.05$).

The diet selection of sheep offered a choice between TC vs. TFC was significantly affected by sub-period; the proportion of food TC selected declined significantly from sub-period TP1 to TP3 (0.813 vs. 0.690, s.e.d. 0.035; $F(1, 15) = 13.30$, $p<0.01$). Sheep in group L2 selected significantly lower proportion of TC in their diet during TP3 compared to TP1;

however, the latter was not the case in the diet selection of sheep in groups H and L1 and this contributed to the formally significant interaction between sub-period and animal state, ($F(2, 15) = 4.90, p<0.05$), (Figure 2.2).

Table 2.4 *The effects of animal state (previous feeding on a high (H) or low (L1 and L2) protein food) on the diet selection of sheep given ad lib a choice between two foods over two sub-periods (TP1 and TP3). The choice was between a food low in protein (T) and food T supplemented with either urea (U), casein (C) or formaldehyde treated casein (TFC) (n=6 per choice); diet selection for each choice was calculated as g of food T/g of total food intake (TFI).*

		Diet selection (g T/g TFI)								
Sub-period		TP1				TP3				
Animal State		H	L1	L2		H	L1	L2		
Food 1	Food 2				Means				Means	
T	TU	0.624	0.637	0.579	**0.613**	0.575	0.576	0.485	**0.545**	
T	TC	0.521	0.630	0.580	**0.577**	0.574	0.636	0.383	**0.531**	
T	TFC	0.898	0.886	0.731	**0.838**	0.650	0.834	0.465	**0.649**	
										s.e.d. 0.051†
Means		**0.681**	**0.718**	**0.630**		**0.599**	**0.682**	**0.444**		s.e.d. 0.054 ‡

Significance of :

Sub-period (TP)	***
Animal State (S)	*
Choice-feeding treatment (CFT)¶	***
TP x S	NS
TP x CFT	*
S x CFT	NS
TP x S x CFT	NS

† For TP x FT interaction; except when comparisons are made with the same level of choice-feeding treatments where s.e.d. 0.039

‡ For TP x S interaction; except when comparisons are made with the same level of animal state where s.e.d. 0.041

¶ Choice-feeding treatment applies to the pairs of food 1 and food 2.

*** $p< 0.001$; * $p< 0.01$; NS, non-significant.

2.4.2.6 Evolution of diet selection

The evolution of diet selection for sheep given access to different choice-feeding treatments during the two sub-periods is given separately on Figures 2.2a, b and c for each animal state. It can be seen that diet selection during TP1 changed over time, with the proportion of the supplemented food (or food TFC, for the TC vs. TFC choice-feeding treatment) in the selected diets increasing over time, but to a different degree for each choice-feeding treatment and animal state. The increase was most evident in sheep of group L2. The interruption of the choice-feeding treatments (sub-period TP2) had little effect on the evolution of diet selection. The temporal change in the diet selection was less obvious during TP3 for all animal states and choice-feeding treatments.

2.4.2.7 Daily live-weight gain

There were no significant differences between the rates of DLWG (g/d) of sheep of the three animal states during the test period (252, 251 and 229, s.e.d. 26 g/d for sheep of groups H, L1 and L2 respectively). However, choice-feeding treatment significantly affected the rate of DLWG of sheep, ($F(3, 71) = 6.26$, $p<0.001$); sheep on the T vs. TU feeding treatment performed best and sheep on the T vs. TC feeding treatment grew at the slowest rate (292, 189, 206 and 289, s.e.d. 30 g/d for sheep in feeding treatments T vs. TU, T vs. TC, T vs. TFC and TC vs. TFC respectively over the whole test period).

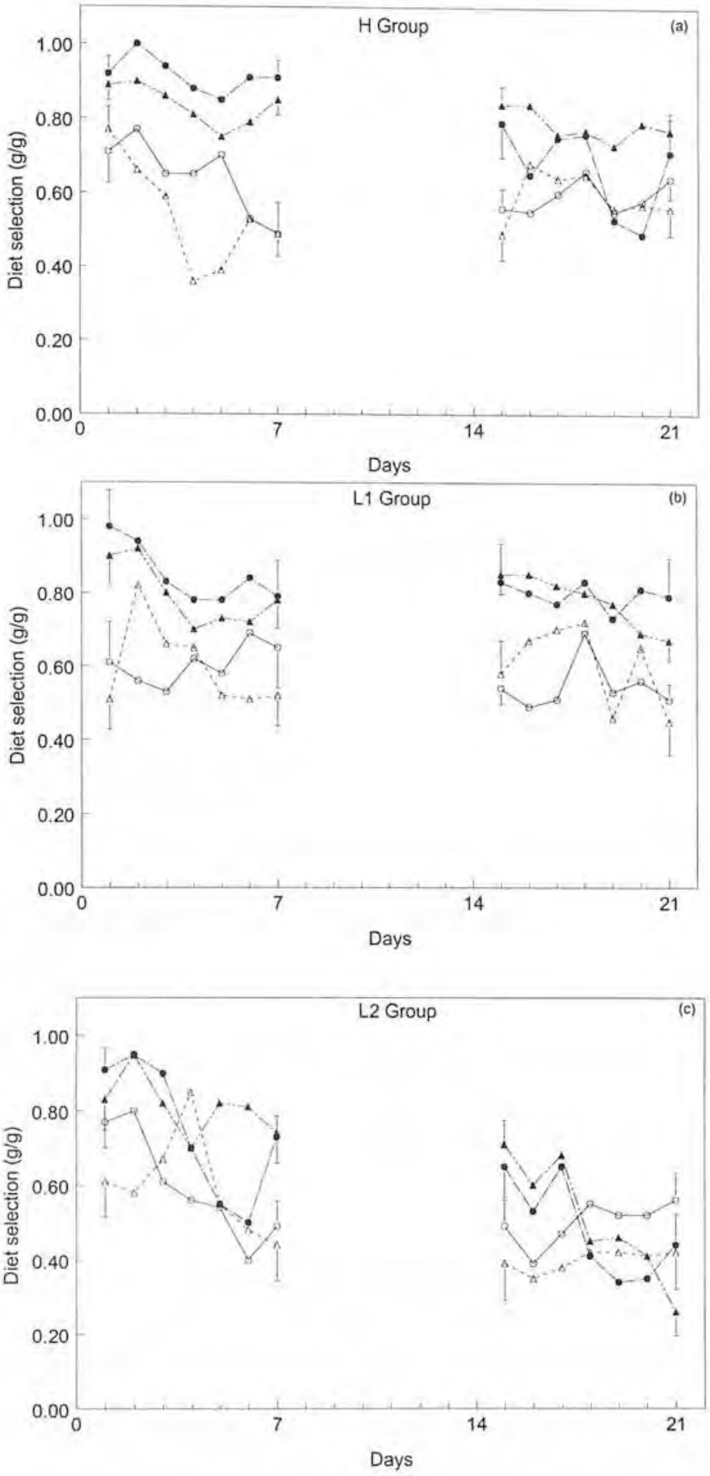


Figure 2.2 Diet selection of sheep from groups H (a), L1 (b) and L2 (c) given ad lib a pair of foods as a choice during two sub-periods (days 1-7 and 15-21 inclusive). The choices were between: (i) a food low in protein, T and T supplemented with either urea (T vs. TU, ○), casein, (T vs. TU, Δ) or formaldehyde treated casein (T vs. TFC, ●) and, (ii) food T supplemented with either casein or formaldehyde treated casein (TC vs. TFC, ▲) (n=6 per choice). Diet selection, for each of the first three choices, was calculated as g of food T/g total food intake (TFI) whereas for choice TC vs. TFC, it was calculated as g of food TC/g TFI.

2.5 Discussion

Feeding sheep on a food low in protein (LP) had the expected consequences on their growth and body composition. The direction of these effects depended on the duration of feeding on this food. Sheep fed the low protein food for 28 days (group L1) were lighter and had lower muscle and fat depths, compared to sheep fed on a high protein food for the same period of time (group H). Conversely, sheep fed food LP for 56 days (group L2) did not differ very much from group H sheep, apart from the fact that they were delayed in their growth and were fatter. Assuming that it is current animal state, rather than how this has been achieved (Kyriazakis and Emmans, 1992a), that affects subsequent feeding behaviour, then sheep of Groups L1 and H differed most in their 'state'; therefore, they were the most appropriate tests of the experimental hypotheses.

We first addressed the existence of an 'unlearned' appetite for protein (nitrogen) in sheep. Given the contribution of microbial protein to metabolisable protein in ruminants, our expectation was that such an appetite would be for N in general, rather than for specific amino acids. However, the short term diet selection, i.e. over the first 30-min, 1h and 24h did not provide any evidence in support of an 'unlearned appetite' for protein by sheep of different states. In fact diet selection was characterised by an overwhelming 'avoidance' of the food supplemented with one of the protein sources used. The lack of an 'unlearned appetite' for protein is consistent with the view of Kyriazakis et al. (1999). They suggested that unlearned appetites should not be the normal means by which an animal selects its diet, given that animals have a requirement for a large number of nutrients in their diet. With so many nutrients it seems to be unlikely that evolution would have equipped them with an unlearned appetite for every single one of them. This, however, does not mean that unlearned appetites for certain nutrients do not exist, as the evidence on salt appetite of sheep suggests (Denton, 1982). This position is a milder and perhaps a more tenable one than the recent extreme view put forward by Galef (2000) that unlearned appetites do not seem to exist for any nutrient.

It is however, possible that our inability to show an unlearned appetite for protein was the outcome of the rather 'mild' protein deprivation imposed on the sheep and the not so large differences in animal state between protein deficient and protein replete sheep. This was despite the fact that differences in the protein content of the two foods were very large. Blair-West et al. (1992), for example, has shown an unlearned appetite for phosphorus in cattle that had been deprived of this nutrient for a period of up to two years. We consider such prolonged states of deprivation rather pathological, and the diet selection of animals under such conditions seems to contribute very little to our understanding of how animals select a diet under more normal conditions. Below, we will put forward a framework that will be able to encompass both the results of our experiment and those in the literature of pathological deprivation.

The diet selection of animals changed over time. The initial avoidance of the food supplemented with one of the protein sources decreased and by the end of the first sub-period, during which animals had the opportunity to select their diet (TP1), they were consuming considerable amounts of the supplemented foods. As there were no differences between animal states in the selected diets, this feeding behaviour can not be accounted for by differences in the degree of novelty between the supplemented and unsupplemented foods (Wang and Provenza, 1996) (Food T can be considered to have been familiar for L1 and L2 sheep, as it was essentially identical to food LP, but not so for H sheep). A more likely explanation for this behaviour is that the protein sources used had some undesirable, non-nutritional properties for the sheep. Although it can be easily assumed that the formaldehyde treatment rendered food TFC with a strong smell, which could be perceived by us, it is less obvious what was the aversive (orosensory) property or properties for foods TU and TC. Avoidance of foods supplemented with urea is common in the literature (Leng and Nolan, 1984; Kyriazakis and Oldham, 1993) but this does not necessary imply that animals are avoiding an orosensory property associated with urea. Nevertheless, the initial feeding behaviour of sheep was identical to that seen by sheep offered access to foods with strong flavours (Augner et al., 1998), and they seemed to follow the proposed rule of thumb suggested to be used by mammalian herbivores: given a choice, avoid foods with strong flavours.

Part of our experimental objectives was to investigate whether, in the absence of unlearned appetites, subsequent diet selection depended upon animal state (degree of previous protein deprivation) and the degree to which food N-source could influence diet selection. For this reason, following the pattern of diet selection over the first week, we decided to give sheep experience on the nutritional consequences of the supplemented foods only. We were expecting that such experience would overcome any predisposition towards the sensory properties of the foods, and perhaps sheep would select their diet on a nutritional basis (Kyriazakis et al., 1991). During this 'experience period' sheep readily consumed the supplemented foods and this was reflected in increased food intakes and LW gains, which were a consequence of the improved nutritional quality of these foods compared to food T. It is, therefore, interesting to note that the rules that underlie the feeding behaviour of animals appear to be different when they are given access to one food, from when they are offered two or more foods as a choice (Kyriazakis and Emmans, 1992b).

This experience period had the following consequences on the diet selection of sheep: (i) animals selected a high proportion of the supplemented food in their diet during the second sub-period when they were given a choice (SP3). However, their diet selection did not change systematically over time, as it had changed during SP1. In the case of sheep offered a choice between food T vs TU their diet selection, although indistinguishable from random, led to LW gains which were very similar to the predicted potential growth of sheep of this LW and breed (Emmans, 1997). It is, therefore, likely that their diet selection reflected their nutrient requirements, and that the calculations upon which we based the rate of urea inclusion (AFRC, 1993) were an overestimate. (ii) sheep fed on the low protein food LP for the longest period of time (group L2) selected a diet which was higher in supplemented food, and consequently had a higher protein content. This is to be expected if sheep were selecting a diet to meet their protein requirements; as L2 sheep were delayed in their growth they would be then expected to have higher protein requirements that the heavier L1 and H sheep (Kyriazakis and Oldham, 1993) and hence select diets higher in protein content. (iii) when animals were given a choice between TC vs TFC they selected a diet that consisted mainly of the former food (approximately 700 g/kg TFI). The 'avoidance' of food TFC was also evident, to a certain degree, in choice T vs TFC, even after the 'experience period'. This avoidance is likely to reflect the persistence of the avoidance of the non-nutritional property(ies) of food TFC, which overrides any potential nutritional benefits arising from its consumption. This persistence was much greater than expected and has been suggested by Forbes and Kyriazakis (1995), and could perhaps account for the occasional failure of the animals to select a diet on the basis of the nutritional properties of foods on offer (e.g. Burritt and Provenza, 1992; Cook et al., 1995).

The above results give very little support to the view that there is an unlearned appetite for protein in sheep, as sheep previously deprived of protein failed to select a protein supplemented diet for a number of days. Instead it is likely that their initial diet selection was determined by the non-nutritional properties of the foods on offer. Selection of considerable amounts of the supplemented food was gradual and 'consistent' only after the animals had experienced considerable consumption of it. This supports the view that control over diet selection is learned. Although not directly addressed and hence not seen in this experiment, the speed of learning to select for an appropriate diet could depend on the extent to which previous feeding has affected the animal's physiological state (Kyriazakis et al., 1999). Animals that have been greatly deprived of a nutrient can be expected to select more rapidly the supplemented food, since under these circumstances the reinforcing properties of feeding behaviour can be very rapid indeed (Capaldi et al., 1991). The effects may be similar to the reinforcement humans seem to derive from drinking after a period of water deprivation (e.g. Kirk and Logue, 1997). This view seems to account adequately for the ability of animals to select an appropriate diet.

Chapter 3

The continuum between preferences and aversions for flavoured foods in sheep conditioned by administration of casein doses

> *"It is one of my most important and verified maxims that nature make no leaps.*
> *This I have called the law of continuity"*
>
> Gottfried Wilhelm Leibniz, (1646 - 1716)

3.1 Abstract

The main objective of the experiment was to investigate whether a continuum links the development of conditioned flavour preferences and conditioned flavour aversions (CFPs and CFAs) in sheep, towards food flavours associated with intraruminal administration of increasing doses of the same nutritive stimulus: casein. A secondary objective was to investigate the effects of dose of casein administration and the number of repeated exposures to casein on the rate of establishment, magnitude and degree of persistence of developed CFPs and CFAs.

The experiment consisted of three conditioning periods each of 8 days. A food with low CP (39.3 g/kg DM) and ME (5.3 MJ/kg DM) contents was used in combination with one of two flavours, orange and aniseed, on a total of 48 Texel x Greyface male, 3 month old sheep. The sheep were conditioned to associate one flavour added to the test food with a particular dose of casein (C) suspended in water (8.75, 17.5, 35 and 52.5 g/animal), or a differently flavoured test food with an equal amount of water. Each dose was administered by gavage through a stomach tube twice daily to each animal. At the end of each conditioning period the preference for the two flavours was measured by a two choice preference test between the two flavoured test foods for 40 min. After the completion of the last conditioning period, three more preference tests (persistence tests), without any intervening exposure to the flavoured test food, were conducted at 7, 21 and 35 days. Preference for the flavoured test food paired with casein was affected by the interaction ($P<0.001$) between the dose of casein and casein association respectively. Association with casein (DPR: intake of a flavoured test food paired with casein/g total intake of flavoured test foods during the preference test) led to CFPs (DPR> 0.70) at the two lowest and to CFAs (DPR< 0.30) at the

two highest doses. CFPs and CFAs were established by the second conditioning and there was no difference in the rate of establishment between preferences and aversions. The persistence tests showed a strong and similar degree of persistence of both formed CFPs and CFAs, since these were not affected by time of persistence preference test or by any of the interactions with time. In view of these results a model is proposed which could account for the conditioned responses of sheep towards administration of increasing doses of the same nutrient. The existence of a continuum between CFPs and CFAs created by the same nutrient given at different doses, could be the basis of how ruminants select a diet which meets their nutrient requirements at a particular point in time, and avoid excess of nutrient intake.

3.2 Introduction

We are interested in how animals learn to predict the nutritional consequences of foods from the characteristics of their sensory properties (e.g. taste, colour, odour). This should help us to understand how animals, and herbivores in particular, select a diet which seems to meet their needs - nutrient requirements - whilst at the same time minimises the consumption of harmful compounds (Provenza, 1995b,c; Kyriazakis, 1997). The prevailing view is that animals form conditioned responses towards the sensory properties, and in particular the flavour, of the foods and their positive or negative post-ingestive consequences (PIC) which result from their consumption (Provenza, 1995c; Kyriazakis et al., 1997 and 1998b). We shall call the former conditioned food (flavour) preferences (CFPs) and the latter conditioned food aversions (CFAs) (Sclafani, 1995).

An attractive hypothesis to consider is that put forward by Provenza (1995c), which suggests that CFPs and CFAs, resulting from the consumption of different doses of the same nutrient, reside along a continuum. Initially the animals develop CFPs as a result of the 'beneficial' PIC that accompany the consumption of a nutrient(s). However, over-consumption of nutrients should be expected to result in CFAs, since eventually excess of any nutrient could be seen as having potential 'toxic' consequences (Kyriazakis, 1997). This hypothesis would be able to account for the observation of ruminants selecting a diet which meets their nutrient requirements at a specific point in time, whilst avoiding over-consumption of nutrient(s) (Kyriazakis and Oldham, 1993).

To date the 'continuum' hypothesis has been tested directly in ruminants by Villalba and Provenza (1997c), where different doses of rapidly degradable nitrogen were used as the nutritive stimulus. The outcome of this study has not given strong support to the hypothesis, mainly because the test doses of nitrogen were almost trivial in amount and hence in consequences, as well as due to other experimental shortcomings. The latter will be discussed in detail later. For this reason we have designed an experiment to investigate whether a source of rumen degradable nitrogen, casein given at different doses, could play a role in the development of CFPs and CFAs in sheep, when the flavour of the food is associated with its administration. Casein seemed to be ideal to test the idea of the continuum, since at lower doses of administration it results in increases of microbial nitrogen available to the ruminant animal, whereas higher doses lead to adverse consequences due to excess ammonia accumulation (Russell et al., 1982; Rooke et al., 1987; Urbaniak, 1995). A complimentary objective of the experiment was to test for the effects of level of casein administration and the number of repeat exposures to it on the development, magnitude and persistence of CFPs and CFAs in sheep.

3.3 Materials and methods

3.3.1 Animals and management

A total of forty-eight Texel x Greyface male sheep, born indoors, were used in the experiment. They had been reared indoors until they were transferred to the individual pens of the experimental unit. On arrival the animals were eight weeks old and had a mean live-weight (M) of 23.4 (s.e. 0.32) kg. The experimental unit was a slatted floor, sheep shed that was naturally ventilated. Temperature was recorded by maximum-minimum thermometers. Each individual pen (1.29 x 2.06 m) was equipped with a trough for the food and either a bucket or an automatic drinker which gave continuous access to fresh water. Prior to the start of the actual experiment, sheep were offered a basal (relatively low in protein) food for a 7 day acclimatisation period (see below). During this acclimatisation week all sheep were vaccinated against different types of clostridia and treated against any possible internal parasites.

3.3.2 Foods and flavours

Two foods with different crude protein (CP) and metabolisable energy (ME) concentrations were used (Table 3.1). The basal food (B), in a meal form, was based on barley, sugar beet pulp and alkali-treated straw (Viton, BOCM PAULS LTD, Ipswich), with a CP content of 95 g and ME content of 9.9 MJ/kg DM. The test (novel) food (T) was of a low quality and low rumen fermentability, based mainly on oat feed (0.91) and was made into pellets. The CP of the test food was 39 g and its ME content 5.3 MJ/kg DM; hence the food was inadequate to supply both the protein and energy requirements of the experimental animals. However, the test food was also imbalanced in terms of its effective rumen degradable protein (eRDP) in relation to its fermentable ME content (eRDP:fME ratio of 3.2 which is well below the optimum recommended by AFRC (1992) ratio of 10). Both foods were intended to be similar and non-limiting in mineral and vitamin contents. The two foods were also made quite different in their physical form so that the sheep could further distinguish between them on the basis of their sensory properties. Samples of both foods offered, were taken every week and a composite sample was analysed for Dry matter (DM), crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), S, Ca and P contents (Table 3.1).

Two flavours (orange and aniseed, International Additives Ltd., Wallasey) formulated on an oil-based, liquid form were used. The flavours were further diluted with vegetable oil (dilution 1:4) and subsequently sprayed on to the test food (pellets) at a level of inclusion of 7.5 g undiluted flavour/kg test food, in the afternoon of the day preceding their use. The two flavours have been previously used and tested on sheep in similar experiments and have been shown to have an equal acceptability by sheep (Kyriazakis et al., 1997). The role of the flavours was to enable sheep to form associations between the test food and administration of casein.

3.3.3 Acclimatisation period

During the 7-day acclimatisation period, sheep were offered the basal food for seven hours, from 9:00 to 16:00 h. This was done in order to maintain a level of feeding motivation in the animals, so that the test food would be readily consumed when offered. In addition, in order to familiarise the animals with the rumen-sampling regime (see below), stomach tubes were introduced during this period to each animal between 13:00 and 15:00 hours three times for three days.

Table 3.1 *Ingredient and chemical composition of the experimental foods*

	Foods	
	Basal food	Test food
Ingredients (g/kg fresh matter)		
Oatfeed	-	914.2
Barley	357.0	-
Sugar beet pulp	300.0	-
Alkali treated straw	272.9	-
Molasses (CMS 20)[†]	50.0	50.0
Salt	-	5.6
Dicalcium phosphate	10.3	11.8
Limestone flour	7.1	10.7
Calcined magnesite	0.2	1.7
Vitamin+mineral premix[‡]		
Scotmin ewe/lamb	2.5	1.6
Scotmin dairy/beef	-	0.4
Sodium sulphate	-	4.0
Analysed chemical composition (g/kg DM)		
Dry matter	873	898
Crude protein	95	39
Gross energy (MJ/kg DM)	16.9	17.3
Ash	84	78
ADF (Acid Detergent Fibre)	201	358
NDF (Neutral Detergent Fibre)	325	709
Calcium	9.0	10.1
Phosphorus	2.4	2.8
Sulphur	3.3	2.5
Calculated protein and energy components (g/kg DM)[¶]		
Effective rumen degradable protein (eRDP)	64	25
Digestible undegradable protein (DUP)	16	8
Metabolisable protein (MP)	57	24
Metabolisable energy ME (MJ/kg DM)	9.9	5.3
Effective metabolisable energy fME (MJ/kg DM)	9.4	5.0

[†] CMS 20 condensed molasses soluble (Intermol, Cobham, Surrey).

[‡] Vitamin and mineral premix supplied by Scotmin Ltd, Ayr, Scotland.

[¶] Calculated according to AFRC (1993).

3.3.4 Experimental Design

The experiment lasted for 59 days and involved three repeated conditioning periods (8 days long each) and three subsequent persistence tests. To achieve the experimental objectives a completely randomised design was conducted. The factors were: 4 different dose levels of casein (nutritive stimulus), associated separately with one of the 2 flavours (orange or aniseed) mixed with the same test food. The sheep were randomly (on the basis of body weight) assigned to one of four groups, which corresponded to a certain dose level of casein (12 lamb/dose). Within each group sheep were further divided into four sub-groups (n=3) and each sub-group received a unique combination of the test food flavoured either with orange or aniseed, associated with the administration of either casein or water (control). Thus, casein order, flavour order and flavour association with casein were also completely randomised within a casein dose.

3.3.4.1 Nutritive stimulus

The doses of casein were 8.75, 17.5, 35 and 52.5 g/animal (providing 1.25, 2.5, 5, and 7.5 g N respectively); the casein used was lactic acid casein (Bacarel and Co Ltd, Ashford, Kent). Each dose was given by gavage through a stomach tube, twice daily at 10:00 and 14:00 hours to each animal, in order to repeat the reinforcement created by the N provision and to minimise the consequences of a casein pulse into the rumen. The casein was suspended in water just before (5-10 min) it was given to the sheep. This was done by mixing 300 ml of warm (35-37 °C) tap water with the pre-weighted amount of casein (8.75 - 52.5 g) in plastic bottles.

The lowest amount of casein (twice 8.75 g) was chosen to supply an amount of protein equal to the estimated metabolisable protein (MP) yield to the test food assuming an intake of 600-650 g/d (i.e. yielding about 9.5g MP/d). The three higher amounts were arranged on a geometric progression, with the exception of the highest amount (twice 52.5 g/d instead of twice 70 g/d). The latter was done to avoid over-excessive accumulation of ammonia into the rumen. Our hypothesis was that the range of casein amounts used had the potential to create both positive (8.75 and 17.5g doses) and negative (35 and 52.5 g doses) PIC. The above casein amounts and assumed intake of test food were expected to lead to calculated eRDP:fME ratios of 4, 6, 12 and 18 respectively.

***Table 3.2** The conditioning and preference test schedules for lambs offered a low quality food (test food) sprayed with one of two flavours (orange or aniseed) which were associated with the administration of either casein or water. This schedule is for 12 lambs on one level of casein dose*[†].

Day	Lambs			
	n=3	n=3	n=3	n=3
	Orange + casein	Orange + water	Aniseed + casein	Aniseed + water
	Orange + casein	Orange + water	Aniseed + casein	Aniseed + water
	Rest (basal food)			
	Rest (basal food)			
	Aniseed + water	Aniseed + casein	Orange + water	Orange + casein
	Aniseed + water	Aniseed + casein	Orange + water	Orange + casein
	40 min preference test (orange vs aniseed)			
	Rest (basal food)			

[†] There were four dose levels of casein and the schedule was repeated three times for each casein dose group.

3.3.4.2 Conditioning schedule and sampling procedure

A conditioning period of 8 days, repeated three times was chosen to be of sufficient duration (Kyriazakis et al., 1997 and 1998b) to allow for the development of CFPs and CFAs as a result of casein administration. Animals were given doses of casein or water (control) by gavage twice daily on days 1 and 2 of each conditioning period as described in Table 3.2, whilst one of the flavoured foods was offered from 09:00 to 16:00 hours. During days 5 and 6 the opposite flavoured test food was associated with the reverse stimulus (water or casein), while the gavaging procedure was identical as in days 1 and 2. The flavoured test food was removed at 16:00 hours and refusals were collected and weighed. Days 3 and 4 of each conditioning period were 'rest' days where sheep received the basal food offered freely from 09:00 to 16:00 hours. It was considered necessary for the sheep to be given access to a relatively sufficient and nutritionally balanced food during this time, in order to correct some of the deficiency and to avoid to a certain extent residual carry over effects, created by the consumption of the low quality test food.

At 16:00 hours of days 2 and 6 of the first two conditioning periods (2 hours after the last casein administration and immediately after the removal of the test food) a sample of about 50 ml of rumen content was obtained in a plastic bottle, through a stomach tube from each lamb. The rumen content samples were withdrawn using a hand-held pump. The time of rumen sample collection was chosen to allow for the development of physiological effects in the rumen following casein administration, in terms of NH_3-N (Russell et al., 1982; Meissner et al., 1993) and rumen pH (Rooke et al., 1987).

3.3.4.3 Preference tests

In the morning of day 7 of each conditioning period all sheep were given a choice between the flavoured test foods (500 g of each) sprayed with either orange or aniseed, and refusals were recorded. Each preference test lasted 40 min and was performed early in the morning (09:00 hours) (see Table 3.2). The duration of the tests was slightly increased in relation to previous experiments (see Kyriazakis et al., 1997); this was deemed necessary to allow animals to consume sufficient amounts of the two low quality flavoured test foods and to show a clear choice. The position of the food troughs containing each flavoured test food was completely randomised within each test. After the completion of each preference test, and for the rest of the day, the animals were given fresh basal food until 16:00 hours.

In order to investigate for the persistence of developed preferences or aversions for the food flavours associated with the administration of casein in the absence of continuous reinforcement (i.e. when casein was no longer administered), three more preference tests (persistence tests) were conducted on days 7, 21 and 35 after the completion of the last (third) conditioning period. The persistence tests also lasted 40 min each, and were performed at the same time in the morning. In the intervening period between the persistence tests, the animals were fed on the basal food without any intervening exposure to the flavoured food.

Preference ratios were calculated from the preference (and persistence) tests as the intake of a flavoured food (orange or aniseed) as a proportion of total flavoured food intake (Flavour Preference Ratio, FPR), or the intake of flavoured food associated with the casein administration as a proportion of the total intake of flavoured food during preference tests (Dose Preference Ratios, DPR).

3.3.4.4 Measurements

Food refusals were removed at 16:00 hours each day, weighed and discarded; food consumption was recorded at the same time. Sheep were weighed at the same day every week during the conditioning periods, and every two weeks after the completion of the last conditioning period. The pH of each rumen sample, was measured immediately after sampling using a glass electrode (model RL 250/pH/ISE meter, EIL Russell Laboratories Ltd., Auchtermuchty), while ammonia-N (NH_3-N) was determined with an ion-selective electrode (model 95-5129, EIL Russell Laboratories Ltd., Auchtermuchty) in an exact amount (25 ml) of rumen sample, diluted with distilled water to 100 ml. Rumen samples were not taken during the third conditioning, in order to minimise the disturbance caused to the animals, since the results from the first two conditioning periods showed clear and consistent effects of casein administration on the rumen pH values and NH_3-N concentration.

3.3.5 Statistical analysis

All statistical analyses were performed using GENSTAT version 5.3 (Lawes Agricultural Trust, 1993). Data were analysed separately for the total test food intake during the days of the conditioning period, the preference ratios (FPR and DPR) and rumen sample parameters (pH and NH_3-N). All data were analysed as a split-plot design in time (Horgan and Sword, 1995) with conditioning periods nested within individual sheep. The effects of conditioning period, casein dose level, association (with either casein or water) and flavour (orange or aniseed) were tested on food intake and rumen sample parameters (pH and NH_3-N).

Regarding the analyses for the preference ratios, FPRs were tested to determine whether preference for a flavoured food (orange or aniseed) was affected by its association with casein, casein dose level or the order of flavour presentation, while DPRs were tested to see whether preference for a flavoured food associated with casein was affected by preference for flavour per se, casein dose level, or order of casein presentation. Each of these effects were tested for interactions with time since preference tests during conditioning and persistence were both repeated three times. Data were tested for compound symmetry (i.e. that the variability in response was the same for all treatments) and to ensure that correlation between two factor levels was the same for all pairs (Horgan and Sword, 1995) before analysis. Where necessary, arsine transformations were used to normalise data before the analysis was carried out.

3.4 Results

Data from one lamb during the third conditioning period were omitted because it was found dead before conditioning was completed. The post-mortem examination suggested that it had died from clostridial enterotoxaemia (toxin type E). This lamb was in the group administered with 52.5 g of casein. The preference ratios, food intake and rumen sample parameters from this lamb during the third period were treated as missing values for the statistical analysis.

3.4.1 Intake of flavoured test food during conditioning periods

The average intakes of the flavoured test food during days when casein or water was administered (mean values for days 1,2 and 5,6) are shown in Table 3.3. There was a highly significant interaction between casein dose and casein association ($P<0.001$). The intake of the flavoured test food was lower when casein was administered, but this was mainly evident at the two highest casein doses. There was also a significant interaction between conditioning period and casein association ($P<0.05$). As conditioning progressed differences between the intakes of the flavoured test food associated with either casein or water became clearer, with intake of flavoured test food being consistently lower when casein was administered (overall means 539 vs 651 g/7 h respectively). There was also a significant main effect of casein dose level ($P<0.001$) and casein association ($P<0.001$), whereas conditioning period had no main effect on the intake of the flavoured test food. There were no effects of flavour or order of flavour presentation on the intake of flavoured test food during conditioning periods (these are not shown on Table 3.3).

Table 3.3 *Average intake of flavoured test food (g/7h) by lambs receiving different doses of either casein (+) or water (-) during each of the three conditioning periods.*

		Casein dose (g)					
Conditioning period	Casein association	8.75	17.5	35	52.5		Means
	+	571	694	534	393		**548**
1st	-	590	673	656	511		**607**
	+	644	677	591	268		**544**
2nd	-	595	722	685	523		**631**
	+	686	640	512	258		**524**
3nd	-	661	778	784	633		**714**
							(s.e.d. 30.4)
Means	+	**634**	**670**	**546**	**306**		**539**
	-	**615**	**724**	**708**	**556**	(s.e.d. 18.9)	**651**
Significance of :							
Conditioning (C)					NS		
Dose (D)					***		
Casein association (CA)					***		
C x D					NS		
C x CA					*		
D x CA					***		
C x D x CA					NS		

* P< 0.05; *** P< 0.001.

3.4.2 Rumen pH and NH_3-N

The mean pH values and NH_3-N concentrations in the rumen samples taken 2 hours after casein or water was administered for the second time within a day are shown in Table 3.4.

Table 3.4 *Mean effects of different doses of either casein (+) or water (-) administration on rumen pH values and ammonia concentration (NH_3-N, mg/l) across conditioning periods. The effects of conditioning period and its interactions were not significant and are not shown.*

	pH		NH_3-N	
Casein dose (g)	-	+	-	+
8.75	6.72	6.60	135	278
17.5	6.82	6.72	133	329
35	6.80	6.64	130	529
52.5	6.82	6.75	125	694
Means	**6.79**	**6.68**	**131**	**457**
s.e.d.	(0.028)		(18.4)	
Significance of[†]:				
Dose (D)	NS		***	
Casein association (CA)	***		***	
D x CA	NS		***	

*** P< 0.001.

[†] Conditioning and its interactions with dose and casein association were not significant.

Rumen pH values were affected only by casein association ($P<0.001$); pH was consistently lower when casein rather than water was administered (6.68 vs 6.79 respectively). Rumen NH_3-N concentrations were significantly affected by both casein dose level and casein association ($P<0.001$). There was also a significant interaction between casein dose level x casein association, indicating that the increase in casein dose levels administered resulted in significant and consistent ($P<0.001$) increases in rumen NH_3-N concentrations (Table 3.4), up to 5.5 times that of the controls (animals dosed with water). All above effects were independent of conditioning period.

3.4.3 Preference ratios during preference tests

Table 3.5 shows the mean preference ratios (DPRs) for the flavoured test food associated with casein as a proportion of total flavoured food intake for all four doses of casein administration, during the three preference tests conducted at the end of each conditioning period. The total amount of flavoured food consumed during preference tests was not different between the four casein dose levels, but was affected significantly ($P<0.001$) by conditioning period: it increased from 151 to 186 to 253 (s.e.d. 33.1) g flavoured food/40 min from the first to the third preference test respectively.

The mean preference ratios (FPRs) for a flavoured food as a proportion of total flavoured food intake were significantly affected by the interaction casein association x casein dose level ($P<0.001$). For the two lower casein doses (8.75 and 17.5 g casein) a flavoured food associated with casein was preferred, whereas for the two higher doses (35 and 52.5 g casein) the opposite was the case (i.e. aversion towards a flavoured food associated with casein). There was also a significant interaction ($P<0.05$) between conditioning period x casein dose level x casein association, suggesting that preferences or aversions for a flavoured food associated with casein administration were reinforced by repeated conditioning.

Table 3.5 *Mean 'Dose Preference Ratios' (DPR, g of the food associated with casein/g total intake of flavoured foods) for the flavoured test food paired with one of the four doses of casein administration during 40-min preference tests performed at the end of each conditioning period.*

	DPR					
	Casein dose (g)					
Conditioning	8.75	17.5	35	52.5		Means
1st	0.470	0.576	0.334	0.472		**0.463**
2nd	0.773	0.824	0.398	0.273		**0.567**
3nd	0.711	0.747	0.371	0.139		**0.492**
						(s.e.d. 0.060)
Means	**0.651**	**0.716**	**0.368**	**0.295**	(s.e.d. 0.103)	**0.507**
Significance of:						
Time of test (T)			NS			
Dose (D)			***			
Dose order (DO)			**			
T x D			*			
T x DO			NS			
T x D x DO			NS			

* P< 0.05; ** P< 0.01; *** P< 0.001.

Flavour order (i.e. flavour presented first or second within the conditioning period) also affected significantly (P<0.01) preference for a flavoured test food, with flavour presented first during each conditioning period being consistently preferred (e.g. FPRs were 0.678 vs 0.433, (s.e.d. 0.073) when orange was presented first or second respectively). None of the interactions with flavour order were statistically significant.

DPRs were significantly affected by the interaction between conditioning period x casein dose level ($P<0.05$); preferences or aversions for the casein associated food were established only after the completion of the second conditioning period, and were reinforced by the third conditioning (Table 3.5). There were no differences in the rates of development of CFPs and CFAs between casein doses. Mean DPRs across conditioning periods were 0.651, 0.716, 0.368 and 0.295 (s.e.d. 0.103; $P<0.001$) for the four casein doses respectively. The same mean DPR values for the last two conditioning periods, where CFPs and CFAs were evident, were 0.742, 0.786, 0.395 and 0.206 respectively.

Dose order had an effect on DPRs, with the flavoured test food associated with casein being preferred when casein dose was presented first rather than water (0.630 vs 0.385, s.e.d. 0.072; $P<0.05$). There were no significant effects of either flavour used (orange or aniseed) or its interactions on DPRs.

3.4.4 Preference ratios during persistence tests

The second objective addressed by the experiment was whether the developed CFPs and CFAs had different degrees of persistence. The effects of the interaction between casein association and casein dose level ($P<0.001$) on the preference ratios for a flavoured food as a proportion of total flavoured food intake (FPRs), persisted throughout tests conducted 7, 21 and 35 days after the completion of the last conditioning period. No other factors affected the formed preferences or aversions throughout these persistence tests.

Similarly the only effect seen on DPRs, preference ratios for a flavoured test food associated with casein as a proportion of total flavoured food intake, was that by casein dose level, which was identical to the one seen on DPRs during preference tests, as can be seen in Table

3.6. Neither DPRs nor FPRs were affected by the time of performing the persistence test or by any interactions with time.

Table 3.6 *Mean 'Dose Preference Ratios' (DPR, g of the food associated with casein/g total intake of flavoured foods) for the flavoured test food associated with casein administration during 40-min persistence tests performed on days 7, 21 and 35 after the completion of the last conditioning event.*

	DPR					
	Casein dose (g)					
Persistence tests (days)	8.75	17.5	35	52.5		Means
7	0.660	0.797	0.299	0.356		**0.528**
21	0.683	0.664	0.257	0.336		**0.485**
35	0.744	0.821	0.267	0.322		**0.539** (s.e.d. 0.040)
Means	**0.696**	**0.761**	**0.274**	**0.338**	(s.e.d. 0.130)	**0.517**
Significance of:						
Time of test (T)		NS				
Dose (D)		***				
Dose order (DO)		NS				
T x D		NS				
T x DO		NS				
T x D x DO		NS				

*** P< 0.001.

3.5 Discussion

The main objective of the experiment was to investigate whether a continuum links the development of CFPs and CFAs in ruminants (sheep), towards food flavours associated with the administration of increasing doses of the same nutritive stimulus: intraruminal administration of casein. A second objective was to investigate the effects of level of casein administration and the number of repeated exposures to casein, on the rate of establishment, magnitude and degree of persistence of developed CFPs and CFAs. To our knowledge, the main hypothesis has been tested for the first time in the same group of animals within the same experiment. For a meaningful interpretation of the results, in the first part of this discussion we address the physiological (PIC) consequences resulting from casein administration. Given these effects, one would expect the animals to develop either CFPs or CFAs, which would reside along a continuum.

3.5.1 Post-ingestive consequences of casein administration

Based on the results reported by Hunter and Siebert (1987), Kyriazakis and Oldham (1993), Urbaniak (1995), Liu et al. (1995), Chowdhury et al. (1997) it was anticipated that for young sheep with a high growth potential, given access to a poor quality food, the administration of casein in four different doses would result in different PIC. Lactic acid casein was chosen because it is a rapidly rumen degradable source of N, but more advantageous and much less toxic than administration of urea or any other NPN source. This is because it is utilised to a greater extent and requires the minimum time for full adaptation of rumen bacteria (Nicholson et al., 1992; Urbaniak, 1995). The created PIC resulting from its administration were determined by measuring two rumen parameters (NH_3-N and pH) relevant to casein administration. This was expected to allow a meaningful interpretation mainly of the CFAs observed during subsequent flavoured food choices, since accumulation of NH_3-N into the rumen could lead to its absorption at a sufficient rate to lead to negative stimuli arising from ammonia toxicity. There were also expected to be positive consequences from casein administration, such as increased supply of N to achieve a maximal yield of microbial protein in the rumen, promotion of higher rates of rumen digestion of fibre and of provision of nitrogenous substrate for certain species of microbes that have an obligatory requirement for pre-formed peptides or amino acids (Hume et al., 1970). However, we did not directly evaluate any positive consequences.

The observed effects on rumen NH_3-N content during the days of casein administration were in the expected direction, i.e. dose dependent. The main effect of casein compared to water administration was significant for each casein dose level administered to sheep (Table 3.4). However, effects on rumen pH did not seem to be dose related, although differences in pH values between rumen samples taken after administration of casein and water (6.68 vs 6.79, respectively, s.e.d. 0.028) were clear, and within the normal pH range (+/- 0.5 pH units, Van Soest, 1994). This difference could well be attributed to a better use of the food's carbohydrates, due to higher availability of N to microbes, which in turn could lead to a higher production of VFAs (Ørskov, 1998) and hence a pH reduction.

The hypothesis that the two low doses of casein would yield preferences for the flavour associated with their administration is related to the possible complementary contribution of casein nitrogen to rumen bacteria. These benefits must also be considered in relation to the low CP content and low degradability of the test food, and the fact that casein was given twice a day (at 10:00 and 14:00 h) and sometime after the flavoured food was offered. It is likely that by that time the rumen environment was more optimal for its breakdown and incorporation into microbial protein (Ørskov, 1998).

In contrast the hypothesis that the two high doses of casein would lead to the development of CFAs is based on the fact that the rumen would be flooded with ammonia, deriving from degradable nitrogen coming mainly from casein administration, but also from food consumption. This could result in an asynchronous supply of N to rumen bacteria and thus lead to an increase of the rumen NH_3-N concentration. The presence of excess NH_3-N could be due to the rate of utilisation by the microflora being exceeded because of the low degradability of the test food (Huber and Herrera-Soldana, 1994). This is supported by the measurements of rumen NH_3-N made over a period of 4 hours in fistulated sheep administered with urea over the same period of time. Kyriazakis and Oldham (1997) suggested that when production of ammonia in the rumen exceeds the optimum value (in terms of the capacity of bacteria to convert it to microbial protein), further ammonia production will be of no value and could be detrimental. It is possible that NH_3-N consequences in the rumen can be conceived in a number of potential modes including effects on rumen motility, inhibition of rumen microflora activity or direct inhibition of digestive functions (Urbaniak, 1995; Egan and Doyle, 1985).

3.5.2 The Continuum between CFPs and CFAs

Information on the factors affecting food preferences and aversions in ruminants could provide important insights on how these animals perceive, value, gain knowledge and evaluate the PIC of the foods they consume (Sclafani, 1995). It is generally accepted that, if an animal is able to ascribe positive or negative PIC resulting from the consumption of different foods, then it may or not incorporate them into its diet and thus be able to attain optimal nutritional choices, by modulating its food intake and diet selection (Provenza and Balphs, 1987 and 1990; Wang and Provenza, 1996; Kyriazakis et al., 1997 and 1998b).

The results of the present experiment clearly demonstrated that sheep are able to distinguish between the positive or negative PIC of different doses of casein administration, and develop CFPs and CFAs respectively. They further demonstrate that a nutritive stimulus is not necessarily singular in its reinforcing properties (such as toxins are, where only CFAs are formed and hence have no upper limit (Booth, 1985), but can be either positive or negative, depending on the levels of its administration. The above suggests that a nutrient in excessive amounts may have aversive properties that overcome any possible positive PIC (Kyriazakis et al., 1991).

A preliminary hypothesis that food preferences and aversions reside along a continuum, where either of these responses can be conditioned by administration of nutrients depending on an animal's requirements and the amounts of nutrient supply, was suggested by Provenza (1995c). He stated that preference increases when nutrients ameliorate (nutritional) needs, and decreases when they satiate needs. The hypothesis of nutrient specific (i.e. protein) CFPs and CFAs and the existing continuum between them was first investigated in rats (Booth, 1985; Campbell et al., 1987; Sclafani, 1995). It can, however, be hypothesised that the continuum between CFPs and CFAs towards the same nutrient administration would be expected to be more difficult to demonstrate in monogastric animals rather than ruminants, since the former seem to have a relatively higher ability to cope with excess of nutrients, such as protein (Booth, 1985; Kyriazakis et al., 1991). On the other hand, the involvement of the modifying role of the rumen in digestion and the various differences in biochemical and physiological processes in ruminants, increases the interest in the issue of the existence of a continuum between CFPs and CFAs. It was, therefore, proposed that by-products of rumen fermentation (e.g. VFAs, NH_3-N) could condition food preferences or aversions, depending on their rate and amount of release (Provenza 1996). As mentioned in the

Introduction, an attempt to test the 'continuum' hypothesis for administration of a specific nutrient in ruminants, has been made by Villalba and Provenza (1997c). These authors claimed that they have demonstrated a continuum between CFPs and CFAs towards increasing administration of degradable nitrogen sources. The results of their experimental series, however, are contradictory to each other, and there is not clear evidence of a continuum or indeed of any CFPs and CFAs created. For example, they contest that sheep developed CFAs when 0.23 g N/d was administered, and CFPs with doses of 0.23g and 0.35 g N/d, whilst an identical food was used. The purported developed preferences (and aversions), when expressed as ratios, are very close to indifference (0.59 maximum in one of the experiments with urea, compared with preference ratios of e.g. 0.75 and 0.13 for CFPs and CFAs in our experiment). Despite this the authors maintain that preferences and aversions have been observed. This could be due to the fact that they have treated the intakes of flavoured foods offered as a choice during preference tests as independent variables in their statistical analysis, whereas they are clearly dependent on each other and should be analysed as such (Elston et al., 1996). Furthermore, there is a substantial quantitative difference between the results found in our experiment and those mentioned by Villalba and Provenza (1997c). The procedures of our study differed from those of Villalba and Provenza (1997c) along several dimensions (e.g. nature of foods, dosing regimes, route of conditioning events, size of animals), and we have used a much wider range of nitrogen (casein) doses. Our levels of N ranged from 3g N/d to 15 g N/d compared to those of Villalba and Provenza (1997c) where the amounts of N dosed were very low: 0.12 g N/d minimum and 0.92 g N/d maximum dose. According to the results of our experiment, the low levels of nitrogen used by Villalba and Provenza (1997c) could not have greatly influenced the feeding behaviour of animals to any extent, even if differences in metabolic weight are taken into account. Previous evidence (Meissner et al., 1993; Liu et al., 1995;

Urbaniak, 1995) provides strong support for our findings regarding the availability and the response of the rumen in young ruminants to cope with exogenous N without any negative PIC, even after administration of degradable nitrogen in considerably higher levels than 0.92 g N/d.

There is no disagreement between the hypothesis developed by Provenza (1995c) and Villalba and Provenza (1997c), and the findings of this paper. The disagreement lies in whether a continuum that links CFPs and CFAs has previously been shown. We suggest that the observed continuum in the formation of CFPs and CFAs created by the same nutrient source, within the same experiment is demonstrated for the first time by our experiment. The process of a continuum which links the development of CFPs and CFAs can be attributed to the fact that different levels of a nutrient provision produce dose related PIC. It seems now appropriate to develop a descriptive model which could account for the feeding behaviour responses of sheep towards administration of increasing doses of the same nutrient. The proposed model shown in Figure 3.1, derives from the observed responses of sheep to food flavours paired with intraruminal administration of different doses of casein, but we believe that it could be applied to account for the administration of any nutrient. The model is based on the assumption of single linear relationships between casein administration and the response of an individual sheep. It offers a suitable explanation of the continuum in the formation of CFPs and CFAs. When neither of the flavoured foods is associated with the administration of a nutrient dose, and other things being equal, the animal is expected to show equal preference for or indifference to both (i.e. preference ratio of 0.5). This behaviour will continue until the animal perceives a significant positive PIC, and hence develops a preference for the flavoured food associated with the administration of the nutrient.

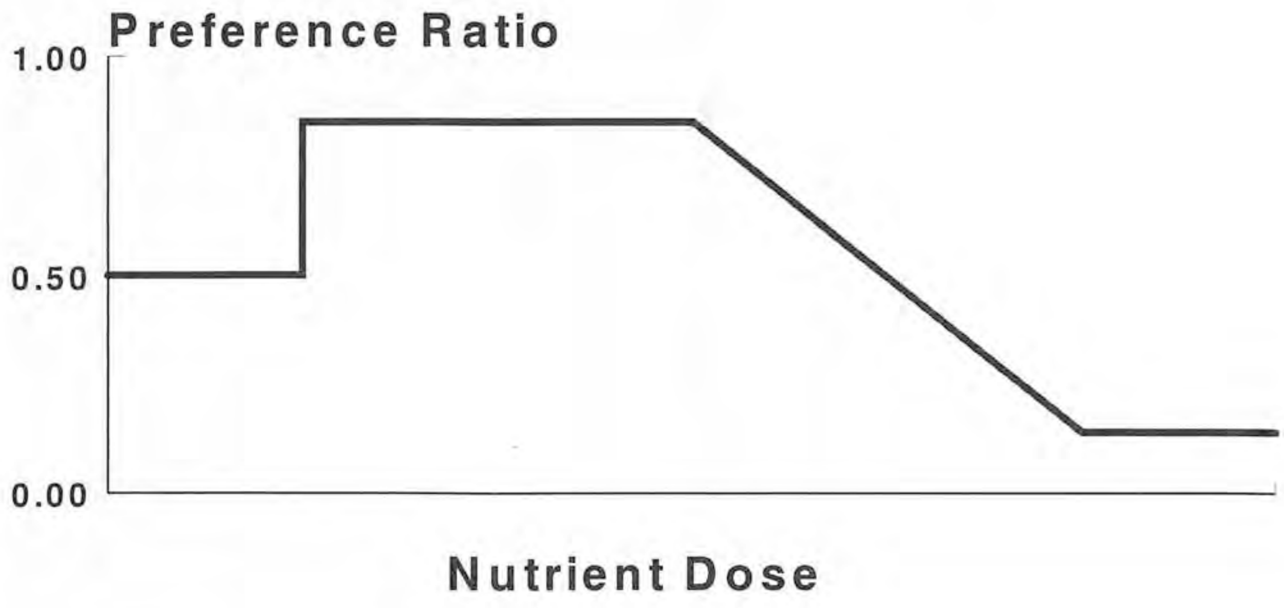


Figure 3.1 The proposed model for the response of sheep to flavoured foods associated with the administration of increasing doses of a nutrient. The response is expressed as a preference (preference ratio) for the flavoured food associated with nutrient administration, as a proportion of total intake (g of the food associated with casein/g total intake of flavoured foods), when sheep are given a choice between the nutrient associated and a non-nutrient associated flavoured foods.

The switch in this behaviour is proposed to be abrupt rather than gradual. The preference created is not absolute, since the animal will continue to consume some of the alternative flavoured food as part of its sampling behaviour; thus the preference ratio will be expected to reach a maximum of 0.8-0.9 (Kyriazakis et al., 1991; Farnsworth and Illius, 1998). Preference is maintained at a maximum until negative PIC start to develop, due to increasing administration of the nutrient. The reduction in the preference for the nutrient associated flavoured food is expected to be gradual rather than abrupt, and dependent on the degree of the negative PIC (Launchbaugh and Provenza, 1994; Kyriazakis et al., 1998b). The preference ratio will be expected to reach a minimum of 0.2-0.1, for the same information constraints suggested for the maximum above.

3.5.3 Rate of development of CFPs and CFAs

A second question asked in the experiment was how quickly sheep form associations between the different flavours and casein doses, which are seen as developed CFPs and CFAs. This could be of interest in establishing how quickly animals learn about the consequences of foods. We expected that the development of CFAs would be more immediate rather than the development of CFPs. This hypothesis was based on previous studies (Provenza, 1996; Kyriazakis et al., 1998b) on the development of CFAs towards administration of toxic compounds, and on suggestions from similar experiments on monogastrics where CFPs seem to be acquired more slowly than CFAs (Booth, 1985; Rozin and Schulkin, 1990). The results of the present study however, do not seem to support this initial expectation. The development of both CFPs and CFAs was not immediate, but nevertheless relatively rapid. During the first conditioning sheep displayed almost a neutral response towards the flavoured food associated with casein, and clear CFPs and CFAs were established only after the second conditioning event. This could be taken to imply that animals needed some time before they could recognise the different PIC of casein administration. They may have also needed some time to be able to associate these effects with the sensory cues of the flavoured test food. These time lags were similar for the formation of both CFPs and CFAs.

The development of CFPs and CFAs towards a food flavour associated with increasing doses of a nutrient, and the continuum between them implies that ruminant animals are able to acquire a degree of flexibility in their dietary habits within a relatively short time. It could be inferred that when preferences are induced for one food then this food will be readily consumed up to a level of consumption where it will be no longer beneficial, i.e. development of negative PIC that result from excesses of nutrient intake.

3.5.4 Persistence of developed CFPs and CFAs

A further objective of the present experiment was to investigate whether the acquired CFPs and CFAs persist in the absence of continuous reinforcement. This would help to explain the persistence in the feeding behaviour in some instances and provide insights on its flexibility. This was tested during persistence tests conducted 7, 21 and 35 days after the completion of the last conditioning period. The duration of the persistence tests was similar to that for the preference tests, because it was considered that any longer exposure to the flavoured test food during these tests could over-familiarise animals with its consumption, in the absence of any reinforcing effect. It could thus lead to the formation of new associations which could overcome former conditioned responses. According to the experimental results, sheep continued to demonstrate preferences towards the flavour that was previously associated with the two low doses of casein and aversions for the flavour associated with the two high doses. The degree of these preferences and aversions was strong and did not change for up to 35 days after the last conditioning (Table 3.6). This may be explained by the fact that CFPs and CFAs were developed quite rapidly (after the completion of the second conditioning), and thus became well established in the following conditioning event.

However, the degree of persistence of developed CFPs and CFAs was larger than anticipated, i.e. CFPs and CFAs were retained for longer than expected. Previous studies in ruminant animals have also shown that there is a degree of persistence of CFPs (e.g. for flavours associated with energy; Ralphs et al., 1995) and furthermore strong persistence of CFAs (Provenza et al., 1994a,b; Provenza 1996; Kyriazakis et al., 1997).The latter is to be expected if CFAs are created towards flavours which are associated with strong PIC. Kyriazakis et al. (1998b) found that CFAs developed through administration of low dose levels of oxalic acid did not persist in the absence of continuous reinforcement, in contrast to CFAs developed towards high oxalic acid dose levels, which had a high degree of persistence. The lack of persistence of CFAs at low dose levels of oxalic acid could be explained by the fact that CFAs were very mild and thus associations were relatively weak and easily extinguished. This was perceived as a flexibility in the feeding behaviour of ruminants, since there would be no advantage to the animal to retain mild conditioned responses for a long time, given possible temporal change(s) in its feeding environment. However, sheep in that experiment (Kyriazakis et al., 1997) were exposed to the flavoured foods, without any intervening reinforcement, for three hours during persistence tests. This could account for the absence of persistence of CFAs given the long exposure during tests. Given the fact that animals had three hours access to the flavoured food it is suggested that they could re-attribute the absence of any negative PIC when consuming small amounts of the previously aversive food, and form gradually new associations. In our experiment the developed conditioned responses however, persisted for up to 5 weeks. It is possible that with our experimental design we actually addressed issues about memory rather than persistence in the absence of reinforcement. Nevertheless, the 5 week persistence of both CFPs and CFAs is surprisingly long, given our knowledge of rapid feeding behaviour adjustments in ruminants in other instances (Tolkamp et al., 1998).

3.6 Conclusion

This study extends previous findings on the development of CFPs and CFAs in ruminants, by demonstrating that sheep are able to distinguish the differential PIC of increasing doses of casein. Whereas small doses of casein lead to CFPs because they are perhaps viewed positively since they could lead to an increased supply of microbial N, high doses of casein are perceived as toxins since they lead to negative PIC (excess rumen NH_3-N) and hence result in CFAs. The rate and the degree of development of such CFPs and CFAs reside along a continuum and are dependent quantitatively on PIC resulting from the nitrogen source administered. Thus the present findings suggest that the feeding behaviour and diet choices in ruminant animals can be influenced quantitatively by nutrient provision. The existence of a continuum between CFPs and CFAs created by the same nutrient, could be the basis of how ruminants select a diet which meets their nutrient requirement at a particular point in time, and avoid excess of nutrient intake.

Chapter 4

Conditioned feeding responses of sheep towards flavoured foods associated with casein administration: the role of long delay learning

"Nature never makes things for means or no uses"

John Locke, (1632 - 1704)

4.1 Abstract

The objective of two experiments was to investigate whether a delayed type of learning, could account for the conditioned feeding responses of sheep towards novel food flavours associated with post-ingestive consequences (PIC) created from the administration at different points in time of a nutritive stimulus (casein). The doses of casein were low (15 g) and high (75 g) for Experiments 1 and 2, previously known to result in positive and negative PIC respectively. Each experiment consisted of 3 conditioning periods, during which sheep were trained to associate one of two novel flavours with either casein or water (placebo) administration. During each conditioning, a novel flavoured food, low in protein and relatively high in energy, was offered for 3 hours (8:00-11:00 h) and was followed by a non-flavoured, nutritionally similar food for the rest of the feeding time (11:00-17:00 h). Sheep were randomly assigned to one of three treatments that were defined by the time when casein or water doses were administered, in relation to the presence of the flavoured food (A= 8:30 and 10:00, B= 11:30 and 13:00 and C= 14:30 and 16:00 h respectively). At the end of each conditioning period preference tests were performed, where sheep were offered a choice between the two flavoured foods. There was no effect of time of casein administration on the conditioned responses towards flavoured foods in either experiment. In both experiments, the proportion of the flavoured food selected was significantly affected by the interaction between preference tests and casein association. For Experiment 1 this was due to an increasing preference for the casein associated food accompanied by a decreasing preference for the water associated flavoured food as a result of repeated conditioning. The degree of such preference was different between flavours used for association with casein or water. For Experiment 2 avoidance of the casein and preference for the water associated food were established after the completion of the 2nd conditioning

and reinforced by the 3rd conditioning. Flavours used had a lesser effect on the conditioned responses of this experiment. The results support the view that sheep develop conditioned responses towards novel food flavours associated with the administration of a nutritive stimulus, even when the PIC resulting from its administration are disassociated in time from the presence of the flavoured food.

4.2 Introduction

Ruminants develop strong conditioned responses towards novel food flavours which are associated with positive or negative post-ingestive consequences (PIC) (e.g. Kyriazakis et al., 1997, 1998b; Chapter 3). It is suggested that such conditioned responses dictate subsequent feeding behaviour, both in terms of the amount of food eaten and the composition of the diet selected (Kyriazakis, 1997). In most cases it is assumed that such associations are formed within the context of a classic learning paradigm, which requires a close temporal contiguity between the novel food flavour and its PIC (Provenza, 1995c). Whilst it is the case that certain PIC are the immediate consequence of food consumption (e.g. osmotic changes within the rumen, Cooper, et al., 1995; Kyriazakis and Oldham, 1997), in most instances the action of digestion and absorption imposes considerable delays to the creation of PIC, which could lead to temporal disassociation between sensory properties and PIC. This would be of particular relevance to ruminants where, due to their gastrointestinal track morphology, PIC can occur up to several hours after food consumption (Forbes, 1995; Weston, 1996). The question then is whether a 'delay learning' could account for such conditioned feeding responses in ruminants.

Garcia et al. (1966) suggested that conditioned responses can be independent of the time lag between the consumption of a novel food and its associated negative PIC. Subsequent experiments on rats gave strong support to this suggestion by demonstrating that if the consumption of a novel food was followed by illness which occurred up to 24 h later, the animal excluded that food or food flavour from its diet (for reviews see Kalat and Rozin 1973; Garcia and Hankins, 1977; Domjan 1985; Capaldi, 1992; Sclafani, 1995). Extension of these studies to ruminants showed that they, like rats, formed conditioned responses to food flavours even when their associated negative PIC were delayed for up to 12 h following food consumption (Burritt and Provenza, 1991; Kronberg et al., 1993; Provenza et al., 1994a,b). In the studies on ruminants the negative PIC were created by the administration of a strong 'toxin', LiCl. One can envisage evolutionary advantages for the existence of conditioned feeding responses arising through delay learning, especially when they are formed from the administration and the consequences of toxins (Rozin, 1977; Garcia et al., 1985).

The question, however, still remains whether a delayed type of learning could lead to conditioned feeding responses towards novel food flavours associated with positive or negative PIC, resulting from the administration of a nutritive stimulus. This question formed the objective of the experiments reported here. The nutritive stimulus used to address it was two levels of casein administration, expected to lead to positive and negative PIC respectively. A further objective was to investigate whether the rate of development and the magnitude of conditioned feeding responses depend on the degree of temporal contiguity between food consumption and PIC.

4.3 Materials and Methods

Two consecutive experiments were carried out; there was a two-week interval between them. The experimental procedures and the conditioned stimulus used were similar to those applied in a previous experiment (Chapter 3), and followed closely the protocol developed by Kyriazakis et al. (1997) to investigate conditioned feeding responses in sheep. Specific design and methods for each experiment are detailed below.

4.3.1 Animals and management

Thirty-six Texel x Greyface wethers, were used. They weighed 38.1 (SD 2.87) kg when obtained at approximately 10 months of age. Prior to the experiments, they were grazing outdoors. The sheep had been vaccinated against different types of clostridia and on arrival they were dewormed and treated for foot rot. They were housed and individually penned in a slatted floor sheep shed that was naturally ventilated. Each individual pen, measured 1.50 x 1.87 m, was equipped with a metal trough for the food and either a bucket or an automatic drinker that gave continuous access to fresh water. During preference tests (see below) the metal trough was removed and replaced with two identical plastic troughs. An initial acclimatisation period which lasted for 14 days was considered long enough to allow sheep to become familiar with their new environment and procedures.

4.3.2 Foods

Three foods (one basal and two test foods) with different crude protein (CP) and metabolisable energy (ME) contents were used. The ingredients and chemical composition of the experimental foods are shown in Table 4.1.

Table 4.1 *Ingredient and chemical composition of experimental foods*

	Test pellets	Test meal	Basal food
Ingredients (g/kg fresh matter)			
Oatfeed	779	805	
Barley	104	129	325
Sugar-beet pulp			202.5
Alkali-treated straw (NIS)			450
Hi-pro Soya	31.7	31.7	
Molasses (CMS 20) [†]	50		
Salt	6.2	6.2	
Dibasic calcium phosphate	9.7	9.7	10.5
Limestone flour	11.6	11.6	9.2
Calcined magnesite	1.4	1.4	0.34
Sodium sulphate	3.5	3.5	
Vitamin+mineral premix [‡]			
Scotmin ewe/lamb	2.2	2.2	2.2
Scotmin dairy/beef	0.34		0.23
Analytical chemical composition (g/kg DM)			
Dry matter	881	886	885
Crude protein	82	73	74
Gross energy (MJ/kg DM)	17.7	17.3	17.9
Ash	81	77	71
ADF (Acid Detergent Fibre)	294	290	263
NDF (Neutral Detergent Fibre)	570	592	521
Calcium	9.2	9.7	9.7
Phosphorus	3.7	4.0	3.7
Sulphur	4.4	4.4	4.5
Calculated protein and energy components (g/kg DM)[¶]			
Effective rumen degradable protein (eRDP)	52	47	52
Digestible undegradable protein (DUP)	14	14	13
Metabolisable protein (MP)	48	44	46
Metabolisable energy ME (MJ/kg DM)	9.7	9.5	9.8
Effective metabolisable energy fME (MJ/kg DM)	9.2	9.0	9.3

[†] CMS 20, condensed molasses soluble (Intermol, Cobham, Surrey).

[‡] Vitamin and mineral premix supplied by Scotmin Ltd, Ayr, Scotland.

[¶] Calculated according to AFRC (1993).

The basal food was formulated as a meal (84 g CP/kg DM and 11 MJ ME/kg DM) while the test food was formulated both as pellets and as meal (82 and 73 g CP/kg DM respectively and 10 MJ ME/kg DM for both). The two physical forms of the test food were considered necessary to allow sheep to distinguish between them (see below), since their composition was very similar. All foods were formulated to be relatively low in protein in relation to energy and inadequate to support the potential growth of the animals (AFRC, 1993); they were similar and non-limiting in mineral and vitamin contents.

4.3.3 Food flavours and hedonic tests

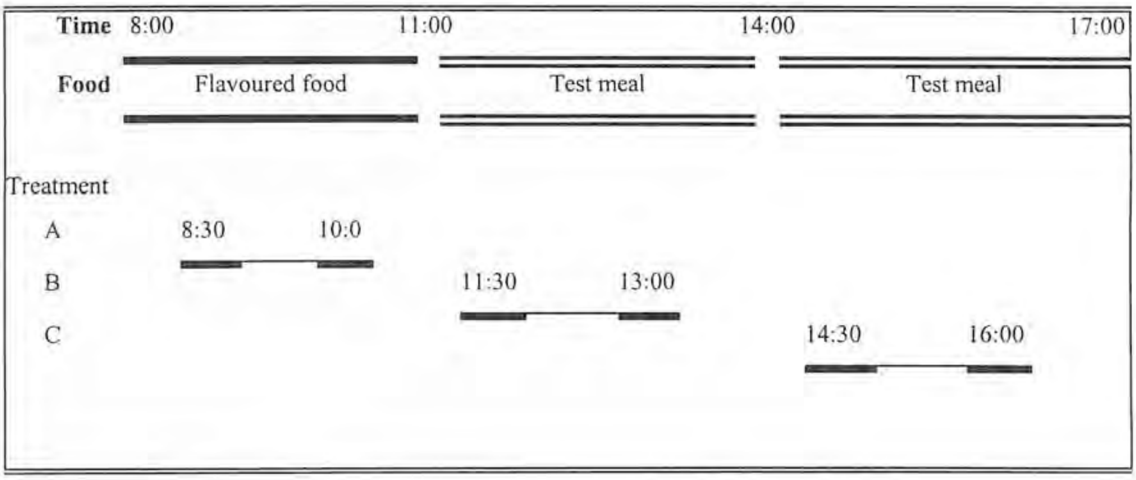
Four artificial flavours in a liquid form (provided by International Additives Ltd., Wallasey) were used. They were further diluted in sunflower oil (orange, aniseed), paraffin oil (garlic) or water (fenugreek) at a level of 1:4, according to manufacturer instructions. Subsequently, the flavoured solution was sprayed onto the test pellets food whilst in a mixer, a day prior to its use. A different set of two flavours was used per experiment. Evidence from both our experiments (unpublished data) and the literature (Hills et al., 1999) suggests that associations between a food flavour and PIC once established can then be retained for extended periods of time and prevent the formations of new associations. The initial acceptability for each set of flavours was tested during a 10-minute hedonic test. These tests were conducted during the acclimatisation period for the set of flavours used in Experiment 1 and during the 2-week interval between experiments for the set of flavours used in Experiment 2. They help to interpret how the initial acceptability of the flavours is modified by subsequent associations. The inclusion rate of the flavours used was 7.5 g flavour/kg food (for details and justification see Kyriazakis et al., 1997; Chapter 3).

4.3.4 General experimental procedures

4.3.4.1 Conditioning

Each experiment consisted of three consecutive conditioning periods, each of which lasted 7 days. The 36 sheep were equally assigned to one of three time related treatments A, B and C. The allocation was such that mean live-weights did not differ significantly between treatment groups. The three treatments were defined by the time when the nutrient stimulus (or water) dose was administered in relation to the presence of the flavoured (test) pelleted food: A= 8:30 and 10:00, B= 11:30 and 13:00 and C= 14:30 and 16:00 h respectively (see Table 4.2).

Table 4.2 *Time schedules for dosing with casein and feeding during days 1,2 and 4,5 of one conditioning period for sheep in treatments A, B and C (n=12). The food sprayed with one of two flavours, was associated at different points in time with the dosing (twice a day) of casein or water. Treatments A, B and C are defined by the time of casein or water dosing and indicated by thick bars on the Table. Identical schedules were used in both Experiments 1 and 2. For the schedule of conditioning, see Table 4.3*



The nutrient stimulus used was lactic acid casein (Bacarel and Co Ltd, Ashford, Kent). Each casein dose was given by gavage through a stomach tube to each animal twice daily for each treatment. This was done in order to repeat the reinforcement created by the provision of N into the rumen. The flavoured food was offered (750 g) for three hours (8:00-11:00 h). Immediately after its removal the test meal was offered in exact amounts (750 g at 11:00 and 750 g at 14:00 h) and any refusals were removed at 17:00 h (Table 4.2). The calculation of the amounts of the foods offered were based on our experience from a previous study with similar sheep (Chapter 3) and on the daily food intake of sheep as measured during the acclimatisation period. Within each treatment sheep were assigned to four sub-groups (n=3), and they were trained to associate a unique combination of a flavoured food that was paired with administration of casein suspension and the consumption of the opposite flavoured food that was paired with administration of a placebo (equal amount of water). The experimental design was balanced for both order of flavour and order of casein presentation (Table 4.3). More specifically, during each conditioning period flavours and casein or water administrations were associated as follows. For half of the sheep, one of the flavoured foods was offered while dosing with casein for the first two days (day 1 & 2). Then followed one rest day (day 3) and for the two subsequent days (day 5 & 6), the opposite flavoured food was offered and sheep were dosed with water. The last two days of the conditioning period (days 6 & 7) were also rest days. In the other half of the sheep, the order of association was reversed with the sheep offered a flavoured food paired with water followed by the opposite flavoured food paired with casein administration.

4.3.4.2 Preference tests

The ability of sheep to learn to associate the flavour of a food with concurrent or delayed PIC created by the administration of casein was evaluated in subsequent preference tests conducted at day 6 of each conditioning period (Table 4.3). These tests lasted for 20 min each and were conducted early in the morning (8:00 h). All animals were given a choice between equal amount (1000g) of the two flavoured foods. The duration of the tests was considered long enough for choices to be made, without causing any significant extinction of the association already formed during the conditioning periods (Kyriazakis et al., 1998b). The amount of foods offered as a choice was well above the intake of sheep over a 20-min period. The position of the troughs and the flavoured foods they contained were randomised within each test. During rest days and immediately after the completion of the preference tests fresh basal food was offered at fixed allowance (1500 g) and for the same period (until 17:00 hours).

Table 4.3 *The conditioning and preference test schedule for one group of sheep (n=12) offered foods sprayed with one of two flavours, which were associated at different points in time (concurrently in treatment A or with delays in treatments B and C) with the administration of casein or water. Identical schedules were used in both Experiments 1 and 2. This schedule is for one time treatment*[†].

Day	n = 3	n = 3	n = 3	n = 3
1	Flavour 1[‡] + casein	Flavour 1 + water	Flavour 2 + casein	Flavour 2 + water
2	Flavour 1 + casein	Flavour 1 + water	Flavour 2 + casein	Flavour 2 + water
3	Rest (basal food)			
4	Flavour 2[¶] + water	Flavour 2 + casein	Flavour 1 + water	Flavour 1 + casein
5	Flavour 2 + water	Flavour 2 + casein	Flavour 1 + water	Flavour 1 + casein
6	20 min preference test			
7	Rest (basal food)			

[†] This schedule was repeated for each group of sheep (time treatment) throughout the two experiments.

[‡¶] For Experiment 1 the two flavours were orange and aniseed and for Experiment 2 were fenugreek and garlic.

The outcome of these choice tests was used as the learning measurement. It was expressed as Preference Ratios. One was calculated on the basis of the intake of a flavoured food (e.g. orange in Experiment 1 and fenugreek in Experiment 2 averaged over casein and water doses) as a proportion of total flavoured food intake, hence called flavour ratio (FRs). The other was calculated on the basis of the intake of a flavoured food associated with casein administration (averaged over both flavours in each time treatment) as a proportion of the total intake of flavoured foods, hence called treatment ratio (TRs).

4.3.4.3 Measurements

Sheep were weighed at the start of each experiment and then on a weekly basis. The intake of the flavoured food was recorded at 11:00 h and the intake of the test meal food at 17:00 h during days when conditioning took place. Intake of basal food was recorded at 17:00 h on rest days. Food refusals were discarded.

4.3.5 Specific experimental design

4.3.5.1 Experiment 1

This experiment was designed to study whether sheep can learn to select for food flavours associated with positive PIC resulting from casein administered either concurrently or after delays in relation to the consumption of the flavoured food. The positive PIC were expected to be the result of stimulation of microbial synthesis in the rumen. The flavours used were orange and aniseed. After the acclimatisation period the animals were allocated into the three treatment groups (n=12 sheep per group) based on their LW (mean of 42.7 kg, (SD 2.85)). A low dose of casein (15 g, providing 2.24 g N) was used as the nutritive stimulus. The underlying assumption was that it would lead mainly to positive PIC, providing complementary N in relation to energy and hence improve microbial activity in the rumen of sheep given a food that was deficient in e-RDP (Leng and Nolan, 1984; AFRC, 1993). In addition, the use of a restricted period of food availability each day (fixed amounts of foods) was expected to reduce differences in food intakes between animals, and to maintain a rumen environment where the release of ammonia from the rapidly degradable N-source (casein) administered would have been more synchronous with the digestion of dietary energy (Sinclair et al., 1995).

4.3.5.2 Experiment 2

The second experiment started two weeks after the completion of the first. The same sheep were used but were re-allocated into three new treatment groups on the basis of their LW which had changed to a mean of 45.9 kg, (SD 2.61). A high dose of casein was used (75 g, providing 11.17 g N) as the nutritive stimulus. This dose was expected to lead to negative PIC, i.e. provide excessive amounts of degradable N in the rumen which would result in accumulation of NH_3-N beyond the conversion capacity of the liver (Van Soest, 1994; Urbaniac, 1995). The set of flavours used were fenugreek and garlic.

4.3.5.3 Measurements of rumen pH and NH_3-N

An extra group of 12 similar sheep (mean LW 49.7 kg, SD 3.94) was used to test for the effects of intraruminal administration of the two doses of casein on rumen content parameters. This was done concurrently with Experiment 2. Our aim was to quantify the PIC resulting from casein administration by measuring two physiological variables (pH and NH_3-N) in samples of rumen content from sheep which were subjected to the same experimental conditions (dosing and feeding treatments) as those used in Experiments 1 and 2. This was expected to allow a meaningful interpretation of the results regarding the flavoured food choices during preference tests in Experiments 1 and 2 respectively. We used the additional group of sheep in order to avoid any adverse or confounding effects, which could be the result of rumen sampling on sheep whilst on the treatments of either Experiment 1 or 2. The 12 sheep were assigned to two sub-groups (n=6), based on their LW. Each sub-group was offered flavoured test food (with either fenugreek or garlic) in association with casein administration (15 and 75 g respectively). A 3x3 Latin square arrangement (3 sheep; 3 treatments) replicated once for each casein dose was used. The three treatments were similar to the time treatments of casein administration in Experiments 1 and 2, as was the feeding regime (see above for details). Each Latin square period was 5 days long with dosing with casein on days 1, 3 and 5 and rest periods on days 2 and 4. Based on our past experience (Chapter 3) and evidence from the literature regarding the degradation rates of casein in the rumen (Urbaniak, 1995), the sampling times were set at 11:00, 14:00 and 17:00 hours and a rumen content sample (approximately 50 ml) was obtained through a stomach tube from all animals. A point sample was expected to show least variation in rumen parameters and was considered sufficient to indicate how the rumen environment (pH and NH_3-N) changed with time in response to casein administration. The pH of each rumen sample was measured immediately after the sampling by using a glass electrode (model RL 250/pH/ISE meter, EIL Russell Laboratories Ltd., Auchtermuchty). The ammonia-N (NH_3-N) was determined just after the end of the sampling period with an ion-selective electrode (model 95-5129, EIL Russell Laboratories Ltd., Auchtermuchty) in an exact amount (25 ml) of rumen sample, diluted with distilled water to 100 ml.

4.3.6 Statistical analysis

All statistical analyses were performed using GENSTAT version 5.3 (Lawes Agricultural Trust, 1993). Data from conditioning periods and preference tests were analysed separately for each experiment, as if they were a split-plot design in time (Horgan and Sward, 1995) with conditioning periods nested within individual sheep. For food intakes (flavoured foods and test meal) during the days of conditioning the nesting structures used were days nested within conditioning periods and conditioning periods nested within individual sheep. The effects of time of casein administration and time of sampling on rumen content parameters (pH and NH3-N) were analysed as a Latin square design; the analysis was performed separately for each casein dose. The initial flavour preference during the hedonic acceptability tests was determined by analysing the intakes of the flavoured foods using a t-test.

Regarding the analyses of the preference ratios, FRs were tested to determine whether preference for a flavoured food was affected by its association with casein or water, the order of flavour presentation and their respective interaction. The expectation was that FRs for a casein associated food flavour will be in the opposite direction for the two experiments: increased FRs, i.e. above random (0.5), when casein dose was low (Experiment 1) and decreased FRs, i.e. below random, when casein dose was high (Experiment 2). TRs were tested to see whether preference for a flavoured food associated with casein was affected by time of casein administration, order of casein presentation, preference for flavour *per se* and their respective interactions. The expectation was that time of casein administration would affect the rate of development and magnitude of preferences for a flavoured food associated with casein. Each of the above effects were tested also for interactions with test since preference tests were repeated three times after the completion of each conditioning period. Data were tested for compound symmetry (i.e. that the variability in response was the same for all treatments) and to ensure that correlation between two factor levels was the same for all pairs (Horgan and Sword, 1995) before analysis. Where necessary, arcsine transformations were used to improve the homogeneity of data before analysis was carried out.

4.4 Results

4.4.1 Animal health

During the acclimatisation period some initial footrot problems were observed and treated. After that, all animals remained in good health throughout both experiments.

4.4.2 Hedonic Acceptability of flavoured foods

During the first hedonic acceptability test (Experiment 1) sheep showed an equal preference ($P>0.05$) for the foods flavoured with either orange (0.47%) or aniseed (0.53% of the total food intake). The actual intakes of the flavoured foods were 30 and 23 (s.e.d. 8.4) g/10 min of test, for the orange and aniseed flavoured foods respectively. However, during the second hedonic acceptability test (Experiment 2) sheep showed a significant ($P<0.001$) preference for the food flavoured with fenugreek (0.76%) over the food flavoured with garlic (0.24% of the total food intake). The actual intakes of the flavoured foods were 274 and 90 (s.e. 18.6) g/10 min of test for the fenugreek and garlic flavoured foods respectively.

4.4.3 Rumen pH and NH_3-N

The effects of casein administration at different times on pH values and NH_3-N concentrations of the rumen samples obtained at three points in time are shown in Table 4.4. There were no significant effects on pH values of rumen samples from the administration of the low dose; the values were unaffected by both time of administration and sampling. However, pH values were significantly affected by sampling time ($P<0.05$) when sheep were administered with the high dose of casein; this was due to the lower pH value observed in the samples taken at 17:00 h.

Table 4.4 *Mean effects of different doses of casein (15 or 75 g) administration on rumen pH values and ammonia concentration (NH_3-N, mg/l). Casein was administered twice at either 8:30 and 10:00, or 11:30 and 13:00 or 14:30 and 16:00 h; rumen samples were taken at three points in time (11:00, 14:00 and 17:00 h).*

		pH				NH_3-N			
		sampling time				sampling time			
Casein dose (g)	Administration times	11:00	14:00	17:00	Means	11:00	14:00	17:00	Means
	08:30 and 10:00	6.15	6.23	6.33	**6.24**	573	433	316	**440**
15	11:30 and 13:00	6.58	6.25	6.18	**6.34**	293	481	396	**390**
	14:30 and 16:00	6.56	6.37	6.42	**6.44**	300	266	463	**342**
	Means	**6.45**	**6.28**	**6.31**	(s.e.d. 0.16)	**389**	**393**	**392**	(s.e.d. 49.4)
Significance of †:									
Administration (A)			NS				NS		
Sampling time (ST)			NS				NS		
AT x ST			NS				***		

		pH				NH_3-N			
		sampling time				sampling time			
Casein dose (g)	Administration times	11:00	14:00	17:00	Means	11:00	14:00	17:00	Means
	08:30 and 10:00	6.26	6.38	6.12	**6.25**	637	798	664	**700**
75	11:30 and 13:00	6.42	6.18	6.05	**6.21**	299	648	710	**553**
	14:30 and 16:00	6.31	6.40	6.00	**6.23**	294	247	549	**363**
	Means	**6.33**	**6.32**	**6.05**	(s.e.d. 0.18)	**410**	**564**	**641**	(s.e.d. 42.2)
Significance of †:									
Administration (A)			NS				***		
Sampling time (ST)			*				***		
AT x ST			NS				***		

† NS, non-significant; * $P< 0.05$; *** $P< 0.001$.

Rumen NH_3-N concentrations were significantly affected by both times of administration and sampling and their respective interactions ($P<0.001$) when sheep were administered with the high casein dose. Only the respective interaction was significant ($P<0.001$) when the low casein dose was administered. Administration of casein led to an increase in rumen NH_3-N concentration of the samples taken after its administration. The increase was persistent for the two samples taken after the administration of the low dose, and for all samples taken after the administration of the high dose. The magnitude of the increase in rumen NH_3-N concentration followed the expected pattern, i.e. was dependent on the amount of casein administration.

4.4.4 Intake of the flavoured and the 'test meal' foods during the days of conditioning

In both experiments, there was no effect of flavour type or flavour order (and no effect of their respective interactions) on consumption of flavoured foods during the days of conditioning.

4.4.4.1 Experiment 1

The mean intake of the flavoured food offered (750 g) from 8:00 to 11:00 h during the days of each conditioning period increased ($P<0.001$) progressively from 576 (s.e. 17.9), to 695 (s.e. 11.3) and to 717 (s.e. 8.6) from the first to the third conditioning period respectively. The mean intake of flavoured food was slightly higher when casein rather than water was administered (581 vs 563, 699 vs 689 and 736 vs 698 g/3h for each of the three conditioning periods respectively), but the effect was non-significant.

The intake of the test meal food offered (1500 g) from 11:00 to 17:00 h also increased significantly (P<0.001) as conditioning progressed from 991 (s.e. 32.2) g/6h in the first conditioning, to 1141 (s.e. 36.8) and to 1255 (s.e. 31.6) g/6h in the second and third conditioning period respectively. There was a significant interaction between conditioning period and casein association (whether casein or water was administered) on the intake of the test meal food (P<0.05). This was due to the lower intakes of test meal when casein rather than water was administered during the first and second, but not the third conditioning period.

4.4.4.2 Experiment 2

Mean intakes of the flavoured food offered (750 g) from 8:00 to 11:00 h during the days of conditioning were 716 (s.e. 8.9), 716 (s.e. 7.4) and 709 (s.e. 10.1) in the first, second and third conditioning period respectively. There were no differences in mean intakes of the flavoured foods when casein or water was administered (717 vs 715, 718 vs 713 and 710 vs 707 g/3h, for the first to the third conditioning respectively).

The intakes of the test meal food offered (1500 g) from 11:00 to 17:00 h during conditioning were 1331 (s.e. 30.5), 1273 (s.e. 34.3) and 1324 (s.e. 32.8) g for the first to the third conditioning period respectively. Time of casein administration significantly (P<0.05) influenced intakes of test meal food; these were 1395 (s.e. 25.1), 1417 (s.e. 20.1) and 1126 (s.e. 42.0) g/6h for casein administration on treatments A, B and C respectively.

4.4.5 Flavour and treatment ratios during preference tests

Table 4.5 shows the results of a split-plot ANOVA model for the FRs calculated from the preference tests performed in Experiments 1 and 2. Similarly, Table 4.6 shows the results of a split-plot ANOVA model for the TRs calculated from the preference tests performed in

Experiments 1 and 2. They are based on FRs and TRs that were transformed (arcsine transformation). The most important findings here are that neither casein dosing time (DT) nor its interactions with the other factors had a significant effect on the mean TRs in either experiment (Table 4.6). Thus, the hypothesis that time of casein administration would affect the rate of development and magnitude of preference for a flavoured food associated with casein was rejected. For this reason, the preference test results are mainly discussed below independently of casein dosing time. The question then is whether association with casein administration affected preference for a flavoured food.

Table 4.5 *Split-plot ANOVA structure and results for 'Flavour Ratios' (FR, g of a flavoured food/g total intake of flavoured foods) calculated from the choices made during 20-min preference tests performed at the end of each conditioning period of Experiments 1 and 2. Lambs were offered a choice between two flavoured foods previously associated with either casein or water administration. Different doses of casein were used in each experiment (in brackets).*

	Experiment 1 (15g)				Experiment 2 (75g)			
Source	*df*	*Mean* Square	*F value*	*P*	*df*	*Mean Square*	*F value*	*P*
Lamb stratum (Between tests)								
Association (A)	1	0.032	0.15	NS	1	0.808	2.83	NS
Flavour order (FO)	1	1.639	7.77	**	1	0.169	0.06	NS
A x FO	1	0.007	0.03	NS	1	0.713	2.50	NS
Error	32	0.210	2.43		32	0.285	4.31	
Lamb x Test stratum (within tests)								
Test (T)	2	0.026	0.30	NS	2	0.261	3.94	*
T x A	2	0.275	3.17	*	2	0.433	6.55	**
T x FO	2	0.094	1.08	NS	2	0.018	0.28	NS
T x A x FO	2	0.039	0.45	NS	2	0.046	0.69	NS
Error	64	0.086			64	0.066		

NS, non-significant; * $P< 0.05$; ** $P< 0.01$.

Table 4.6 *Split-plot ANOVA structure and results for 'Treatment Ratios' (TR, g of the food associated with casein/g total intake of flavoured foods) calculated from the choices made during 20-min preference tests performed at the end of each conditioning period of Experiments 1 and 2. Lambs were offered a choice between two flavoured foods previously associated with either casein or water administration. Different doses of casein were used in each experiment (in brackets).*

	Experiment 1 (15g)				Experiment 2 (75g)			
Source	*df*	*Mean Square*	*F value*	*P*	*df*	*Mean Square*	*F value*	*P*
Lamb stratum								
(Between tests)								
Dosing time (DT)	2	0.155	0.64	NS	2	0.131	0.41	NS
Flavour (F)	1	0.100	4.12	*	1	1.359	4.21	*
Casein order (CO)	1	1.639	6.74	*	1	0.223	0.69	NS
DT x F	2	0.043	0.18	NS	2	0.006	0.02	NS
DT x CO	2	0.230	0.95	NS	2	0.077	0.24	NS
F x CO	1	0.007	0.03	NS	1	0.605	1.88	NS
DT x F x CO	2	0.031	0.13	NS	2	0.004	0.01	NS
Error	24	0.243	2.58	NS	24	0.323	4.62	NS
Lamb x Test stratum								
(within tests)								
Test (T)	2	0.274	2.92	NS	2	0.027	0.39	NS
T x DT	4	0.006	0.07	NS	4	0.054	0.78	NS
T x F	2	0.026	0.28	NS	2	0.265	3.79	*
T x CO	2	0.094	1.00	NS	2	0.117	1.67	NS
T x DT x F	4	0.130	1.38	NS	4	0.130	1.87	NS
T x DT x CO	4	0.028	0.29	NS	4	0.063	0.90	NS
T x F x FO	2	0.038	0.41	NS	2	0.158	2.27	NS
Error	52	0.094			52	0.069		

NS, non-significant; * $P< 0.05$.

4.4.5.1 Experiment 1

The across tests mean TRs for time of casein dosing treatments in Experiment 1 were 0.528, 0.513 and 0.428 (s.e.d. 0.096) for treatments A, B and C respectively. The mean TRs for time of casein dosing treatment after the third conditioning (final test) were 0.572, 0.548 and 0.442 (s.e.d. 0.13) for treatments A, B and C respectively.

***Table** 4.7 Mean Preference Ratios (g of the flavoured food associated with casein or water/g total intake of flavoured foods) during preference tests performed at the end of each conditioning period of Experiment 1.*

Test	Casein (15 g)			Water			
	Orange	Aniseed	Means	Orange	Aniseed	Means	
1	0.493	0.330	**0.411**	0.670	0.507	**0.588**	
2	0.625	0.449	**0.537**	0.551	0.375	**0.463**	
3	0.575	0.467	**0.521**	0.533	0.425	**0.479**	
Means	**0.564**	**0.415**	**0.490**	**0.585**	**0.436**	**0.510**	(s.e.d. 0.059)

Table 4.7 shows the mean Preference Ratios for the flavoured food associated with either casein or water in Experiment 1. Mean FRs were significantly affected by the interaction (P<0.05) between time of test and casein association, indicating that during repeated conditioning preference for the casein associated flavoured food was increasing, while preference for the water associated flavoured food was decreasing. However, the degree of such a preference was different between flavours used for association with either casein or water, since flavour used had a significant effect (P<0.05) on TRs irrespective of association (see Table 4.6). For example, overall orange was preferred over aniseed flavour (0.564 vs 0.415, s.e.d. 0.078) when each was associated with casein dosing. Flavour order (i.e. flavour presented first or second within conditioning period) also affected significantly (P<0.01) the

preference for a flavoured food, with the flavour presented second during each conditioning being consistently preferred. For example, FRs for the food associated with casein were 0.460 vs 0.689 (s.e.d. 0.073) when orange was presented first or second respectively. None of the interactions with flavour order was statistically significant. The mean total intakes of both the flavoured foods during the three preference tests were 568, 755 and 952 (s.e.d. 28.9) g/ 20 min respectively; the increase in intake was highly significant (P<0.001). No other factor or interaction affected the total intake of both flavoured foods during preference test.

4.4.5.2 Experiment 2

The across tests mean TRs for time of casein dosing treatments in Experiment 2 were 0.474, 0.375 and 0.391 (s.e.d. 0.099) for treatments A, B and C respectively. The mean TRs for time of casein dosing treatment after the third conditioning (final test) were 0.478, 0.393 and 0.332 (s.e.d. 0.12) for treatments A, B and C respectively.

Table 4.8 *Mean Preference Ratios (g of the flavoured food associated with casein or water/g total intake of flavoured foods) during preference tests performed at the end of each conditioning period of Experiment 2.*

Test	Casein (75 g)			Water			
	Fenugreek	Garlic	Means	Fenugreek	Garlic	Means	
1	0.680	0.381	**0.531**	0.619	0.320	**0.470**	
2	0.386	0.365	**0.376**	0.635	0.614	**0.625**	
3	0.483	0.300	**0.392**	0.700	0.517	**0.609**	
Means	**0.516**	**0.349**	**0.433**	**0.651**	**0.484**	**0.568**	(s.e.d. 0.050)

Table 4.8 shows the mean Preference Ratios for the flavoured food associated with either casein or water in Experiment 2. The mean FRs for a flavoured food as a proportion of total flavoured food intake were significantly (P<0.01) affected by the interaction between time

of test and casein association; increased preference for a flavoured food associated with water (or a decreased preference for a flavoured food associated with casein) was established only after the completion of the second conditioning period and was reinforced by the third conditioning (Table 4.8). There was also an effect of time of test ($P<0.05$) on the mean FRs for a flavoured food, which was due to a difference in preference shown during the first test compared to the other two.

TRs were significantly affected by the type of flavours used ($P<0.05$). There was an initial preference for the fenugreek over garlic flavoured food associated with casein administration, in the preference test conducted at the end of the first conditioning (0.680 vs 0.381, s.e.d. 0.096). These differences in preference for the two flavoured foods were similar to the results of the hedonic acceptability test. However, following repeated conditioning the effects of flavour decreased, indicating that any initial preference for a particular flavour was overcome, most likely as a consequence of the negative PIC created from casein administration. The interaction between time of test and flavour on the mean TRs was formally significant ($P<0.05$). The mean intakes of both the flavoured foods during the three preference tests were 895, 942 and 1017 (s.e.d. 36.3) g/20 min respectively; the increase in intake was highly significant ($P<0.01$). No other factor or interaction affected the total intake of both flavoured foods during preference tests.

4.5 Discussion

The objective of this study was to test the hypothesis that a delayed type of learning could lead to conditioned responses towards novel food flavours associated with PIC resulting from intraruminal administration of a nutritive stimulus in sheep. Two particular issues were addressed: (i) the relative role of the temporal contiguity between the consumption of novel flavoured food and associated positive or negative PIC on the formation of conditioned responses, and (ii) the rate of development and magnitude of such conditioned responses. Most research into delay learning has concentrated on the development of conditioned responses towards a novel food that has been associated with illness induced by a toxin, such as LiCl (e.g. Provenza, 1996; Sclafani, 1997). The time lag between the presentation of the novel food and the toxin administration has varied between several minutes (Lindberg et al., 1982; Zahoric et al., 1990; Sclafani, 1997) to several hours (Olsen et al., 1989; Burritt and Provenza, 1991; Kronberg, 1993).

The LiCl experiments have been successful in demonstrating a delayed type of learning involvement, and it has been argued that such a response would be of an adaptive significance to the animal. It would enable it to attribute adverse PIC to the consumption of a novel food, irrespective of their temporal contiguity (Garcia et al., 1985; Provenza et al., 1994a,b). In the present study, we investigated delay learning by the use of a nutritive stimulus, instead of a toxin. Our aim was to mimic animal responses that occur in nature and to give some further consideration to how ruminants learn to regulate their feeding behaviour. We used a nutritive stimulus at doses that were expected to lead to both negative (high dose) and positive (low dose) PIC. Our expectation was that a delay learning response would at least lead to conditioned responses towards a novel flavoured food associated with the high dose. This was because at the limit nutrient excesses can be seen acting as toxins (Kyriazakis, 1997). We considered our experiment a severe test of the delay learning paradigm for two reasons: first because it was tested on ruminant animals; the modifying role of the rumen has always been considered as a barrier for the development of associations between foods and their resulting PIC (Westoby, 1974; Zahoric et al., 1990; Forbes, 1995). Second, we used delays up to 5 hours to (dis)associate the consumption of the novel food and the administration of the nutritive stimulus. In the past time delays have been of a much shorter span (see below).

Irrespective of whether a high or a low casein dose was used the results show that all time delay treatments were equally effective in conditioning responses towards food flavours associated with their administration. When a high dose was used (Experiment 2), which was presumed to lead to negative PIC, the results were consistent with our expectations. Sheep avoided the flavoured food associated with casein irrespective of time of its administration. We presume that one of the reasons for this response was the consequence of the marked increase in rumen NH_3-N concentration that was sustained by the administration of a repeated high dose of casein. The high concentration of rumen NH_3-N and its persistence was verified by a parallel experiment that was conducted on a similar group of sheep. These measurements showed that NH_3-N concentration most likely 'peaked' at about one hour after dosing with casein, and apparently persisted for more than 6 hours. Therefore, the conditioned responses are consisted with the notion that sheep attempt to avoid high levels of rumen ammonia, and maintain their rumen conditions within a certain physiological range (Cooper et al., 1995). The conditioned responses are also consisted with previous findings obtained with the use of toxins whose PIC have been disassociated in time with the consumption of a novel food in ruminants. Burritt and Provenza, (1991) demonstrated that preference for a novel food was markedly decreased when it was associated with illness created by administration of LiCl within time intervals of up to 4 hours after its consumption. In experiments on cattle, Olsen et al. (1989) found that they too can learn to avoid a novel food, if it was associated with the intraruminal administration of LiCl 2 hours after consuming it. Kroneberg et al. (1993), found similar responses in cattle by imposing delay intervals up to 12 hours. Therefore, it can safely be concluded that a delay learning paradigm also accounts for the conditioned responses of ruminants towards foods which are associated with excessive amounts of nutrients.

There was no relationship between the time lags imposed and the magnitude and rate of development of conditioned responses associated with the administration of the high casein dose. There were, however, clear interactive effects between repeated casein administration and the formation of conditioned responses, i.e. sheep needed more than one conditioning period before they started to exhibit the development of conditioned responses. This, however, has not been the case for conditioned responses developed towards food flavours associated with delayed negative PIC resulting from LiCl administration. They have been established after only a single LiCl dose (e.g. Ralphs and Cheney, 1993; Provenza, 1996). This contrast is perhaps an indication of the difference in the strength of PIC created by the administration of a nutrient and those resulting from a toxin (Kyriazakis et al., 1998b). We based the high dose of casein used in this experiment on our past evidence from the development of conditioned responses of sheep (Chapter 3). In that experiment the magnitude of the conditioned responses was rather high, since sheep avoided significantly the flavoured food associated with casein administration (i.e. preference ratios of 0.29). In contrast, the responses observed in the current experiment were lower (i.e. preference ratios of 0.40). This can be due to several differences between the two experiments, one of which was the relatively lower pH ranges observed in the current experiment, which would have reduced the rate of NH_3-N absorption from the rumen wall (Van Soest, 1994) and hence lead to less dramatic negative PIC.

The idea that conditioned responses could be the outcome of food associations with either positive (resulting from appropriate amounts of nutrient intake) or negative (resulting from toxin or excess of nutrient intake) PIC has been prevalent in the literature dealing with the feeding behaviour of animals (Sclafani, 1995 and 1997; Kyriazakis and Day, 1998). Since it has been shown that delay learning can lead to conditioned responses resulting from negative PIC, the question then is whether a similar learning process applies to conditioned responses resulting from positive PIC. Attempts to test this hypothesis directly have been previously made only on laboratory animals, and the resulting evidence suggests that they are also able to learn to prefer food flavours associated with low to moderate nutrient doses, whose administration was disassociated from novel food consumption by 10-60 minute intervals (Baker and Booth, 1989a; Capaldi et al., 1987; Perez et al., 1995). These imposed time lags are rather trivial when compared to those applied for the disassociation between negative PIC and novel food consumption: up to a 24h delay. We tested the hypothesis that delay learning could also apply to conditioned responses resulting from positive PIC, by imposing time disassociations of up to 5h in Experiment 1. The outcome of these tests, however, do not give an unequivocal support to our hypothesis. Although the observed conditioned responses followed the pattern expected and were unaffected by the temporal contiguity between novel food consumption and PIC, they were rather mild in their magnitude. We offer three explanations to account for these results. The first one is that this type of learning is indeed very difficult to demonstrate where positive PIC are involved. Positive PIC are not acute and distinctive, and may also have a slow onset (Capaldi, 1992), compared to negative PIC resulting both from excess nutrient intake and more importantly administration of toxins (Domjan, 1985; Capaldi, 1992; Sclafani, 1997). Although we do not have a direct evidence on the temporal development of the positive PIC resulting from the administration of the low casein dose, it would be safe to assume that these were indeed gradual. The second explanation, which is not independent of the first, is that the dose of casein administered was not sufficiently high to lead to strong PIC, and thus readily allow animals to form strong conditioned responses. It is known that the strength of resulting PIC could influence the rate of development of conditioned feeding responses (Kyriazakis et al., 1997; Chapter 3). The fact that repeated conditioning strengthened the observed feeding responses supports this suggestion. It is possible, therefore, that the magnitude of conditioned responses would have further increased had the conditioned periods been extended. The third and most likely explanation is that the observed responses were the outcome of combined PIC and the artificial flavours used. It is likely that sheep have different predisposition for specific flavours (Ramirez, 1995; Augner et al., 1998; Early and Provenza, 1998). Such a predisposition leads to selective associations when such flavours are used and the strength of the associated PIC is not sufficient to overcome them.

In Experiment 1 the results from the initial hedonic test indicated that there were no significant differences in the acceptability between aniseed and orange flavoured foods. However, the results of the preference tests that followed conditioning periods, showed that the effect of flavours was overwhelming. We do not know whether the difference in response between these two tests lies, but it is possible that they are reflecting the difference in the flavour. Sheep selected predominantly for flavoured foods associated with casein only when the orange flavour was used. When the aniseed flavour was used preference ratios were not significantly different from random (i.e. 0.50). We suggest therefore, that the effect of flavours *per se* can be overcome, at least after repeated conditioning, when the associated PIC are strong or distinctive, so that in these cases feeding behaviour is dictated by nutritional rather than associated cues (Forbes and Kyriazakis, 1995). This was the case in Experiment 2 where the initial predisposition against the garlic flavour continued to dominate the conditioned responses of sheep during the first preference test, but was not evident in subsequent tests.

In conclusion the results of both experiments give evidence for the relevance of delay learning on the development of conditioned responses in ruminants. Sheep appear to be able to associate the flavour of a novel food with PIC resulting from the administration of a nutritive stimulus, even if these PIC become apparent after considerable delays following the consumption of the novel food. Admittedly, we have not been able to demonstrate unequivocally the formation of conditioned responses from delayed positive PIC, but we have offer explanations to account for them. We trust that the explanations we offer regarding the significance of delay learning will lead to better understanding of the ability of ruminants to predict the consequences of foods through their sensory properties.

Chapter 5

Conditioned feeding responses of sheep towards flavoured foods associated with the administration of ruminally degradable and/or undegradable protein sources

"Yet nature cannot be contravened, but preserves a fixed and immutable order"

Benedict Spinoza, (1632 - 1677)

5.1 Abstract

The main objective of the experiment was to investigate the conditioned responses of sheep towards food flavours associated with the administration of ruminally degradable protein (RDP) and ruminally undegradable, but readily digestible protein (DUP) sources given either alone or in combination. The experiment consisted of three consecutive periods during which sheep were conditioned to associate a flavoured food with a nutritive stimulus (or water, W). Two foods (basal and novel test) with different CP (92 and 64 g/kg DM respectively) and similar ME (≅ 9 MJ/kg DM) contents were used on a total of 48 Texel x Greyface female sheep. The basal food was offered during non-experimental (rest) days whereas the test food was used in combination with two flavours, orange and aniseed, during experimental days. Food was presented for 8 h (09:00 to 17:00) daily throughout the experiment. Two nutritive stimuli (casein, C, and formaldehyde treated casein, FC) were chosen such as to provide major contrasts in their RDP and DUP contents, on isonitrogenous basis. Each dose (50 g) of a particular nutritive stimulus was administered by gavage through a stomach tube twice daily (at 10:00 and 14:00 h). Sheep were randomly assigned to one of four (C *v.* W, FC *v.* W, C *v.* FC, C *v.* FC+C) treatments (n=12 per treatment). For the first two days (days 1 & 2) of each conditioning period half of the sheep within each treatment were offered one flavoured food paired with the administration of C (treatments C *v.* W, C *v.* FC and C *v.* FC+C) or FC (treatment FC *v.* W). The other half were offered the opposite flavoured food paired with the administration of water (treatments C *v.* W and FC *v.* W), FC (treatment C *v.* FC) or C+FC (treatment C *v.* FC+C). There followed two days (days 3 & 4) of rest and for the two subsequent days (days 5 & 6) received the flavoured food whilst the order of association between food flavours and stimuli was reversed. In the morning of day 7 sheep were offered a choice between the two flavoured

foods for 20 min. After the completion of the preference test sheep were offered the basal food. The same procedure was followed for each of three conditioning periods (i.e. each animal followed the same flavour/stimulus association throughout the experiment). The design was balanced for order of flavour and stimulus presentation. Sheep preferred the flavoured food associated with C ($P<0.05$) or FC ($P<0.01$) over the opposite flavoured food associated with water in C *v.* W and FC *v.* W treatments respectively. In the C *v.* FC treatment sheep showed a strong preference for food flavours associated with the administration of FC to those associated with C ($P<0.05$). In the C *v.* FC+C treatment sheep showed equal preference towards the food flavours associated with either stimuli. These results: (i) support the view that sheep are able to form learned preferences for food flavours associated with the administration of protein, and (ii) suggest that sheep are able to distinguish between food flavours associated with the administration of both RDP and DUP sources. Sheep preferred flavours associated with DUP administration only over flavours associated with RDP administration only; however, such preferences did not develop when DUP was administered concurrently with RDP. Given the learned responses of sheep towards flavours associated with RDP and DUP the expectation is that they may be able to select their diet on the basis of these qualities when they are offered a choice.

5.2 Introduction

We are interested in how ruminant animals learn to associate the orosensory properties of a food with its positive or negative post-ingestive consequences (PIC), and subsequently develop conditioned preferences or aversions towards such properties. This is because it has been suggested that the existence of such processes would enable animals to select a diet that either minimises nutrient deficit or meets nutrient requirements whilst minimising excess nutrient intake (Provenza, 1995c; Kyriazakis, 1997; Kyriazakis et al., 1999). There is now consistent evidence to support the view that ruminants are able to form strong learned associations between a food flavour and PIC which result from the administration of a rumen degradable protein (RDP) source (Villalba and Provenza, 1999a; Chapters 3 and 4); these subsequently lead to conditioned responses towards the food flavour. The question then is whether ruminants can also develop such associations between a food flavour and PIC created by the administration of a ruminally undegradable, but readily digestible protein (DUP) source.

The question is important on several counts. First of all, it relates to the issue of the basis of diet selection for protein by ruminant animals. The argument of whether ruminants select their diet on the basis of either RDP or DUP contents of the foods available as a choice remains unresolved (for different suggestions see Kyriazakis and Oldham, 1993; Tolkamp et al., 1998; Tolkamp, 1999; Kyriazakis et al., 1999). Second, it is linked to the question of whether certain PIC are valued more than others for the formation of such associations by ruminants. Currently, it is unknown whether the ruminant animal gives equal or different weights to the PIC resulting in the ruminal or post-ruminal digestive tract. Given the evolutionary history of ruminants one reasonable expectation is that they might rely more on feedback from the former rather than the latter PIC (Tolkamp et al., 1998). Third, it elucidates the relative role of the rumen and post-ruminal digestive tract in the underlying mechanisms that account for the development of associations between food flavours and PIC. This is because RDP administration results in a relatively close temporal contiguity between food flavours and consequent rumen PIC (Chapter 3). On the other hand, PIC resulting from DUP arise after some considerable delay and hence might be expected to be disassociated from food flavours (Chapter 4).

To date, the question of whether specific conditioned feeding responses can develop from the administration of a DUP source has received little attention; the exception is the preliminary and inconclusive study of Villalba and Provenza (1997c). The objective of the experiment described here was threefold: (i) to test whether sheep are able to form specific associations between food flavours and the PIC induced by the administration of a DUP source; (ii) to investigate the relative importance of RDP and DUP sources and their consequent PIC in the development of such associations; and (iii) to test whether such associations can be formed when DUP is administered concurrently with a RDP source. The effects of type of PIC and the number of repeated exposures to them on the development and magnitude of conditioned responses were also investigated. We hypothesised that learned associations would be dictated by the PIC attributed to RDP rather than DUP administration. This seemed to us to be consistent with the evolutionary history of ruminants.

5.3 Material and Methods

5.3.1 Animals and management

Forty-eight 7 month old Texel x Greyface female sheep were used. They had a mean live-weight (LW) of 49.5 (SD 4.93) kg when obtained. The sheep were moved from grazing outdoors, into the individual pens of the experimental unit that has been described previously in Chapter 4. On arrival in the experimental unit all sheep received an oral dose of an anthelmintic, Oramec (Ivermectin, 200 pg/kg LW; MSD AGVET, Hoddesdon, Herts). An acclimatisation period was provided for 2-weeks prior to the start of the actual experiment; this was considered long enough to allow sheep to adapt to their new environment. The sheep were weighed in the morning of the same day each week, prior to feeding. Natural lighting and ventilation were used.

5.3.2 Foods and feeding procedure

Two foods, a basal and a novel, test were used. Their ingredients and chemical composition are shown in Table 5.1. The two foods were different in their crude protein (CP) content but similar in their calculated metabolisable energy (ME) supply. The basal food was made in a meal form (92 g CP and 9.1 MJ ME/kg DM), while the test food was made as pellets (64 g CP and 9.0 MJ ME/kg DM). The test food was made with as low a CP content as possible in relation to energy, with the consequence of being imbalanced in terms of RDP content (effective RDP:fermentable ME ratio of 4.1 which is well below the optimum ratio of 10 recommended by AFRC, 1993) and metabolisable protein (MP) yield in relation to ME. Both foods were intended to be similar and non-limiting in minerals and vitamins. The animals were fed for eight hours, from 09:00 to 17:00 h daily, throughout the experiment.

Table 5.1 *Ingredient and chemical composition of the experimental foods*

	Test food	Basal food
Ingredients (g/kg fresh matter)		
Oatfeed	380	310
Wheat	75	
Citrus pulp	444	366
50% Fat premix	25	
Barley		300
Molasses (CMS 20) †	50	
Salt	8.3	5.1
Dibasic calcium phosphate	12	9.0
Limestone flour		4.8
Calcined magnesite	0.5	0.3
Sodium sulphate	1.9	2.3
Vitamin+mineral premix ‡		
Scotmin ewe/lamb	2.5	2.2
Scotmin dairy/beef		0.3
Analytical chemical composition (g/kg DM)		
Dry matter	863	867
Crude protein	64	92
Gross energy (MJ/kg DM)	17	17.1
Ash	78	62
ADF (Acid Detergent Fibre)	281	190
NDF (Neutral Detergent Fibre)	402	308
Calcium	12	11
Phosphorus	3.4	3.8
Sulphur	4.4	4.5
Calculated protein and energy components (g/kg DM)		
Effective rumen degradable protein (eRDP)	52	65
Digestible undegradable protein (DUP)	10	11
Metabolisable protein (MP)	43	52
Metabolisable energy ME (MJ/kg DM)	9.0	9.1
Effective metabolisable energy fME (MJ/kg DM)	8.7	8.8

† CMS 20, condensed molasses soluble (Intermol, Cobham, Surrey).

‡ Vitamin and mineral premix supplied by Scotmin Ltd, Ayr, Scotland.

¶ Calculated according to AFRC (1993).

The basal food was fed during the acclimatisation period and then during the 'rest days' of the conditioning periods (see below for details). The test food was offered flavoured during specific days of each conditioning period. Both basal and test foods were offered freely to sheep over the 8 h period and food refusals were weighed daily at 17:00 h and discarded. During the whole experiment weekly samples of both foods were taken regularly in order to obtain a composite sample for chemical analysis.

5.3.3 Food flavours and nutritive stimuli

Two artificial flavours, orange and aniseed (International Additives Ltd., Wallasey), formulated on an oil-based liquid form, were used. They were further diluted in sunflower oil at a level of 1:4, according to manufacturer instructions. Subsequently, the flavoured solution was sprayed onto the test food whilst in a mixer, at a level of inclusion of 7.5 g undiluted flavour/kg test food (for details and justification see Kyriazakis et al., 1997; Chapter 4).

Two nutritive stimuli were used: lactic-acid caseinate (C) and formaldehyde treated sodium-caseinate (FC), (Bacarel and Co Ltd, Ashford, Kent). The nutritive stimuli were chosen to provide major contrasts in their RDP and DUP contents, on an isonitrogenous basis. It was expected that C administration would lead mainly to RDP supply and thus its PIC would be attributed to the involvement of the rumen; i.e. enhancement of microbial protein synthesis and promotion of fibre digestion in the rumen (Wallace et al., 1999). On the other hand, FC administration was expected to increase substantially the supply of DUP (Ketelaars and Tolkamp, 1991) and thus its PIC (i.e. greater availability of amino acids to the animal for metabolism, Froetschel, 1996; McBride et al., 1998) would arise mainly at a post-ruminal level. The contribution of FC to RDP supply via N-recycling was expected to be very small, given the protein content of the test food used. The amount of each dose was set to 50 g for both types of casein (both providing 7.3 g N). It was calculated on the basis of RDP supply of C. The assumption was that contribution of C to the amounts of RDP and fME provided by the predicted consumption of the test food alone, would lead to a calculated optimum ratio eRDP:fME of 10 (AFRC, 1993). Each dose of the nutritive stimuli was suspended in water (about 300 ml) and administered directly into the rumen, using a stomach tube, of each animal twice daily. Sodium chloride was added into the water used for the suspension of lactic acid caseinate. This was considered necessary to equalise the amount of Na available across all treatments and thus avoid any bias in feeding responses (Hills et al., 1999).

5.3.3.1 Formaldehyde-treated Na-caseinate

The sodium-caseinate was protected from microbial degradation in the rumen with formaldehyde treatment, using the dry method described by Ketelaars and Tolkamp (1991). For each kg of Na-caseinate 50.4 ml of commercial formalin containing 37.1% formaldehyde was sprayed onto 34.2 g of a very fine silica powder (Sipernat 22 S, Degussa, Winterton). After the mixing of silica and formalin, Na-caseinate was gradually added in a container and the container was then firmly closed and placed in a 30°C oven for two days. Its contents were mixed again without opening the container and were replaced in the oven for one more day. The degradability of the FC was determined by an *in situ* procedure using a group of four heifers fitted with rumen fistulas. The procedure used was that described by Ørskov (1988). Samples of 6 g of FC were weighed into polyester bags (pore diameter 41 μm, Sericol Ltd., Broadstairs, Kent) and incubated in the rumen of the heifers (two bags per animal for each incubation time). Incubation times were 1, 2, 4, 8, 16 and 24 h. These bags, together with control ones (0 h incubation) were subsequently washed, dried and the contents removed for N analysis. The FC had an apparent degradability of 14% and it was expected to have a digestibility of approximately 85% in the small intestine (for further details see Ketelaars and Tolkamp, 1991). The *in vitro* digestibility was tested with the method of Tilley and Terry (1963); it was found to be 92%.

5.3.4 Experimental design

The experiment, which lasted for 38 days, included a 2-week acclimatisation period and three consecutive conditioning periods, each of 8 days. The 48 sheep were assigned to one of four treatments (n=12 per treatment). The allocation was such that mean LW did not differ significantly between treatment groups.

5.3.4.1 Conditioning

A conditioning period of 8 days was chosen, as it was found to be of sufficient duration for the development of conditioned feeding responses in sheep in previous experiments (Kyriazakis et al., 1997; Chapter 3). Flavours and stimuli were associated as follows. For half of the sheep in each treatment, one of the flavoured foods was offered from 09:00 to 17:00 h, whilst dosing with a stimulus for the first two days (days 1 & 2). Administration of the stimuli occurred at 10:00 and 14:00 h. This was done in order to strengthen the reinforcement created by the administration of the nutritive stimulus, in the presence of flavoured food. Then followed two days of rest (days 3 & 4). For the two subsequent days (days 5 & 6) the opposite flavoured food was offered and sheep were dosed with a different stimulus (for details see Table 5.2). In the morning (9:00 h) of day 7, sheep were offered a choice between the two flavoured foods for 20 min. For the rest of day 7 and during day 8 sheep were offered the basal food. In the other half of the sheep, the order of association was reversed. The same procedure was followed for each conditioning period (i.e. each animal followed the same flavour/stimulus association throughout the experiment).

Table 5.2 *The conditioning and preference test schedules for one group of sheep ($n=12$) offered a low protein food (test food) sprayed with one of two flavours (orange, O, or aniseed, A) which was associated with the administration of either casein or water (treatment C v. W)*[†]

	Sheep			
Day	no.=3	no.=3	no.=3	no.=3
1.	Casein (Orange)	Water (Aniseed)	Casein (Aniseed)	Water (Orange)
2.	Casein (Orange)	Water (Aniseed)	Casein (Aniseed)	Water (Orange)
3.	Rest (basal food)			
4.	Rest (basal food)			
5.	Water (Aniseed)	Casein (Orange)	Water (Orange)	Casein (Aniseed)
6.	Water (Aniseed)	Casein (Orange)	Water (Orange)	Casein (Aniseed)
7.	20 min preference test (choice between test food flavoured with either Orange or Aniseed)			
8.	Rest (basal food)			

[†] The schedule was identical, in terms of flavour and nutritive stimulus presentation, for all four treatments: C *v.* W, FC *v.* W, C *v.* FC, C *v.* FC+C and was repeated three times. The only difference between treatments was the different stimuli used.

The above design was balanced for order of flavour and stimulus dose presentation. There were four treatments: (i) C *v.* W: this was a control treatment. The nutritive stimulus was C and it was expected that sheep offered a choice, after conditioning, would prefer the flavoured food previously associated with C over the opposite flavoured food associated with water. (ii) FC *v.* W: the nutritive stimulus was FC. This treatment was designed to test whether sheep are able to form specific learned associations between food flavours and PIC induced by the administration of a DUP source. According to our hypothesis the PIC induced by its administration should not lead to conditioned responses in sheep. (iii) C *v.* FC*:* the purpose of this treatment was to elucidate the relative importance of isonitrogenous RDP and DUP sources in the development of conditioned flavour preferences, by testing whether their subsequent PIC are valued differently by sheep in the development of such responses. (iv) C *v.* FC+C: this was designed to address a similar question to treatment C *v.* FC, by ensuring that DUP was the only variable to change between the two nutrient stimuli. It was anticipated that the conditioned responses of sheep towards flavoured foods associated with nutritive stimuli that differ in their RDP content and/or DUP yield in the last two treatments would be dictated by the PIC attributed to their RDP content only.

5.3.4.2 Preference tests

The ability of sheep to learn to associate the flavour of a food with PIC created by the administration of different stimuli was evaluated in preference tests conducted on day 7 of each conditioning period (Table 5.2). The preference tests were conducted early in the morning (9:00 h) and lasted 20 min each. All animals were given free access to 1000g of each of the two flavoured foods in separate troughs. This amount was well above the intake of sheep over a 20-min period. The position of the troughs with the flavoured foods they contained was randomised for each test. Immediately after the completion of the preference test fresh basal food was offered for the rest of the day (until 17:00 h).

The outcome of the preference tests was used as the learning measurement. It was expressed as a preference ratio (PR), which was calculated as the intake of a flavoured food (e.g. orange) as a proportion of total intake of flavoured food (orange + aniseed, g/g).

5.3.5 Statistical analysis

All statistical analyses were performed using GENSTAT version 5.3 (Lawes Agricultural Trust, 1993). Data were analysed separately for each of the four main treatments, as if they were from a split-plot design in time (Horgan and Sward, 1995). For the total intakes of the flavoured test food during the days of conditioning (mean values for days 1+2 and 5+6), the structure used was days nested within conditioning periods and conditioning periods nested within individual sheep. These were tested for the effects of repetition (T), association (AS) with a stimulus (e.g. with either casein or water for treatment C *v.* W) and with flavour (F) (i.e. with either orange or aniseed).

PR values were arcsine transformed before the statistical analyses were carried out. The structure used was conditioning periods (repetitions) nested within individual sheep to test whether preference for a flavoured food was affected by association with a nutritive stimulus (AS), order of flavour presentation (FO) and repetition (T). Association tested whether the proportion of the orange flavoured food selected was greater when it was associated with a nutritive stimulus rather than a control. The nutritive stimuli were assumed to be C for treatments C *v.* W, C *v.* FC, C *v.* FC+C and FC for treatment FC *v.* W. We also compared the intake of the orange flavoured food, as a proportion of total intake in the preference tests, to 0.5 using all of the data from the experiment (3 repetitions for each of 48 animals). This was to test whether our *a priori* assumption, of no general preference for one of the flavours used over the other, was justified.

5.4 Results

Data from one animal in the C *v.* W treatment were omitted because of its very poor performance and irregular intakes throughout the experiment. The preference ratios and food intake from this animal were treated as missing values for the statistical analysis.

Table 5.3 *Average intake by sheep of flavoured food (g/8 hrs) associated with a dose of one of two different stimuli during each of three conditioning periods (repetitions). The nutritive stimuli were casein, C and formaldehyde treated casein, FC.*

	Treatment							
	C *v.* W		FC *v.* W		C *v.* FC		C *v.* FC+C	
Association	C	W	FC	W	C	FC	C	FC+C
Repetition								
1st	908	978	971	1030	838	982	1047	1015
2nd	967	1223	1226	1173	1072	1166	1134	1132
3rd	1043	1292	1248	1211	1093	1212	1206	1218
Means	**973**	**1164**	**1148**	**1138**	**1001**	**1120**	**1129**	**1122**
	s.e.d. 35.1		s.e.d. 25.3		s.e.d. 19.7		s.e.d. 16.4	
Significance of :								
Repetition (T)	**		***		**		**	
Association (AS)	***		NS		***		NS	
T x AS	NS		NS		NS		NS	

NS, non-significant; **P< 0.01; ***P< 0.001.

5.4.1 Intake of the flavoured test food during conditioning periods

Table 5.3 shows the average intakes of the flavoured test foods during the days of conditioning (mean values for days 1+2 and 5+6) for each of the four treatments. The consumption of the flavoured test food for all four treatments increased significantly ($P<0.001$ for FC *v.* W and $P<0.01$ for the other treatments) with repeated conditioning. The type of the nutritive stimulus (C or FC) had a significant effect ($P<0.001$) on the intakes of the flavoured food associated with its administration in treatments C *v.* W and C *v.* FC respectively. In both cases the intake of a flavoured food was reduced when it was associated with C administration. However, this was not the case for the other two treatments. Flavour used had a significant effect (not shown in Table 5.3) only in FC *v.* W ($P<0.05$) treatment, whereas its effect tended to be significant ($0.05<P<0.1$) in C *v.* W treatment. In both cases the orange flavour was preferred over aniseed; 1103 *v.* 1034 (s.e.d. 35.1) in treatment C *v.* W and 1173 *v.* 1114 (s.e.d. 17.9), in treatment FC *v.* W respectively. None of the interactions between repetition, association with a nutritive stimulus and flavours affected significantly the intake of the flavoured test foods during the days of conditioning periods.

5.4.2 Preference ratios during preference tests

Preference ratios (PR) for orange as a proportion of total food intake were not different from 0.5. PR for orange were not affected by either repetition or treatment. The backtransformed PR means were: 0.593, 0.543, 0.506 and 0.521 g orange / g total food intake for treatments C *v.* W, FC *v.* W, C *v.* FC and C *v.* FC+C respectively. This confirmed our hypothesis that no general preference for one of the flavours used over the other was to be expected.

Table 5.4 *Preference Ratios (PR) for orange flavoured food (g/g total intake of flavoured foods) previously associated with either a nutritive stimulus or water (W). The nutritive stimuli were either C or FC for treatments C v. W and FC v. W respectively. The PR were calculated from preference tests conducted at the end of each conditioning period and were repeated three times.*

	Treatment			
	C *v.* W		FC *v.* W	
Association	C	W	FC	W
Repetition				
1st	0.562[†] (0.465-0.657)	0.437 (0.342-0.534)	0.697 (0.604-0.782)	0.566 (0.469-0.661)
2nd	0.680 (0.587-0.767)	0.538 (0.440-0.633)	0.691 (0.598-0.777)	0.386 (0.294-0.482)
3rd	0.846 (0.770-0.910)	0.531 (0.433-0.627)	0.743 (0.654-0.823)	0.304 (0.219-0.397)
Means	**0.704** (0.647-0.757)	**0.502** (0.442-0.561)	**0.711** (0.655-0.764)	**0.418** (0.360-0.478
Significance of:				
Repetition (T)	NS		NS	
Association (AS)	*		**	
T x AS	NS		NS	

NS, non-significant; *P< 0.05; **P< 0.01.

[†] Backtransformed means with upper and lower limits of 95% confidence intervals in parentheses below each mean.

Table 5.4 shows the backtranformed mean PR associated with the nutritive stimulus and the controls for treatments C *v.* W and FC *v.* W during the three preference tests conducted at the end of each conditioning period. Table 5.5 shows the corresponding backtranformed mean PR for treatments C *v.* FC and C *v.* FC+C. The comparison between PR associated with a nutritive stimulus *v.* controls was not affected by repetition in any of the four treatments, although there appeared to be some trends in response to repeated conditioning.

Treatment C *v.* W*:* PR were significantly ($P<0.05$) affected by C association; sheep preferred the flavoured food associated with C over the opposite flavoured food associated with water. The flavour presented first within a conditioning period was preferred ($P<0.01$) over that presented second (0.757 *v.* 0.443). There was also a significant interaction ($P<0.01$) between flavour order and association on PR. This interaction reflected the fact that the flavoured food associated with water and presented first within a conditioning period was preferred over that presented second, during the preference test at the end of each conditioning period. This was not the case for the casein associated flavour (0.768 *v.* 0.235 and 0.745 *v.* 0.662, for the flavoured food associated with water and casein respectively). The mean total intakes of the flavoured foods during the three 20 min preference tests were 412, 455 and 371 (s.e.d. 45.3) g.

Treatment FC *v.* W*:* PR were significantly ($P<0.01$) affected by FC association; sheep preferred the flavoured food associated with FC over the opposite flavoured food associated with water. There was also a flavour order effect on PR, with the flavour presented first within a conditioning period being preferred ($P<0.001$) over that presented second (0.796 *v.* 0.322 for flavour presented first and second respectively). A significant interaction ($P<0.05$) between flavour order and association with FC on PR, reflected the fact that the flavour associated with FC and presented first within a conditioning period was preferred over that presented second, in the preference test at the end of each conditioning period. This was also the case, but to a lesser extent, for the water associated flavour (0.956 *v.* 0.362 and 0.559 *v.* 0.282, for the flavoured food associated with FC and water respectively). The mean total intakes of the flavoured foods during the three 20 min preference tests were 388, 368 and 418 (s.e.d. 27.1) g.

Treatment C *v.* FC*:* PR were significantly (P<0.05) affected by FC association; sheep preferred the flavoured food previously associated with FC over the opposite flavoured food associated with C (0.653 *v.* 0.351) administration. The flavour presented first within a conditioning period was consistently preferred (P<0.001) over that presented second (0.793 *v.* 0.211 respectively). The interaction between flavour order and FC association was not significant. The mean total intakes of the flavoured foods during the three 20 min preference tests were 374, 369 and 388 (s.e.d. 20.6) g.

Treatment C *v.* FC+C*:* There was no significant effect of either association or its interactions on PR. As for previous treatments, there was a significant (P<0.001) effect of flavour order; the flavour presented first within a conditioning period was preferred over that presented second (0.929 *v.* 0.133). A significant (P<0.05) interaction between repetition and flavour order on PR indicated that the initial flavour order effect was decreased with repeated conditioning. None of the other main effects or interactions was statistically significant. The mean total intakes of the flavoured foods during the three 20 min preference tests were 396, 410 and 448 (s.e.d. 27.7) g.

Table 5.5 *Preference Ratios (PR) for orange flavoured food (g/g total intake of flavoured foods) previously associated with one of two different nutritive stimuli. The nutritive stimuli were C and formaldehyde treated casein, FC, in treatment C v. FC and C and FC+C in treatment C v. FC+C respectively. The PR were calculated from preference tests conducted at the end of each conditioning period and were repeated three times.*

	Treatment			
	C *v*. FC		C *v*. FC+C	
Association	C	FC	C	FC+C
Repetition				
1st	0.414[†] (0.320-0.511)	0.496 (0.399-0.592)	0.578 (0.481-0.672)	0.515 (0.418-0.611)
2nd	0.357 (0.267-0.452)	0.716 (0.625-0.799)	0.617 (0.521-0.709)	0.558 (0.461-0.653)
3rd	0.284 (0.201-0.375)	0.738 (0.648-0.818)	0.573 (0.476-0.668)	0.467 (0.371-0.564)
Means	**0.351** (0.295-0.409)	**0.653** (0.595-0.709)	**0.590** (0.531-0.648)	**0.514** (0.454-0.573)
Significance of:				
Repetition (T)	NS		NS	
Association (AS)	*		NS	
T x AS	NS		NS	

NS, non-significant; *P< 0.05.

[†] Backtransformed means with upper and lower limits of 95% confidence intervals in parentheses below each mean.

5.5 Discussion

The overall objective of the experiment was to address the principles underlying the conditioned feeding responses of ruminants towards food flavours associated with the administration of different protein sources. A specific objective was to investigate the (relative) importance of RDP and DUP sources in the development of such associations. Our initial hypothesis was that the formation of such conditioned responses would be dictated by the PIC attributed to the RDP rather to the DUP source; this was based on our knowledge of the evolutionary history of ruminants.

The outcome of conditioning to distinguish between two food flavours that were associated with either casein or water, a treatment designed as a 'control', clearly demonstrated that sheep learned to prefer the flavoured food associated with a nutritive stimulus that supplied mainly RDP. This was in agreement with our initial hypothesis and the outcomes of previous studies (Villalba and Provenza, 1999a; Chapters 3 and 4). In addition, it has been previously demonstrated that the formation of conditioned responses towards food flavours associated with the administration of RDP sources is unaffected by the temporal contiguity between the consumption of food and its resulting PIC (Chapter 4). On the other hand the outcome of treatment FC *v.* W was contrary to our initial hypothesis. Sheep learned to prefer the food flavour associated with the nutritive stimulus which was high in DUP, over the food flavour associated with water. In addition, there did not seem to be any difference in the rate of establishment and the magnitude of the conditioned responses developed through the above two treatments. It seems, therefore, that the position that the formation of conditioned responses by ruminants is dictated *only* by PIC resulting in the rumen, needs to be modified. In view of our results a more tenable position would be that learning can arise from PIC resulting in both the ruminal and the post-ruminal digestive tract, as suggested also by Provenza (1995c). This would be consistent with the outcome of studies on the formation of learned food preferences associated with the supply of amino acids in the small intestine of non-ruminants (e.g. Baker and Booth, 1989b; Gietzen et al., 1992; Perez et al., 1995 and 1996; Fromentin et al., 1998; Di Battista and Mercier, 1999). The fact that PIC in the post-ruminal digestive tract are likely to arise after some considerable delay from the consumption of the flavoured food by ruminants, should not be seen contradictory to the above position. We have discussed above that the formation of conditioned responses is, within limits, independent of such temporal contiguity.

The above suggest that PIC arising from both RDP and DUP sources are involved in the formation of learned responses towards protein in ruminants. However, the outcomes of the above two treatments provide little information on the relative importance of RDP and DUP sources in the development of such responses. This issue was further investigated by the treatment C *v.* FC, where sheep were conditioned to associate a flavoured food with the administration of RDP and an opposite flavoured food with the administration of isonitrogenous DUP source. The results clearly show that sheep are able to learn to discriminate between these two flavoured foods. They learned to prefer the flavoured food previously associated with the administration of DUP. The results could be viewed as an indication of the general principle that ruminants give more weight to the PIC resulting in the post-ruminal rather to PIC from the ruminal digestive tract. This seems plausible, in view of our modified position, as microbial protein resulting from RDP, and DUP contribute to the same pool of amino acids in the small intestine (Oddy et al., 1997). The suggestion would also be consistent with the argument advanced for the outcome of treatment FC *v.* W, above. On the other hand, it is possible that the observed conditioned responses are not general, but due to some specific effects of the two protein sources used in this experiment. For example, the quality of DUP (e.g. in terms of specific bio-active peptides, Froetschel, 1996) or the negative consequences arising from RDP (e.g. in terms of sudden peaks in rumen ammonia concentration, Ørskov, 1988) could account for the direction of the observed conditioned responses. Thus, a general position could be reached only by comparing the relative conditioned responses of ruminants to various protein sources in future experiments.

The final treatment, C *v.* FC+C, was an elaboration upon treatment C *v.* FC, as it too was designed to investigate the relative importance of RDP and DUP source in the development of conditioned responses by sheep. Sheep, however, did not show any preference for either flavoured food associated with its respective stimuli. It is difficult to accept that sheep were indifferent to the PIC created by the additional administration of FC, especially in view of the conditioned responses observed in treatments FC *v.* W and C *v.* FC. It is more likely that the observed response is part of a continuum that links the conditioned responses towards combined administration of C and FC. We have shown that a continuum from flavoured food preference to aversion links the responses of sheep towards the PIC created by increases doses of casein (Chapter 3). Others have also suggested that a critical variable in the formation of conditioned food flavour preferences is the concentration of the associated nutritive stimuli (Mackintosh, 1973; Sclafani, 1995 and 1997; Provenza, 1995c). It can, therefore, be hypothesised that as we move from treatment C *v.* FC to C *v.* FC+C to C *v.* FC+C+C an initial preference towards FC is diminished by additional doses of casein.

In this experiment we balanced for the order of flavour and nutritive stimuli dose presentation across sheep, according to the experimental protocols we have developed to study the conditioned feeding responses of ruminants (Kyriazakis et al., 1997; Chapter 4). We also used flavours that were expected, given our previous experience, to have an equal acceptability by sheep (Kyriazakis et al., 1998b; Chapter 3). Nevertheless, we have found that the development of conditioned responses was influenced to a certain extent by these issues. In all treatments the effect of the order of flavour presentation on preference ratios was highly significant. These effects raise issues associated with the design and interpretation of conditioning experiments; it has been suggested that such effects are consequences of the experimental protocols (Lawrence and Illius, 1997). The effect of flavour order is observed in many conditioning experiments on sheep (Augner et al.,, 1998; Hills et al., 1999; Chapter 4) and laboratory animals (Gibson and Booth, 1986; Ramirez, 1995; Myers and Hall, 1998). Although it is difficult to predict in quantitative terms how these effects account for the observed conditioned behaviour, there is evidence that they can be overcome, following repeated conditioning (Hills et al., 1999; Chapter 4).

As asserted in the **Introduction**, we are interested in the development of conditioned feeding responses of animals because it is generally believed that they affect subsequent feeding behaviour (Kyriazakis et al., 1999). Animals use the orosensory properties of the foods as predictors of their nutrient composition, which allow them to select a diet that either minimises nutrient deficit or meets nutrient requirements. The evolution of such a process has an adaptive significance, given the amount of time and effort expended by the foraging animal in the process of diet selection (Krebs and Inman, 1992; Edwards et al., 1997). The widely held view is that ruminants have evolved to respond to PIC that result in their rumen from the consumption of protein (Tolkamp et al., 1998; Tolkamp, 1999; Faverdin, 1999); the implication is that conditions that arise *only* from such PIC influence feeding behaviour. This is because PIC in the rumen are likely to correlate highly with PIC resulting in the post-ruminal digestive tract (Leng and Nolan, 1984; Ørskov, 1988). The absence of a response, in terms of feeding behaviour, to post-rumen provision of protein and manipulations of the intestinal amino acid equilibrium, is often accounted for by the absence of the development of conditioned responses towards the PIC arising from such manipulations (Faverdin, 1999). Although this was the starting hypothesis in our investigations, the experimental outcomes led to its rejection. Sheep were actually able to distinguish between flavoured foods associated with the administration of both DUP and RDP, and hence develop conditioned responses towards them. We are inclined to agree with the suggestion of Kyriazakis et al. (1999) that the ruminant animal does not differ from the non-ruminant one, in the process of development of conditioned flavoured food responses that are associated with PIC arising in the post-ruminal digestive tract. Although the two classes of animals have evolved differently in terms of their digestive physiology, the need of the tissues for N supply is similar to both (Oldham et al., 1997; McBride et al., 1998). It should not, therefore, be surprising that the processes underlying feeding behaviour share common characteristics. Given the learned responses of sheep in the current study towards flavours associated with both RDP and DUP, the expectation is that they may be able to modify their feeding behaviour on the basis of these qualities when selecting a diet.

Chapter 6

Variation between individuals and the consequences for diet selection by groups of animals

"Only daring speculation can lead us further, and not accumulation of facts"

Albert Einstein, (1879-1955)

6.1 Abstract

A model to predict the diet selection of a population of animals is described. Its two components are assumptions about the characteristics that matter to each individual in a population, and a description of their variation in the population. The individual has only three characteristics: a requirement for a single nutrient (protein is taken as an example), a limited ability to discriminate between foods of different nutrient (protein) contents and a need to collect information about both foods by sampling them continuously. It is implicit that the foods are such that the energy requirement will be met and the model predicts the composition of the diet. The animal is given access to two foods. In experiments one is always a low protein food while the protein content of the other is the treatment variable. The animal is assumed to select a diet that avoids both a deficiency and an excess of protein with no error, where this is possible. It may not be possible only because of a failure to discriminate, or a need to sample, or the compositions of both foods being either above or below the requirement. To construct the population two further assumptions are made. The first is that the values of each of the three characteristics are drawn from uncorrelated normal distributions subject to the values being logically possible. The second is that, for different mean values for the population, the standard deviation is directly proportional to the mean so that the coefficient of variation is independent of the mean. The model was used to predict the outcomes of six experiments where the means and coefficients of variation of the three parameters were systematically varied. In each experiment the protein content of the higher protein food was systematically varied. There were 100 individuals on each treatment in each experiment. The shape of the population response was different to that for any individual. The quantitative effects of varying either the mean value of the parameters, or their variation, on both the mean composition of the diet selected, and on its

variation, were not possible to predict without using the model. The attempt to combine a simple description of the characteristics of individuals with a description of the structure of a population was useful. Extensions to the model may be able to increase its relevance to practical issues of diet selection.

6.2 Introduction

Frameworks, from a wide variety of disciplines and perspectives, have been developed to try to explain and predict the feeding behaviour and diet selection of animals. In the context of natural systems the usual theoretical approach is to collapse the nutritional complexity of the diet to a single resource, almost invariably energy (Westoby, 1974; Krebs et al., 1981; Stephens and Krebs, 1986; Lemon, 1991). It is then assumed that the animal uses some rule, or set of rules, to decide its feeding behaviour and the scientific problem is to correctly conjecture what these rules might be (Rozin and Schulkin, 1990; Emmans, 1991; Day et al., 1998). The set of rules may include the idea that the animal has incomplete information about the foods (Bateson and Kacelnik, 1995 and 1997). At the other extreme from natural systems are animals in the highly controlled environments of either the laboratory or intensive animal production systems. In these cases the conditions are usually such that the total intake of energy will be sufficient to meet all of the requirements of the animal, and its intake over time can be predicted on this basis (Parks, 1970; Emmans, 1997). Where two or more foods of different composition are freely offered the new problem is that of predicting the composition of the diet that will be selected, rather than just the quantity (of energy) that will be eaten (Emmans, 1991). There are also models that are intended to predict diet selection in these more controlled environmental conditions (Howard et al., 1994; Simpson and Raubenheimer, 1996; Raubenheimer and Simpson, 1997 and 1999).

For both the natural and controlled systems, and for the problems of both energy intake and diet selection, it is important to distinguish between two levels of organisation. The first is the behaviour of the *average* animal, or of the population of animals as an *average* value. For example: what will be the feeding behaviour of *the* oystercatcher, *the* rat or *the* pig under particular conditions? The second level is concerned with the *variation* in the behaviour of the individual animals that comprise the population. There are cases where consideration of the variation in behaviour is necessary to predict the mean responses of the population (Fisher et al., 1973; Slater, 1981). Zach and Smith (1981) felt that 'variability of foraging skills deserves further attention' and Caldow et al. (1999) point out that 'in recent years, there has been an increasing awareness of the need to quantify individual variation'. Perry and Pianka (1997) state that 'stochastic dynamic modelling and individual-based models promise to have greater relevance to complex, real-life situations'. It is on such variation between individuals, to the extent that it is heritable, that selection (both natural and human) can operate.

If the system is such that the nutritional environment can be treated as the single dimension of energy then the description of the feeding environment can be at the level of the food items that provide that single resource. Where more food resources, and possibly many food items that differ in composition, need to be considered then the level of complexity is considerable (Westoby, 1974; Parsons et al., 1994; Armstrong et al., 1997a,b; Freer et al., 1997). Models that seek to deal with such complex systems deal, perhaps necessarily, only with the average animal and the problem of the variability within a population of like animals is neglected. It is a challenge to extend the recognition of individual variation to more complex ecological processes (Sutherland, 1996; Sherratt and MacDougall, 1995; Kacelnik and Abreu, 1998). There are at least some instances where the differences between the responses of individual animals are likely to be systematic and may be large enough to be of importance for accurate prediction. A nice example is that of Fisher et al. (1973; see also Curnow, 1973) for laying birds, who showed how nutritional assumptions about all individuals could be combined with a description of the structure of a population to predict the population response to nutritional treatments. Ferguson et al. (1997) used a similar approach to deal with the variation in food intake and performance in a population of growing animals. Goss-Custard (1981) considered the problem of an individual bird faced with a food resource that was a mixture of small and large worms. From assumptions about optimal behaviour he deduced that, depending on the density of large worms, the bird should take either all or none of the small worms on offer. He concluded that the failure of the data to be consistent with this prediction could have come about because 'the stepwise response of individuals could be masked by averaging the data for all the birds'. In these examples selection for the first limiting nutrient from two or more foods is not dealt with, but only total (energy) intake.

The objective of this study was to develop a model that would account for the feeding behaviour of a population of animals when given access to two foods that differ in their concentration of the first limiting nutrient. The main aim was to see how assumptions about the characteristics of the individuals in the population could be combined with descriptions of the way in which the members of the population vary, in order to predict the behaviour of the population. Both parts of the model were kept simple in order to achieve this aim.

6.3 Description of the model

6.3.1 The individual animal

We start, as did Fisher et al. (1973) and Curnow (1973), by stating the characteristics that are assumed to apply to all individuals. The final structure of the model is in Figure 6.1. Its elements will be described in turn.

For our model the characteristics are a requirement for a nutrient, an ability to discriminate between the two foods of a pair, and a sampling rule. Each individual has a nutrient requirement expressed as a concentration in the diet. The 'nutrient' used here is protein of a given quality and the requirement is *R* g/g. We assume that, where the protein contents of the diets of the pair allow it, the animal will select a diet with a protein content of *R* (Emmans, 1991; Kyriazakis, 1997; Kyriazakis et al., 1999) with no error. This is equivalent to the animal selecting a diet that avoids both deficiency and excess, and doing both perfectly. It is implicit that the foods are such that enough food will be eaten in total to meet the energy requirement so that it is only the composition of the diet that is of interest. Even where the compositions of the foods of the pair are such as to allow the diet selected to have a protein content of *R*, a diet with this composition may still not be selected because of two other effects that may operate.

The first effect arises because the animal is assumed to have only a limited ability to discriminate between the two foods of a pair (Forbes and Kyriazakis, 1995; Farnsworth and Illius, 1998). This ability is described by a discrimination ratio, here called *D*. As the compositions of the two foods become closer to being the same a point is reached at, and beyond, which the animal is unable to distinguish one from the other. As the food compositions are made more nearly similar than this, the animal will eat exactly one half of each. There are two straightforward ways of describing how the foods need to differ, in order to be just distinguishable. One is as an absolute difference and the other is as a ratio. We have chosen the assumption that there is a maximum ratio of the protein contents of the two foods that can just be distinguished. This is a version of the Weber-Fechner law, recently used in a similar context by Bateson and Kacelnik (1995) and further discussed by Kacelnik and Abreu (1998), for which there is empirical evidence from other fields. We assume that this law holds here. With PC_L and PC_M the protein contents of the lower and higher protein foods that are offered respectively, this is equivalent to stating that when PC_L/PC_M is greater than or equal to D then the two foods cannot be distinguished.

The second effect that may cause the animal to fail to select a diet that exactly meets its protein requirement, even where the compositions of the two foods of the pair would allow this, is the assumption that it has a continuing need to sample both of the available foods (Goss-Custard, 1981; Forbes, 1995; Day et al., 1998). The sampling is needed for the animal to continuously update the information that it has about the foods on offer. Where p_L is the proportion of the total diet selected as the food L it is assumed that the animal uses the sampling rule that both p_L and $(1- p_L)$ must be equal to or greater than S, the sampling ratio.

Each individual animal thus has three characteristics: a requirement for protein, R, an ability to discriminate, D, and a need to sample, S.

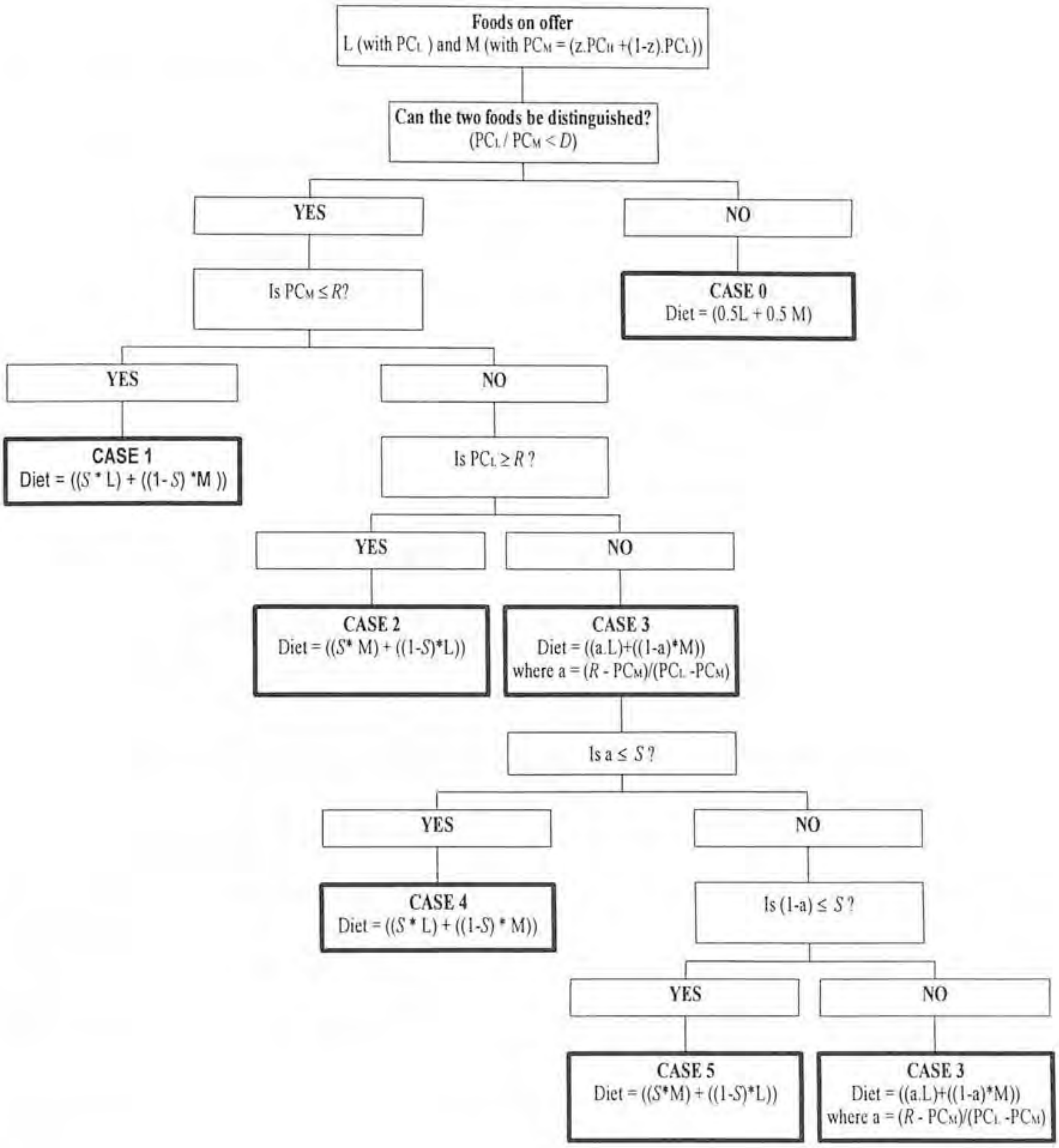


Figure 6.1 Flow diagram showing the basic assumptions made for the proposed model. The animal is offered a choice between a fixed food L, with low protein content PC_L , and a second food M, which is L diluted with a proportion z of a food H with higher protein content, PC_H. The protein content of M is given by $PC_M = z.PC_H + ((1-z).PC_L)$. The discrimination ratio is D, S is the sampling ratio and R the protein requirement, g protein/g of food (see text for further details). The animal is expected always to select a diet that exactly meets its requirement (R, g protein/g food) providing only (i), that it is able to discriminate $((PC_L/PC_M) \leq D)$ between the two foods on offer and, (ii) that it will always show sampling behaviour so that p_L and $(1- p_L) \geq S$. Where these two conditions are met the protein content of the diet selected is $a.L + (1-a)\ PC_M$ where $a = (R - PC_M)/(PC_L - PC_M)$.

6.3.2 The population structure

We construct a population of individuals by making two assumptions. The first is that the values of each of the three characteristics are normally distributed, subject to one restriction. With some values of the parameters of the model the assumption of normality leads to the prediction of values of the characteristics for some individuals that are logically impossible because they are not in the range 0 to 1. Sherratt and MacDougall (1995) faced the same difficulty and solved it by setting upper and lower positive values. Kacelnik and Abreu (1998) had a similar problem with their model and solved it by simply ignoring such values. While there is no ideal solution we have preferred to re-set such values to be either zero or one rather than to ignore them. Sherratt and MacDougall (1995) saw their approach, of using truncation, as being 'preferable to considering a normal distribution in a less familiar and less understood preference index' such as a logarithmic scale. We have also chosen not to transform our characteristics. The second assumption that we made was that, for different mean values for the population, the standard deviation is directly proportional to the mean. This has the consequence that the coefficient of variation (CV = standard deviation/mean expressed as a fraction) is independent of the mean. The justification of this assumption is that it is consistent with the Weber-Fechner law described above.

The linear correlation coefficients between the distributions of the three characteristics for the individuals are assumed to be zero for the present purposes. The model can thus be seen as a special case of a more general one where the correlation coefficients between the characteristics are allowed to be other than zero. An individual animal of the population is defined by the values drawn at random for each of the three characteristics from the specified distributions. The default values for the parameters of the model are shown in Table 6.1.

***Table 6.1** Default values for the parameters of the model*

Parameter	Mean	CV
Crude Protein content of food L (PC_L g/g)	0.10	
Crude Protein content of food H (PC_H g/g)	0.20	
Protein requirement (R, g/g)	0.16	0.15
Discrimination ratio (D)	0.85	0.09
Sampling ratio (S)	0.15	0.30
Dilution step size (z)	0.01	

6.3.3 Experiments

For each nutritional experiment we drew 100 individual animals from the defined distributions of R, D and S. Each animal was then the subject of n treatments in each of which it was given a choice of two foods. The animal was assumed to be in a stable state so that its condition did not change between experimental treatments. The n treatments were to give pairs of foods where one was always L, with protein content PC_L, and the other M, with protein content PC_M. The food M was thus a mixture of foods L and H with z as the proportion of food H in the mixture. The protein content of M, PC_M, was given by $PC_M = ((1-z).PC_L+(z.PC_H))$. When $z = 0$ then $PC_M = PC_L$ and when $z = 1$ then $PC_M = PC_H$. The value of z was increased from 0 to 1 in n steps. At the start of each of the six experiments on population structure (varying the mean and the CV values for each of the three characteristics in turn) a new sample of 100 individual animals was drawn. The ranges in the values used in the experiments, shown in Table 6.2, were chosen to be relevant to real physical and biological values for birds and mammals (Emmans and Fisher, 1986).

6.3.4 Mechanics

The structure of the model is shown in Figure 6.1. The first question is whether the animal can distinguish between the two foods (L with a low protein content and M with an intermediate one) that it is offered. If it cannot then it will eat one half of each food (Case 0). If it can distinguish between the two foods then it will attempt to select a diet with a protein content that is as close as possible to its requirement, R. This will not be possible when the protein content of M is below the requirement (Case 1). Nor will it be possible when the protein content of L is above the requirement (Case 2). Given that it can distinguish between the two foods it will eat to meet its requirement subject only to eating at least S of both of the foods on offer. The further three possible outcomes where the two foods are able to be distinguished are shown in Figure 6.1 as Cases 3, 4 and 5. The model is programmed using the MINITAB statistical software (Minitab Inc., 1996) and is available in the appendices. The first part of the program draws an individual from the population with values of R, D and S. In the second part the compositions of the diets that will be selected by that individual are predicted for each of the n experiments described above. The set of outcomes for a given individual is deterministic. A second individual is then drawn and the same treatments repeated. Initial simulations suggested that the estimates of both the mean and the standard deviation were sufficiently reliable when 100 individuals were used. The results from the 100 individuals on each of the treatments were used to calculate the mean and standard deviation of the proportion of food L in the diet selected, p_L. So each experiment consisted of n treatments applied to the same 100 individuals. The individuals were not the same between experiments but, with samples of 100, this had little effect on comparisons between experiments.

Table 6.2 *The values of the parameters used in the experiments*

Parameter	Value of mean		Value of CV	
	min/max	step size	min/max	step size
Requirement (R g/g food)	0.06/0.24	0.02	0.06/0.24	0.03
Discrimination ratio (D)	0.65/1.00	0.05	0.03/0.15	0.02
Sampling ratio (S)	0.03/0.27	0.03	0.10/0.50	0.05

6.4 Results and discussion

The model predicts that the composition of the diet selected by any one individual animal will vary with z as shown for four individuals in Figure 6.2.

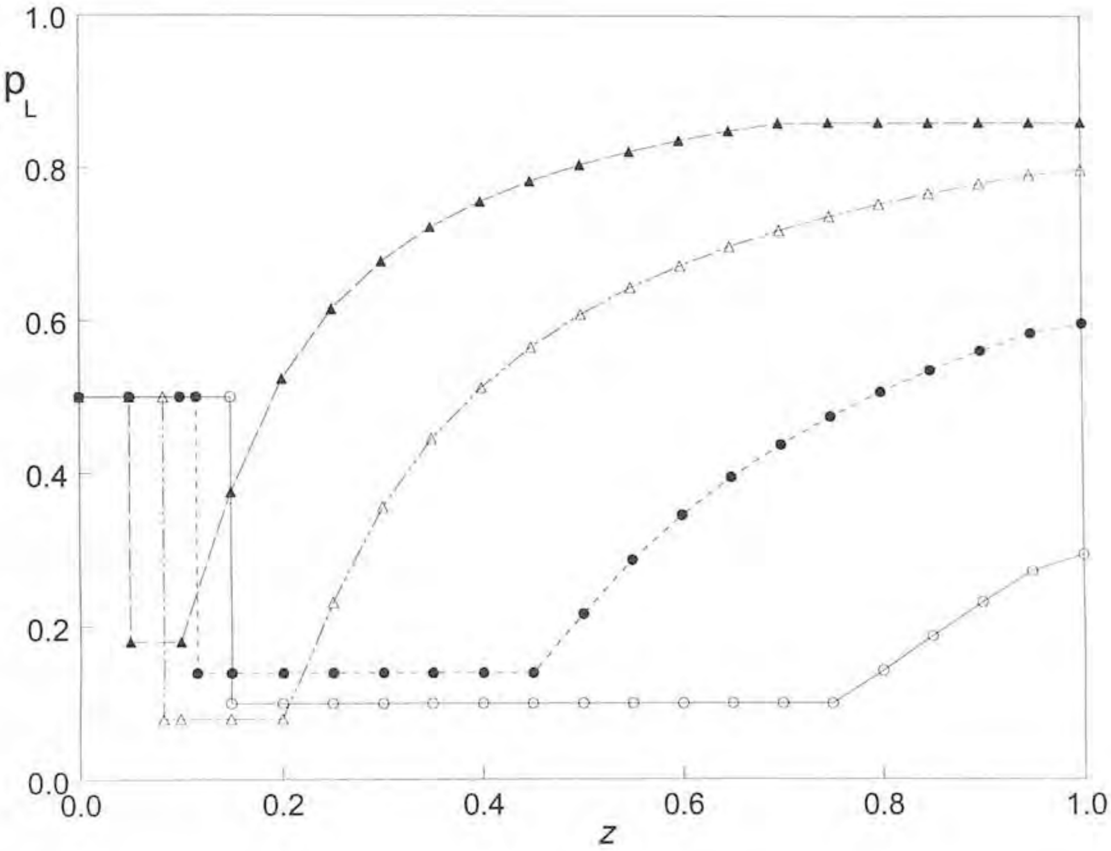


Figure 6.2 Schematic presentation of the diet selection of four individual animals drawn from a hypothetical population predicted by the proposed model shown in Figure 6.1. The animals are all offered a choice between a fixed food L, with a low protein content of PC_L g/g, and a second food M, which is L diluted with a proportion z of a food H with higher protein content, PC_H. The proportion of L in the diet selected is p_L. The protein content of M is given by $PC_M = z.PC_H + ((1-z).PC_L)$.

At low values of z the composition of the second food differs little from that of L and the animal cannot distinguish between them; the diet selected will be 0.5 of each food. As z increases a value occurs above which the diet selected changes abruptly to be that which meets R subject to the proportion of L in the diet, p_L, exceeding S. It continues to select a diet such that R is met, subject to $(1- p_L)$ exceeding S. For the population of 100 individuals the mean response will be smoothed, compared to that of the individual, because of the variation that is present in the values of the three characteristics, Figure 6.2.

In the sections that follow the effects of varying the mean and CV values for R, D and S are described in turn. As, in some cases, the outcomes are similar to those that could have been readily predicted, and all of the results are shown in Figures, not all outcomes are commented on.

6.4.1 Varying the requirement

Figure 6.3a shows how the composition of the mean diet selected changed as z was varied for five different levels of R and Figure 6.3b the effect on the standard deviations for the same experiments. When R had a mean value below, or at, PC_L, the proportion of L in the selected diet increased with z from 0.5 towards the mean default value of $(1- S) = 0.85$. The standard deviation, *sd,* firstly increased towards about 0.18 and then decreased steadily towards about 0.04. With the value of R between PC_L and PC_H (the case for values of R of 0.14 and 0.18) the proportion of L in the diet selected steadily decreased from 0.5 to about 0.2 as the value of z increased, and then increased at values of z above about 0.5. For $R =$ 0.14 the standard deviation increased steadily to reach a roughly stable value of 0.20 to 0.24. In marked contrast, for $R = 0.18$, the pattern in the *sd* was much more complex. It had a maximum at $z = 0.15$ followed by a minimum at $z = 0.4$. It then increased steadily as z

increased further. Where the mean R was above PC_H, with a value of 0.22, the proportion of L in the diet selected fell steadily from 0.5 to the mean value of S as z increased. The *sd* increased steadily, then decreased and then slightly increased again.

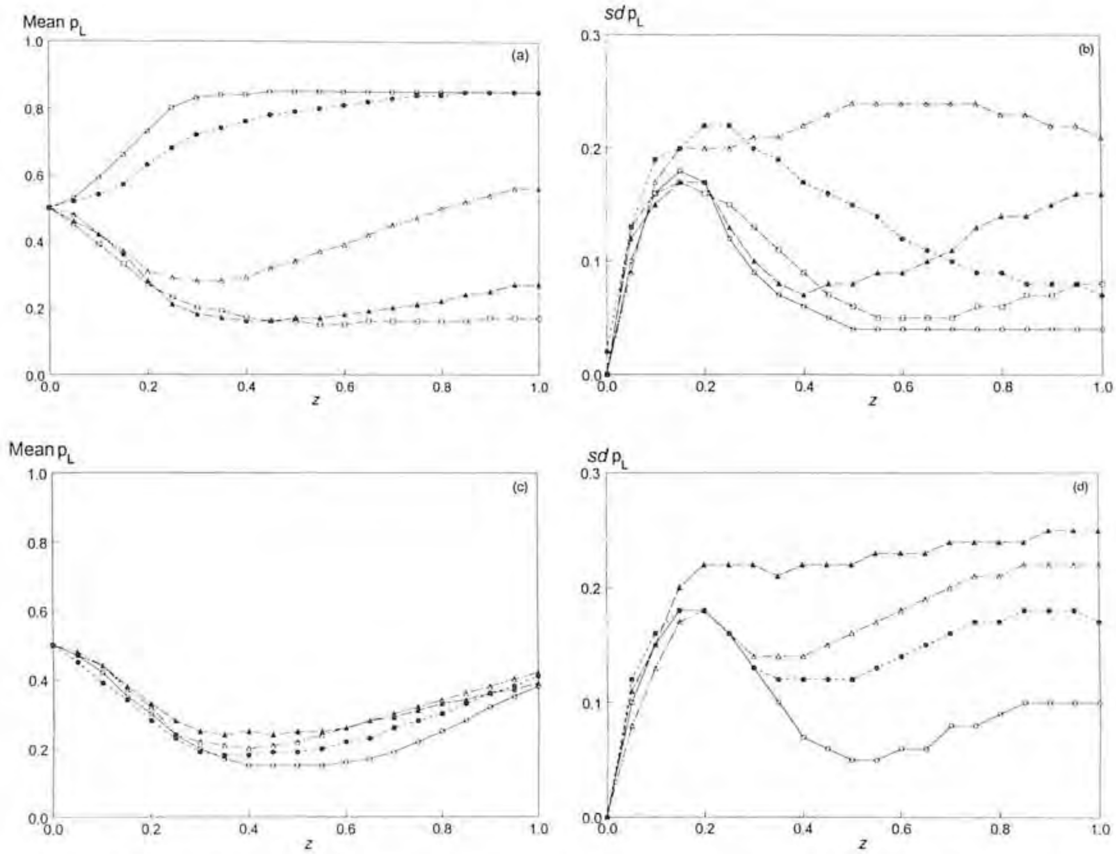


Figure 6.3 The predicted effects of varying z (the proportion of the food H in food M; see legend to Figure 6.2), on p_L, that is the proportion of the low protein food, L, in the diet selected by animals given a choice between foods L and M. The lines show the effects of different values of R, the mean protein requirement, (○, ●, △, ▲, □, for R = 0.06, 0.10, 0.14, 0.18 and 0.22 g/g respectively), in (a) and (b), and its coefficient of variation (○, ●, △, ▲, for CV = 0.06, 0.12, 0.18 and 0.24 respectively), in (c) and (d). Figures (a) and (c) show the effects on the mean value of p_L, and Figures (b) and (d) the effects on its standard deviation. All other parameterers are set to their default values shown in Table 6.1.

For a given value of z i.e. a treatment with a particular diet pair, the responses in p_L and its *sd*, were far from linear to changes in the mean value of R, Figures 6.3a and 6.3b. The shape of this response, predicted by the model, could not have been predicted intuitively.

The effects of varying the *CV* of *R* are shown in Figures 6.3c and 6.3d. The effects on the mean composition of the diet selected were small. Only at intermediate values of *z* was there any appreciable effect. However, there were large effects on the *sd*, but only when the value of *z* was such that most of the animals were eating to meet their requirements.

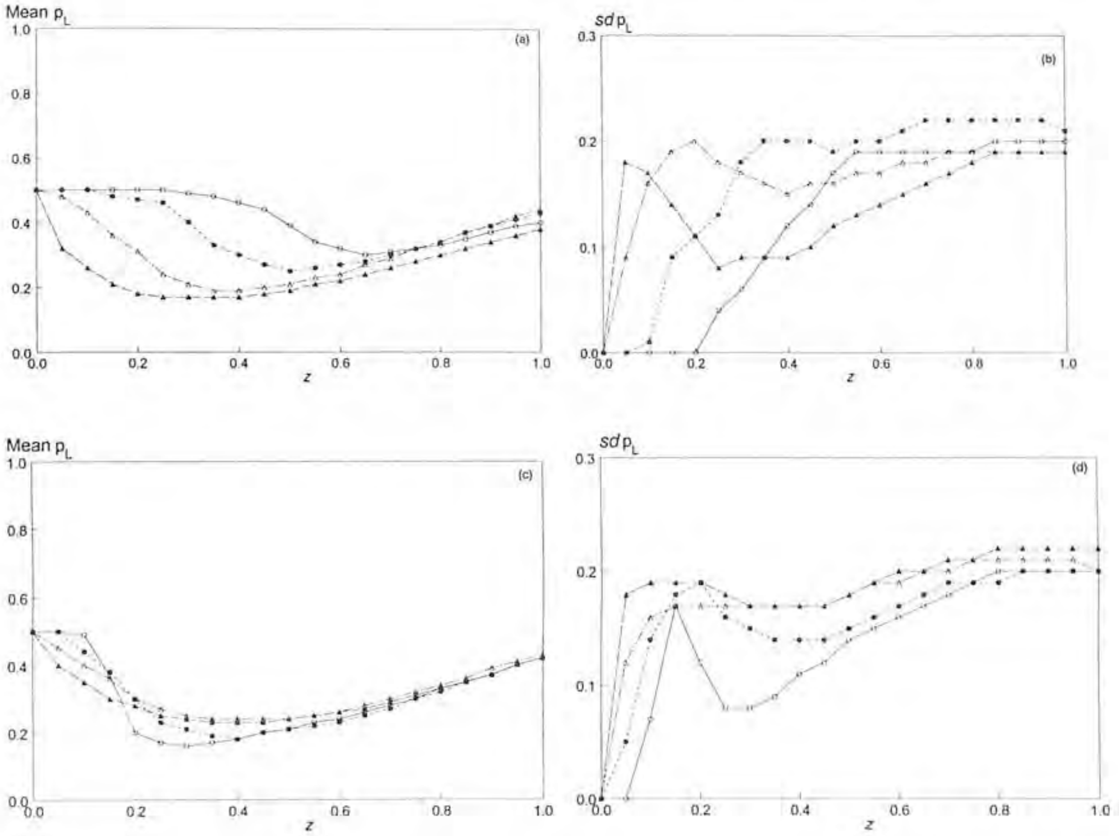


Figure 6.4 The predicted effects of varying z (the proportion of the food H in food M; see legend to Figure 6.2), on $p_{L,}$ that is the proportion of the low protein food, L, in the diet selected by animals given a choice between foods L and M. The lines show the effects of different values of *D*, the discrimination ratio, (○, ●, △, ▲, for $D = 0.65$, 0.75, 0.85 and 0.95 respectively), in (a) and (b), and its coefficient of variation (○, ●, △, ▲, for CV = 0.03, 0.07, 0.11 and 0.15 respectively), in (c) and (d). Figures (a) and (c) show the effects on the mean value of p_L, and Figures (b) and (d) the effects on its standard deviation. All other parameterers are set to their default values shown in Table 6.1.

6.4.2 *Varying the value of* D

Figure 6.4a shows the expected effect of varying *D*. At particular values of *z* below about 0.55 there were large effects of the mean value of *D* on p_L. But, at higher values of *z*, it had virtually no effect at all. The patterns of response in the *sd* to varying *z* at different values of *D* is shown in Figure 6.4b. These were complex and unpredictable without running the model.

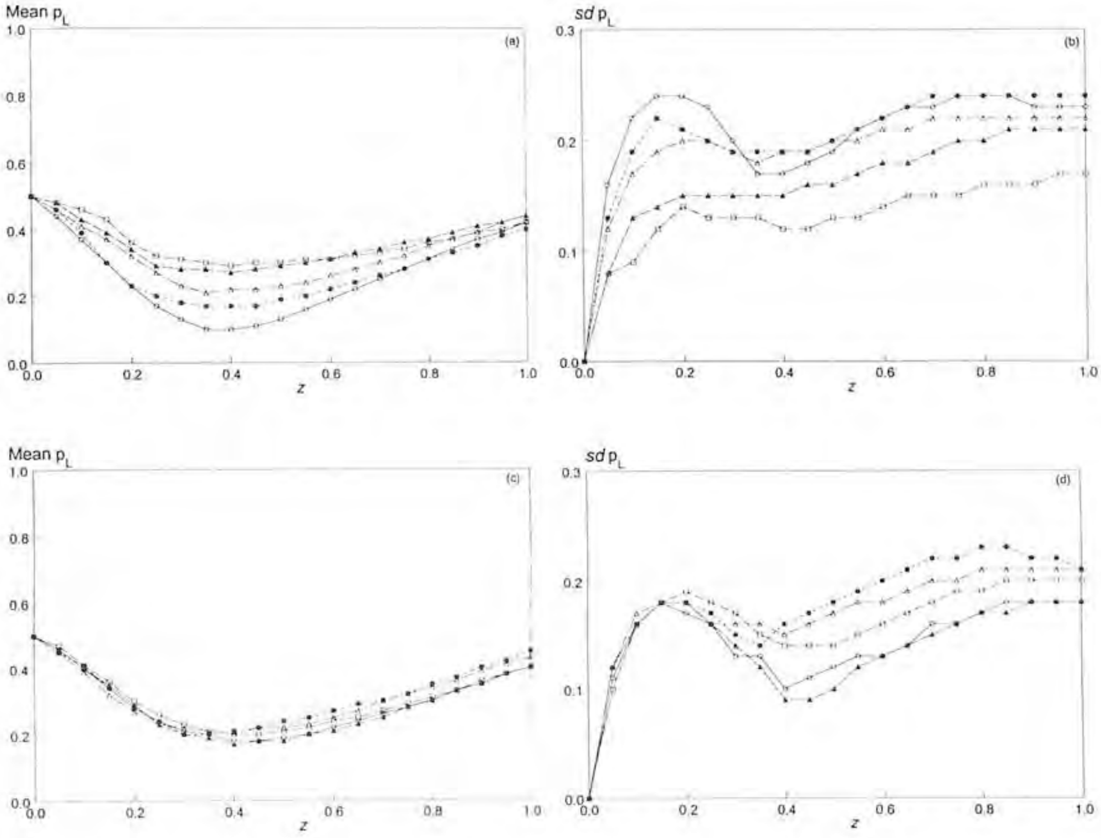


Figure 6.5 The predicted effects of varying *z* (the proportion of the food H in food M; see legend to Figure 6.2), on p_L, that is the proportion of the low protein food, L, in the diet selected by animals given a choice between foods L and M. The lines show the effects of different values of *S*, the sampling ratio, (○, ●, △, ▲, □, for *S* = 0.03, 0.09, 0.15, 0.21 and 0.27 respectively), in (a) and (b), and its coefficient of variation (○, ●, △, ▲, □, for CV = 0.10, 0.20, 0.30, 0.40 and 0.50 respectively), in (c) and (d). Figures (a) and (c) show the effects on the mean value of p_L, and Figures (b) and (d) the effects on its standard deviation. All other parameterers are set to their default values shown in Table 6.1.

The composition of the mean diet selected was not at all sensitive to varying the *CV* of *D*, as can be seen from Figure 6.4c. As expected, the level of variation in the composition of the diet selected was sensitive to the *CV* of *D* only at the low values of z, for which *D* is relevant, as can be seen from Figure 6.4d.

6.4.3 Varying the value of S

As z was increased p_L decreased and then increased again for all of the values of *S* used as shown in Figure 6.5a. At intermediate values of z the effect of different values of *S* on p_L was appreciable and non-linear. As z was either increased or decreased from these intermediate values the effects became progressively closer to zero. At all values of z the *sd* was inversely related to the mean value of *S*, Figure 6.5b, with the effect greatest at lower values of z.

There were only very small effects of variation in *S* on the composition of the mean diet selected, Figure 6.5c, but the effects on the *sd* were considerable particularly at higher values of z, Figure 6.5d.

6.5 General Discussion

6.5.1 Description of the model

The model described here is, at the level of the individual animal, as simple as we thought it could be made while still being interesting. Each individual animal has just three characteristics: a requirement for a single nutrient (protein is taken as an example), a limited ability to discriminate between foods of different nutrient (protein) contents and a need to collect information about both foods by sampling them continuously. In the list of 'Factors affecting foraging behaviour' given by Perry and Pianka (1997, Box 3) three kinds of factors are identified – external, internal and historical (phylogenetic). One of the internal factors is 'nutrient requirements', which we have in the model. Another is 'learned experiences'. Our sampling behaviour does not relate directly to learning, as there is no feedback in the model of the information gathered by sampling to the composition of the diet selected. Sampling behaviour is a necessary precursor to introducing learning into the model as is intended in later versions. Among their historical (phylogenetic) factors are 'sensory limitations' which can be seen as corresponding to our discrimination ratio. The point is not that the three characteristics in our model can be related to a more general consideration of foraging but, rather, that we directly consider *only* two of the 15 factors listed by Perry and Pianka (1997). Our aim in using such a simplified model for an individual was to be able to see clearly the effects of variation in the characteristics on the mean compositions of the diets selected by the population and their variation. The aim was not to decompose complex population responses but to see how they could arise from a simple set of assumptions, about both individual animals and population structures.

It is possible to see how the model of the individual animal could be extended, and we deal with such extensions later. There would remain the problem of maintaining the connection with the sub-model dealing with population structure. We see our model as growing in the direction of that described by Goss-Custard et al. (1995a,b) who produced and tested a model of the foraging behaviour of oystercatchers. It predicted (energy) intake, rather than diet composition and, in the view of the authors it 'was regarded as a suitable starting point for exploring how the proportion [of the population] failing to acquire enough food, and either emigrating or starving, is affected by population size'.

We assumed that the variation between the members of the population would be proportional to the mean and that the values of the three characteristics would not be correlated with each other. The first assumption was made partly because it seemed to be reasonable and partly because it was convenient; the behaviour of the model is not qualitatively sensitive to it. The second assumption of zero correlation was made because it simplified the population description and because we had no reason a priori to assume anything else. Technically it is straightforward to extend the description to deal with correlations that are not zero. However, if many more than three characteristics are included in the model, (the 15 of Perry and Pianka (1997) for example), then the problem of quantifying the covariances could rapidly become overwhelming. An analogy is with the problem of extending a description of the potential growth of an individual to a population of like animals (Emmans and Fisher, 1986; Ferguson et al., 1997). We see the level of description of the population that we have chosen as being a reasonable first step.

We chose to truncate normal distributions in order to avoid having any logically impossible values for the characteristics i.e. outside the range 0 to 1. We were concerned that some of the predicted consequences might arise simply because we had chosen to do this. We tested this possibility in two ways. Firstly, we compared those cases where illogical values were extremely unlikely to be drawn with those where they were likely to be drawn. The absence of any qualitative difference suggested that the results from the model were not arising from the truncation. Secondly, and more critically, we cut out the truncation steps in the model and allowed logically impossible values (outside the range 0 to 1) to be selected in order to keep the distributions normal. There were quantitative differences between the two versions of the model for the predicted values of both the means and the standard deviations. But the two versions gave results that were qualitatively similar and, in particular, the non-linearity in the responses remained.

We have chosen to make the problem that the animal faces to be one of selection for a nutrient concentration. This is mainly because this has been a problem of long-standing interest to us (Emmans, 1977 and 1991; Kyriazakis et al., 1990), but also because it makes a change from considering energy intake as the only dimension of importance to the animal. Where the foods available are such that energy is abundant, and where they vary in their ratios of nutrients to energy, then the animal's problem is that of getting a diet that has nutrient rather than energy adequacy (Westoby, 1974; Belovsky, 1990) and, perhaps, of avoiding excesses (Kyriazakis et al., 1990; Kyriazakis and Oldham, 1993). Only one requirement is in the model, that for protein of a given quality, but the individual animal part of the model could be expanded to consider a set of requirements. The experiments, that would then be designed to be the simplest to reveal the consequences of the assumptions that comprise the model, would need to be made more elaborate with more than two foods being available (Emmans, 1991). Where the number of requirements exceeds the number of foods it will be necessary to deal with the rules that the animal uses to decide the trade-off that it will make between ensuring adequacy of one resource while avoiding excess of another (Kyriazakis et al., 1999). As the number of requirements, foods, and dimensions needed to describe a food, are all increased so the model can be seen as becoming more realistic for at least some actual cases. But the costs of the increased realism are the necessary loss of clarity in seeing how the model behaves and the problem of getting proper estimates of the values of its parameters, particularly at the level of the population. Our aim was to produce a model that dealt with a base case of diet selection by a population of animals that showed variation.

The model deals with variation between individuals along three continua – R, D and S. In the nature of the model there can be no *qualitative* variation present, although this has been observed between individual animals in foraging behaviour (Sherratt and MacDougall, 1995; Bateson and Kacelnic, 1997; Caldow et al., 1999).

6.5.2 Experiments and the performance of the model

One of the great advantages of models over reality is that very much higher levels of replication can be used than is usually practical in reality so that variation can be accurately estimated. Another is the ability to consider a simplified version of the real world. The outcomes of the experiments on the model shown in the Figures are simply the consequences of the assumptions made and the parameter values chosen. This is so with all models. But not all of the responses were predictable in advance. As can be seen from Figures 6.3-6.5 there were large changes in the predicted level of variation as the value of z was changed. While it was intuitively expected that changing the *variation* in the parameters would affect the *variation* in the diets selected (as it did in Figures 6.3d, 6.4d and 6.5d) it was not so obvious that this would also affect the mean outcomes (Figures 6.3c, 6.4c and, particularly, 6.5c). The effects of varying the mean values of the parameters on both the mean diet composition selected, and on its variation, were not at all easy to predict without running the model. With more characteristics, the use of such a model in making clear, explicit and quantitative predictions of the effects of the assumptions being made becomes of even greater value.

The predictions of the model show how assumptions of stepped responses (Figure 6.2) for any one individual will give smoothly varying population responses because of the variation between individuals. This connects with the model of Fisher et al. (1973) and with the case discussed by Goss-Custard (1981). It confirms the view that step-wise responses of individuals could be masked by averaging the data from animals which are treated the same.

With only one nutrient considered the structure of the experiments needed to reveal the behaviour of the model could be made quite simple. With one food of the pair always the same the composition of the other food was varied in a single dimension by diluting the first food with one with a higher protein content. This technique, the 'diet dilution technique', has been used in real experiments where many treatments could be given (Fisher and Morris, 1970; Fisher et al., 1973) as single foods. It has also been used successfully in diet selection experiments (Kyriazakis et al., 1990) to test theoretical predictions.

6.5.3 Extending the model

The model is likely to have relevance to 'real' problems only under considerably curtailed circumstances because of the emphasis on simplicity. Nevertheless the attempt to combine a skeletal description of the characteristics of any individual with a description of the structure of the population can be seen as being of relevance to practical issues of diet selection. The challenge lies in how to extend the model to make it more realistic.

As well as dealing with more nutritional dimensions, as discussed above, we see three extensions as being useful. The first is to relax the current assumption that S has a value, which is independent of both the number of foods available, and on how different they are from each other. Kyriazakis et al. (1990) for pigs, and Kyriazakis and Oldham (1993) for sheep, produced data that indicated that S might not be independent of the compositions of the foods of the pair that were offered. The data indicated that S might vary with the extent to which both foods deviated from the requirement. Such a rule can be quantified and incorporated in the current model. The second extension is to make the model dynamic so that it can deal explicitly with learning and with temporal changes in the values of the three parameters. This would enable the model to deal with changes in animal state as well with the ability of the animal to learn. There is evidence that animals deprived of some nutrient become more able to discriminate between diets with different levels of it (Simpson and Raubenheimer, 1996). It is widely recognised that the requirements of animals change as they develop (AFRC, 1993; NRC, 1998) and such changes could be quantitatively described (Emmans and Fisher, 1986; Ferguson et al., 1994). Such an extension would be in agreement with the suggestion of Perry and Pianka (1997) that 'stochastic dynamic modelling and individual-based models promise to have greater relevance to complex, real-life situations'. The third extension is to allow *D* to vary with the nutritional dimension. In the current model the only nutritional dimension is 'protein'. When the model is extended to more dimensions a decision will need to be made on the value of *D* for these different dimensions. There would seem to be no reason a priori to assume that it will be constant for different nutrients and the model will need to allow it to vary.

In its present form the model has clear limitations but we do feel that it has sufficient nice properties for us to be encouraged to go on to extend its scope. Extensions, such as those described above, may be able to increase its relevance to practical issues arising from the need to understand and predict diet selection.

Chapter 7

General Discussion

"Science is perhaps the only human activity in which
errors are systematically criticised and, ... in time corrected"

Karl Popper, (1902 - 1994)

7.1 Introduction

The main objective of this thesis was to investigate how sheep select for the protein content in their diet. It was achieved by following two lines of research. The emphasis of the first line of research has been on the question whether sheep have an inherited, 'unlearned' ability to select for an appropriate protein content in their diet or whether they have to learn to do so. It was addressed by an initial experiment (Chapter 2) that was designed to test the hypothesis (Hughes, 1979) that as animals possess a series of unlearned appetites for many specific nutrients that allow them to select a food rich in a particular nutrient, in which they are deprived, almost instantaneously and without any previous experience on it. The results of this experiment (Chapter 2) did not provide any evidence for an unlearned appetite for protein in sheep under the experimental conditions used; the outcome was in favour of the view that control over this aspect of diet selection is learned. Hence, the next step was to define the role of learning.

The second line of research (Chapters 3-5) investigated the proposition (Provenza and Balph, 1990; Provenza, 1995c; Kyriazakis et al., 1999) that animals have developed behavioural mechanisms that allow them to recognise foods on the basis of their nutritional, as well as other properties, and that such learning depends largely on the extent of the post-ingestive consequences (PIC) induced by foods. In particular, the question how sheep learn to predict the nutritional consequences of foods from their flavour, and the effectiveness of different protein sources on such learned responses, was investigated. A classical conditioning methodology was used to carry out this line of research.

It has been emphasised in the General Introduction (Chapter 1) that the identification of factors that affect the ability of animals to reach decisions related to their feeding behaviour should help us to arrive at more robust predictions of both food intake and diet selection. Hence, in Chapter 6 an attempt has been made to develop a model that would account for the diet selection of a population of animals, by making simple assumptions about the characteristics of the individuals that comprise the population, including the variation between them. Although the model was developed for an 'abstract' animal, it was expected to be also relevant to the selection for dietary protein by sheep.

The purpose of this final Chapter is to bring together findings of the previous Chapters (Chapters 2-6) and to further discuss them with reference to current literature. The Chapter deals first with the particular question of how sheep (and ruminants in general) reach decisions related to their selection for dietary protein. Subsequently, the various findings of this thesis are reviewed in an attempt to discuss the implications for the study of feeding behaviour of ruminant animals and their relevance on the diet selection of animals under more natural conditions. Finally, a number of issues raised by this thesis are discussed together with some suggestions for themes for future research.

7.2 Diet selection for protein by sheep

7.2.1 The importance of animal state

Kyriazakis et al. (1999) suggested that the diet selection of animals should be considered within a framework of feeding behaviour that views both food intake and diet selection as an outcome of the animal's internal state and knowledge of its feeding environment (see Figure 7.1).

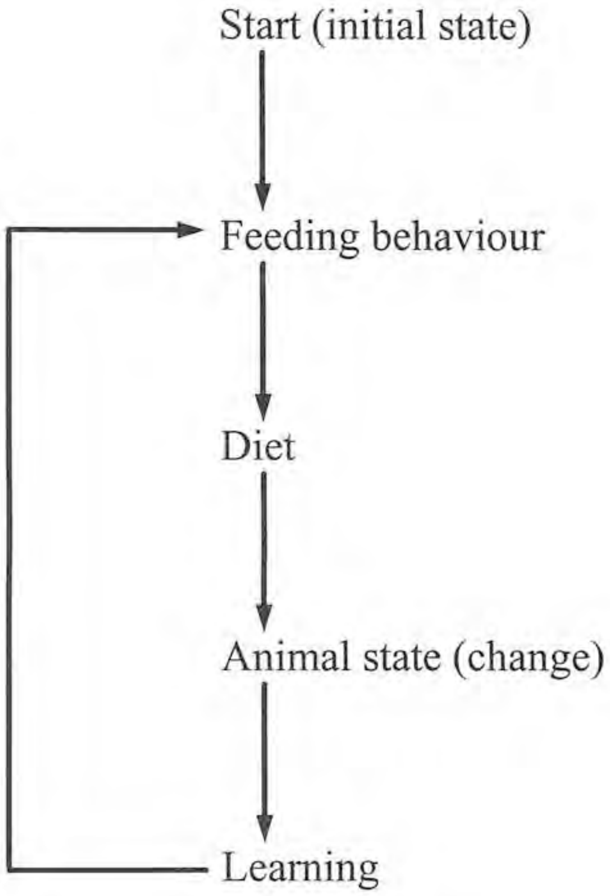


Figure 7.1 A framework for considering the way by which learning and animal internal state affect feeding behaviour (after Kyriazakis et al., 1999).

It is assumed in that framework that the rate at which animals learn about foods, and how long this knowledge is retained, depends largely on the extent of the animal's deficiency (deviation from its 'desired' state) and the strength of the PIC induced by the foods. In the experiment described in Chapter 2 it has been concluded that an unlearned appetite for protein is unlikely to exist in sheep. However, as already discussed in Chapter 2 this might simply be a reflection of the degree of protein deprivation of sheep induced in that experiment. The question of whether sheep would respond differently after a more severe protein deprivation, albeit a less relevant one, still remains unclear. It is interesting, however, that Denton (1982, p. 549) in his classic study 'The hunger for salt' stated that *"within a particular species such as sheep....given the same environment and dietetic conditions from birth, and these for example involving low but adequate salt intake in food and water, some animals when given free access to sodium solutions behave as salt gluttons, whereas others ingest little or no salt"*. Denton also stated (1982, p. 605) that *"even in a herbivorous creature such as sheep, whose highly developed appetite organization has been extensively investigated, it is obvious that learning mechanisms become richly superimposed on innately generated drives"*.

It has been shown in Chapter 2 that sheep selected a diet that reflected their protein requirements, which is in agreement with previous finding on the ability of sheep to regulate the protein content in their diet and select a diet that supports their potential rate of growth (see section 1.2, for appropriate references). However, the results of the experiment described in Chapter 2 indicated that the *initial* diet selection of sheep was greatly affected by the 'non-nutritional properties' of foods on offer. This was independent of the differences in animal state, induced by different protein feeding regimes. The importance of such 'non-nutritional properties' of foods in diet selection was discussed in Chapter 2 and more recent evidence (e.g. Villalba and Provenza 2000a,b) is in agreement with the conclusion reached in section 2.5, that eventually the nutritional consequences of food seem to be more important than its 'non-nutritional properties'.

I therefore suggest that the fact that diet selection for protein changed systematically over time, and that the sheep needed some time before selecting a diet, is in agreement with the framework of diet selection proposed by Kyriazakis et al. (1999). However, there are aspects of learning that the experimental design in Chapter 2 did not allow us to compare: these include the rate of learning, i.e. if animal state plays a role in the rate that animals learn about foods. This issue has not been elucidated any further by this thesis, as in the second line of the research followed (Chapters 3-5) there were no imposed differences in animal state between sheep that were subject to the same or different training schedules. The issue of whether animal state, and degree of protein deprivation in particular, affects the rate of learning is a fruitful question to be addressed in future research.

7.2.2 The role of learning

On the assumption that ruminants are likely to require cues in order to be able to distinguish which food is associated with which specific PIC (Chapter 1), the question of how sheep reach decisions related to their selection for dietary protein was addressed in a series of four experiments (Chapters 3-5). In all experiments discussed, sheep were individually penned and tested to assess the development of learned responses towards a flavoured food that had been previously associated with the administration of a protein source versus a flavoured food associated with the administration of water or a different protein source. The idea was that the methodology used would enable sheep to use flavour as a cue to learn about the PIC of the foods on offer (as for example suggested by Rescorla and Wagner, 1972; Provenza, 1995c). The experimental design thus did not allow us to address issues related to how social environment influences learning about foods (for an appropriate review on this see Ralphs and Provenza, 1999).

The results of these four experiments suggest that learning plays an important role in the control of diet selection for dietary protein by sheep. Despite the peculiarities of their digestive system, as described in Chapter 1, sheep are able to learn to predict the PIC of foods, attributed to their protein content, from the characteristics of their sensory properties, i.e. flavour. In the experiment described under Chapter 3, it has been suggested that the continuum hypothesis, indeed accounted for the ability of sheep to distinguish the differential PIC of increasing doses of a rumen degradable protein (RDP) source. Sheep showed conditioned flavour preferences (CFPs) and conditioned flavour aversions (CFAs) towards food flavours associated with positive or negative PIC respectively, resulting from administration of an RDP source (casein). It has been suggested in Chapter 3 that feeding behaviour and diet choices in ruminant animals can be influenced quantitatively by protein (RDP) provision. It was further concluded (section 3.6) that the ability of sheep to develop CFPs and CFAs, created by the same nutrient (protein), could be the basis of how ruminants select a diet which meets their nutrient requirement at a particular point in time, but also avoid excess of nutrient intake. The latter issue is a pertinent one in the development of any theory that aims to predict the feeding behaviour of farm animals.

The next question of interest in this line of research (see section 1.3.5.2), whether and to what extent the time-interval between food consumption and its PIC can affect the formation of learned responses, was addressed in the experiments described in Chapter 4. It has been shown that the development of CFPs and CFAs from the PIC induced by the administration of an RDP source in ruminants could be established also through delay learning (i.e. when the two are disassociated in time). The formation of CFPs through delay learning by ruminants was the main novel finding in this Chapter. However, it is still unclear whether the ruminant animal gives equal or different weights to the PIC occurring in the rumen and within the post-ruminal digestive tract on the formation of such conditioned feeding responses (see Chapter 1). Given that foods may vary in their RDP and digestible undegradable protein (DUP) yield, the final experiment covered by this thesis (Chapter 5) was an attempt to assess specifically the effects of RDP and DUP yield of foods on the development of conditioned feeding responses. The experimental design followed allowed for such a direct test. It has been shown that the ability of sheep to develop CFPs is not unique to RDP administration but that the DUP yield of food is also involved. This should not be surprising given the possibility that ruminants, like non-ruminant animals, have to depend on the potential metabolisable protein yielded by the foods. However, the results of this experiment (section 5.5) revealed that there are some conditions that have to be met for DUP to lead to the formation of CFPs. It has been suggested that the PIC induced by the DUP content of foods may have different relative importance under different circumstances, depending on factors such as the RDP contents of the foods on offer. For example, when differently flavoured foods are associated with adequate RDP, any additional protein (RDP or DUP) may be perceived by the animal as 'too much of a good thing' which results to its avoidance.

Throughout the experiments covered by this thesis (Chapters 2-5) I have tried to present many of the components that together account for how sheep and ruminants in general learn about the protein content of foods. However, how sheep will then use such learning when they select for the protein content in their diet is a question that merits further investigation. The issue of feeding behaviour is certainly much more complicated than the form of learning developed here and much more is yet to be done to reach at a satisfactory theory (model) of feeding behaviour. As already pointed out by other workers in the field, knowledge about the nutritional content of food alone is insufficient to motivate diet selection and assessment of how animals reach such knowledge alone is insufficient to understand or even predict feeding behaviour (Day et al., 1998; Kyriazakis et al., 1999). Another important determinant is the current need of the animal and it has been suggested that learning is useful as a parameter within the context of a larger framework (Kyriazakis et al., 1999).

Overall, the results of this thesis suggest that sheep should be able to modify their feeding behaviour based on the qualities of dietary protein (both the RDP and DUP) when selecting a diet. What is more difficult is to predict to what extent such learned associations can assist animals in their diet selection in real life situations; this will be further discussed in a later section. Furthermore it was outlined in Chapter 6 that a better understanding of the rules underlying such selection can assist us in the development of feeding behaviour predictions.

7.2.3 The basis of diet selection for protein by ruminants: the debate will continue

Since the work of Kyriazakis and Oldham (1993) a large body of experimental evidence (see Oldham et al., 1997; Kyriazakis et al., 1999; Tolkamp, 1999; Faverdin, 1999) has been accumulated on the ability of ruminants to select for the protein content in their diet. This has been resulted in considerable debate. Tolkamp (1999) stated that there are particular problems in interpreting experimental results of diet selection for protein by ruminants (see also section 1.2); hence, it is not always possible to reach at a consistent view of how ruminants select for protein in their diet.

The previous section (section 7.2.2) provided strong evidence that sheep are able to predict the PIC of foods in relation to their protein content by developing CFPs and CFAs. However, these findings on the learning and discriminatory capabilities of sheep for the

RDP and DUP content of foods, simply suggest which rules they might use in their decision making. They do not always indicate clearly what is the basis of diet selection for protein. For example, one possibility, as discussed in Chapter 5 is that the PIC induced by the DUP source used, formaldehyde treated casein, could have been specific to it because of its particular qualities (see also section 1.3.5.3). Although it was appreciated that there is some 'priority' given to the PIC induced by the RDP content of food, the view taken in this thesis is that current evidence does not provide a clear answer to the question of what is the basis for protein selection by ruminants. The results of the experiment described in Chapter 5 leave open questions about the relative importance of the rumen and the post-ruminal digestive tract in the selection for dietary protein by ruminants. I therefore suggest that the debate on what is the basis of diet selection for protein by ruminants will most likely continue. A general position could be reached only after further experiments using various DUP sources and by combining learning experiments with diet selection experiments. Proposed experiments that could address the former have been put forward and they are part of ongoing investigations in the laboratory where the work described in this thesis was conducted.

7.3 Understanding the feeding behaviour of ruminant animals

It was emphasised in the General Introduction that the absence of a theory that can both explain and correctly predict the feeding behaviour farm animals is widely recognised. The aim of the following three sections is to bring together the various findings and ideas which have been described throughout the preceding Chapters and to discuss their possible importance and relevance to the feeding behaviour of ruminants.

7.3.1 Advantages and limitations of using a conditioning methodology

Since the time of Pavlov, it has been well documented that conditioned stimuli (cues) predicting the delivery of food can come to condition a series of distinctive behavioural changes. The idea that classical conditioning provides a methodological and conceptual tool for dealing with questions about how animals control their feeding behaviour has been reinforced by recent studies (e.g. Sclafani, 2000; Davidson et al., 2000). For example Davidson et al. (2000) stated that the same mechanisms that enable animals to form learned associations in laboratory experiments relate also to normal feeding.

The central problem addressed by the experiments described in Chapters 3-5, has been the question of how sheep learn to predict the PIC of foods (here attributed to protein) from their flavour. This problem was approached by adopting a classical conditioning methodology. The assumption was that this methodology is the most effective in evaluating the learning abilities of animals (see section 1.3.2) and it has been proven successful in previous experiments with sheep (e.g. Kyriazakis et al., 1997). In addition, as discussed by Kyriazakis et al. (1999), the findings of such studies should be general and would, therefore, apply to 'more complex natural environments'.

One important characteristic of the adopted methodology is that the ability of sheep to form learned associations between food flavours and PIC, created by the administration of a nutrient source (see Chapters 3-5), was evaluated in preference tests following a training period. Learning, considered here as the modification of feeding behaviour through conditioning, was measured at the level of individual animals. Thus, the importance of the individual animal within a group was given particular emphasis. Although not reported in detail (learning measurements were averaged between the animals of a group) in the experiment described in Chapter 5 (section 5.4.2) there were considerable differences between individual animals within specific treatment groups.

Thibault and Booth (1999) stated that unless there is a sensory cue to the presence of a particular nutrient in a food, no mechanism is available by which ingestive behaviour could lead to selective intake. In all the experiments of this thesis where a conditioning methodology was employed (Chapters 3, 4 and 5), we balanced for the order of flavour and nutritive stimulus/stimuli dose presentation across sheep and we also used artificial flavours that were expected to have equal acceptability by sheep. As mention in section 4.5 and further discussed in section 5.5 the development of conditioned feeding responses was influenced to a certain extent by these issues. Taken together, however, the present results (Chapters 3-5) provide strong evidence that such differences were diminished by repeating the training schedule which is in agreement with other recent studies (Augner et al., 1998; Hills et al., 1999; Villalba and Provenza, 1999a and 2000a,b).

Another possible limitation of such a methodology is the argument that the methodology used represents an artificial situation not encountered by ruminants in natural habitats (Duncan and Gordon, 1999). For example, these authors suggest that such methodology cannot represent the feeding responses of grazing animals, because there is little evidence that such animals restrict their diet to individual food items for any length of time. The question that arises then is how such animals are expected to reach decisions related to their diet in more complex situation? However, more recently, Villalba and Provenza (2000b) have stated that although the diet of a grazing ruminant may contain many plant species (i.e. more than 100), the bulk of the diet is typically composed of 3 to 5 different ones. The suggestion put forward here is that the methodology used and the results presented are, therefore, applicable to more complex, less artificial situations. We will address this issue in detail in a later section dealing with some practical implications of CFPs and CFAs.

7.3.1.1 Application of the above principles to selection for other nutrients

The literature on conditioned feeding responses in laboratory animals generally acknowledges that the phenomenon involves a broad category of nutrients and that accounts to a large extent for the modification of feeding behaviour by such animals (Capaldi, 1992; Ramirez, 1996; Sclafani, 1995 and 1997; Capaldi et al., 1997; DiBattista and Mercier, 1999; Myers and Hall, 2000). Characterising the qualitative and/or quantitative differences between the PIC induced by different nutrient sources, now appears to be an area of research likely to provide valuable insight into the development of conditioned feeding responses. In particular emphasis should be given on the ways that multiple PIC induced by different nutrients become co-ordinated and converge to affect feeding behaviour (for example, see reviews by Berthoud, 2000; Sclafani, 2000; Spector, 2000).

In all experiments covered by the second line of research in this thesis (Chapters 3-5) sheep were trained to form conditioned feeding responses towards food flavours that were associated with PIC attributed to the administration of different forms of casein as a protein source. It was mentioned in the General Introduction (section 1.1.1), that the prevailing view is that farm animals are able to select a diet that meets their requirements for any particular nutrient when offered a choice between appropriate foods. The possibility that sheep can also learn through the PIC induced by other nutrients (or nutrient sources) has been recently addressed in a series of experiments, i.e. for sulphur by Hills et al. (1999) and by Villalba and Provenza for starch and casein (1999a), for starch (2000a) and for milo grain (2000b). The fact that sheep can discriminate between the PIC arising from energy and protein sources respectively and subsequently develop CFPs, is of particular importance given the interaction between energy and nitrogen metabolism in the rumen (see section 1.2.1) and the existing controversy regarding the relative importance of energy and protein in food selection by ruminants (see Villalba and Provenza, 1999a). I am, therefore, tempted to suggest that learning by association (CFPs and CFAs) should be relevant to selection for other essential nutrients in their diet. However, there are many important issues and lines of research that can be developed to address this. For example, what is the relevance of a continuum response to increasing amounts of nutrients that are unlikely to lead to negative PIC (e.g. excessive amounts of water soluble vitamins).

7.3.2 Practical implications of learned food preferences and aversions

7.3.2.1 Yet another debate

The idea of using the food aversion model as the basic paradigm to account for feeding behaviour of ruminants animals has generated a significant body of literature that elucidates, to a certain extent, how ruminants reach decisions related to their diet selection (e.g. Ralphs, 1992; Launchbaugh et al., 1993; Provenza, 1996). At the same time this position has resulted to some practical applications, such as training animals to avoid poisonous plants (e.g. see Ralphs and Provenza, 1999). However, it has been emphasised in the General

Introduction (section 1.3.3) that considerable debate has also been generated by this idea. The major argument is that it represents an artificial situation that is not encountered by ruminants in natural habitats and therefore its application to such systems is unclear (see Duncan and Gordon, 1999). Animals should be able to learn from negative, but also from *positive* PIC. As mentioned in a previous section (section 7.3.1) Duncan and Gordon raised a similar argument to the use of learned food preferences as the basis for diet selection in free-grazing systems.

This thesis suggests that the idea of learned food aversions (Provenza, 1996; Ralphs and Provenza, 1999) as the basis of diet selection of ruminants is not a tenable one. Learned food preferences (CFPs) are of equal importance to learned food aversions (CFAs) to the ruminant animal when selecting its diet. More recent studies by Villalba and Provenza (1999a,b; 2000a,b) seem to be in agreement with this modified and more general view. The latter studies, together with the results of the experiments covered by this thesis (Chapters 3-5) provide now considerable evidence about the ability of ruminants to form CFPs and CFAs for food flavours associated with PIC induced by the administrations of several nutrient sources. But how can we link the ability of ruminants to form CFPs and CFAs under controlled situations to their feeding responses in nature? This question epitomises the challenge. Although the development of CFPs and CFAs apply literally where foods are pure to individual nutrients I suggest that in nature, where diets are relatively more mixed, the implications of learning can be virtually analogous: the selection for the nutrient composition in the diet, each near an optimum level, through the development of CFPs and CFAs would permit 'tuning' of the amounts of nutrient intake towards actual needs. This is analogous to selection for the first limiting nutrient in the diet proposed by Emmans (1991).

7.3.2.2 Acquisition and persistence of learned preferences and aversions

Optimal foraging theory predicts that animals will consume foods with the highest nutritional yield at the lowest energetic cost (Stephens and Krebs, 1986; Krebs and Inman, 1992). If grazing animals can locate and subsequently discriminate between food sources using cues in a predictive manner, such ability would save them the energetic cost of search time and would provide flexibility in their feeding behaviour. For example, Kronberg and Malechek (1997) stated that in free ranging situations *"it is possible that ruminants can quickly learn which feeds offer more positive post-ingestive feedback that is associated most with protein or energy and then seek out those feeds during the day that offer the nutrients they desire"*. As such, the development of learned food preferences and aversions could be a factor contributing to the evolutionary fitness of an individual (see also Krebs and Inman, 1996; Kyriazakis et al., 1999) and hence favoured by evolution.

In the last few years there has been an intense research effort put to determine how grazing ruminant use cues related to food (i.e. visual cues or flavours) in locating and discriminating among plant differing in their nutritional quality (Scott and Provenza, 1998 and 2000; Van Tien et al., 1999; Ralphs and Provenza, 1999; Concha and Nicol, 2000; Howery et al., 2000; Dumont et al., 1998 and 2000). These studies revealed that both visual and flavour cues can be detected at a long distance and on a wide range of circumstances. The overall conclusion of the above studies (see also section 1.3.2.1) is that the development of behavioural mechanisms used to find food sources is of great value because it may enable grazing animals to predict the PIC of the available foods, before an amount of these foods can be ingested. When an animal encounters a food that is associated with positive PIC it is likely that it will increase its consumption. In the case of learned food aversions the outcome is the reverse.

An important issue is the persistence of learned food preferences and aversions in the absence of continuous reinforcement. For example the results of Chapter 3 indicate that sheep can remember learned associations even after a relatively long period of time (up to 5 weeks). There are different views about the length of time for which ruminants can retain such learned associations, and about whether or not their persistence over long periods of time is beneficial (Zahoric and Houpt, 1981; Ralphs, 1992; Provenza, 1995a; and 1996; Krebs and Inman, 1996; Kyriazakis et al., 1997 and 1998; Ralphs and Provenza, 1999; Duncan and Gordon, 1999). Under natural foraging the persistence of aversions to flavoured foods previously associated with negative PIC could be viewed as both beneficial and non-beneficial. The former would be seen as a protective mechanism for the grazing ruminant, enabling it to avoid toxic plants in future. Such long term CFAs have been demonstrated for relatively long periods of time without any intervening exposure to the toxic compound and its PIC (Kyriazakis, et al., 1997; Ralphs and Provenza, 1999). Conversely, the persistence of food aversions for long periods of time can be viewed as imposing limitations on the sampling behaviour of animals. Kyriazakis et al. (1998b) suggested that in nature CFAs should have a 'flexible' occurrence allowing the grazing animal to sample potentially toxic foods and thus to detect changes in their PIC. This learning hypothesis helps to explain how new food items are identified in the environment, and how animals monitor changes in the PIC of their food resource. A general principle guiding the inclusion of a novel food into the diet, is that no negative PIC follow from initial consumption. The fact that novel foods are potentially dangerous, leads to food neophobia which, however, disappears when repeated samplings of a novel food are not followed by negative PIC; novel foods become an accepted part of the diet. Furthermore, the suggestion that ruminants generalise over sensory cues seems to be another mechanism involved in discriminating between novel foods which enhances the applicability of learning considerably (see also Augner et al., 1998). It has been suggested that for the grazing ruminant that is being introduced to a new feeding environment, many foods are novel and can elicit a neophobic response. During this transitional introductory period, the adaptive value of the neophobic response must be weighed against the animal's need for variety in the diet (Ganskopp and Cruz, 1999). Ralphs and Provenza (1999) also suggested that food aversions may be difficult to maintain in new environments. They concluded that *"social facilitation is the most important factor preventing widespread application of aversive conditioning"*.

So far the majority of grazing studies have concentrated on the study of feeding behaviour as a function of animal morphology and plant structure, with very little reference to the potential importance of plant biochemical composition as a factor controlling intake rate (Illius and Hodgson, 1996; Illius and Gordon, 1999). Recently Duncan et al. (2000) demonstrated that exposure to diets containing particular secondary plant compounds can lead to increased rates of detoxification of secondary plants compounds by rumen microbes. If this is the case, then grazing ruminants would be expected to sample small amounts of novel, potentially toxic, plants but following continued ingestion the induction of detoxification mechanisms could enable them to increase their consumption of such plants without detrimental effects. It has been suggested that rumen microbial detoxification of poisonous plants might have been as important as rumen microbial degradation of cellulose for the evolutionary development and ecological expansion of ruminants as herbivores (Freeland and Janzen, 1974; Van Soest, 1994; Launhbaugh, 1996). Although, it is possible that such a mechanism could also account for the extinction of learned food aversions, and for the ability of free-grazing animals to cope with plant toxicity, it is clear from this section that further research is needed.

7.3.3 The problem of prediction: individual versus group diet selection

The idea of explaining and predicting diet selection, by accounting for the ability of individual animals to select an appropriate diet has been extensively used in designing and interpreting diet selection experiments (Emmans and Kyriazakis, 1995). In all the experiments described in this thesis (Chapters 2-5), a common factor was that diet selection and learning measurements were at the level of individual animals. It was found that diet selection different significantly between and within individual sheep of different groups. However, as mentioned also earlier these differences were diminished over time. For example, the conditioning methodology in the second line of research in this thesis (Chapters 3-5) allowed the greater part of variation in feeding responses between individuals to be diminished very quickly.

It was mentioned in the General Introduction, that a large body of literature suggests that substantial individual variability in food intake and diet selection exists in many experiments with ruminant animals (see section 1.3.6). It was also emphasised within that section that the issue of individual versus group diet selection remains far from being fully understood. This is important, given the recent emphasis in ecological studies of extrapolating upwards from individual-level phenomena to population responses (e.g. Goss-Custard et al., 1995a,b; Fahse et al., 1998; Wilson, 1998; Caldow et al., 1999; Ruel and Ayres, 1999).

The last part of this thesis (Chapter 6) was an attempt to deal with this issue. Of course, the phenomenon that population means do not match feeding behaviour of individuals is well known (see for example, Dove, 1935; Slater, 1981; Krebs and Inman, 1992, Caldow et al., 1999) and it should be made clear here that the model described in Chapter 6 was not intended to merely explore this obvious fact. The idea was to start with a minimum set of assumptions about the feeding responses of individuals (see Figure 6.1). Individuals were characterised by three parameters with biological relevance: a nutrient (protein) requirement (*R*), an ability to discriminate between foods of different protein contents (*D*) and a need to collect information about both foods (*S*). Each animal was assumed to select a diet that avoided both a deficiency and an excess of protein, where this was possible. Having established this set, we needed to use a model in order to see what the effect of variation in the individual characteristics would be on the predicted population outcomes. The main point was not whether population means reflect individual behaviour correctly, but whether population means could be 'generated' from assumptions about individuals, combined with further assumptions about the variation between the individuals in a population. All of the assumptions in the model were supposed to apply to all individuals. The model was completely deterministic at the level of the individual once it has been assigned values for the parameters *R, D* and *S* that are drawn from the defined distributions. At the population level variation in the values of the three 'constants' produced the more complex forms of response shown in the other Figures of Chapter 6. The latter is part of the crucial distinction between the proposed model and other similar approaches (e.g. Ruel and Ayres, 1999).

It was accepted in Chapter 6 (section 6.5.3) that the model in its present form has clear limitations. However, particular extensions of the model were suggested as the most appropriate way to modelling nutritional decisions, which at the same time would increase its biological relevance. In the following sections, a particular emphasis is given to the issues raised by this thesis which merit further investigation in this respect.

7.4 Issues raised and suggestions for future research

Two particular points have been especially highlighted in this thesis: the first was that sheep are able to develop learned associations between the flavour of a food and PIC induced by the administration of a nutrient source and that such learned associations can be influenced quantitatively by nutrient provision. The second was the fact that a simple model could be used to link the individual behaviour with population-level responses. Individuals were characterised in the model by their values of a minimum number of parameters, chosen for their biological relevance. By varying the values of these parameters it was possible to track the diet selection of individuals whereas by manipulating the variability among individuals in a given parameter an important link was made between individual and population-level. In my view these two points merit priority in further investigation. In the preceding sections I have provided suggestions for other fruitful lines of research that arise from the outcomes of the studies pursued. I will not elaborate any further on these.

7.4.1 The type of post-ingestive consequences

Both positive and negative PIC appear to be involved and to be equally important for the formation of learned associations towards food flavours associated with the administration of protein sources. The involvement of both the rumen and the post-ruminal digestive tract in the development of conditioned feeding responses has been demonstrated. However, the kind of PIC that are involved in the development of CFPs and CFAs by ruminants towards food flavours associated with the administration of nutrients is largely a matter of speculation. Future work could expand the current findings in a number of ways. For example, it could be interesting to investigate whether other DUP sources would lead to similar outcomes of learned feeding behaviour. This would help us to make more general statements about the importance of the 'rumen' versus that of the 'post-ruminal' digestive tract in the ability of ruminants to control their feeding behaviour. Research in this direction would also enable us to appreciate how a multitude of PIC will lead to the selection of an appropriate diet (section 7.2 from this Chapter). On this account another unresolved issue is whether and how differences in animal state affect the formation of CFPs and CFAs.

7.4.2 Modelling diet selection

It can be claimed that one understands a phenomenon only when one is able to make accurate predictions about it (Emmans and Kyriazakis, 2000). In Chapter 6 it was emphasised that the challenge now lies in how to extend the rather skeletal model in order to make it more realistic and hence more accurate in its predictions of feeding behaviour. Within section 6.5.3 three possible extensions were proposed in an attempt to establish what a 'realistic' model might include. These extensions have been put forward for improving the model, but have not been dealt specifically. For example, the assumption that the animal has a continuing need to update the information about the foods on offer, the sampling ratio (*S*), can be further elaborated by the incorporation of different sampling rules. Such elaboration seems a necessary precursor to introducing a learning component to the model which will 'allow' the individual to fix on one food type or a mixture after a run of sampling. It may be useful to divide up the process of sampling into two sampling ratios, one as a minimum (Sampling A) and another dependent on the differences between the nutrient content of the foods on offer (Sampling B) which could account for learning. It seems reasonable that such learning can be included when working on the proposed extensions of the model (Section 6.5.3), or introducing new ones. The latter view has evolved considerably as I have reviewed the findings of this thesis and I see modelling of diet selection as an important theme for future research.

Finally, I hope that the thesis will provide a significant resource for those interested in understanding and hence predicting feeding behaviour of farm animals.

Bibliography

Adolph, E.F. 1947. Urges to eat and drink in rats. *American Journal of physiology*, **151:** 110-125.

Agricultural and Food Research Council, AFRC. 1993. *Energy and Protein Requirements of Ruminants. An advisory manual prepared by the AFRC Technical Committee on Responses to Nutrients,* pp. 1-106. CAB International, Wallingford.

Armstrong, H.M., Gordon, I.J., Grant, S.A., Hutchings, N.J., Milne, J.A. and Sibbald, A.R. 1997a. A model of the grazing of hill vegetation by sheep in the UK. 1. The prediction of vegetation biomass. *Journal of Applied Ecology*, **34:** 166-185.

Armstrong, H.M., Gordon, I.J., Hutchings, N.J., Illius, A.W., Milne, J.A. and Sibbald, A.R. 1997b. A model of the grazing of hill vegetation by sheep in the UK. 2. The prediction of intake by sheep. *Journal of Applied Ecology*, **34:** 186-207.

Arnold, G.W. 1966. The special senses in grazing animals. III. Smell, taste, touch and dietary habits in sheep. *Australian Journal of Agricultural Research,* **17:** 531-542.

Arnold, S.J. 1981. The microevolution of feeding behavior. In *Foraging Behavior, Ecological, Ethological and Psychological Approaches* (ed. A.C. Kamil and T.D. Sargent), pp. 409-454. Garland STMP Press, New York.

Augner, M. Provenza, F.D. and Villalba, J.J. 1998. A rule of thumb in mammalian herbivores? *Animal Behaviour,* **56:** 337-345.

Baker, B.J. and Booth, D.A. 1989a. Preference conditioning by concurrent diets with delayed proportional reinforcement. *Physiology & Behavior,* **46:** 585-590.

Baker, B.J. and Booth, D.A. 1989b. Genuinely olfactory preferences conditioned by protein repletion. *Appetite,* **13:** 223-227.

Baker, B.J., Booth, D.A., Duggan, J.P. and Gibson, E.L. 1987. Protein appetite demonstrated-learned specificity of protein-cue preference to protein need in adult-rats. *Nutrition Research,* **7:** 481-487.

Bateson, M. and Kacelnik, A. 1995. Accuracy of memory for amount in the foraging starling, *Sturnus vulgaris*. *Animal Behaviour*, **50:** 431-443.

Bateson, M. and Kacelnik, A. 1997. Starlings' preferences for predictable and unpredictable delays to food. *Animal Behaviour*, **53:** 1129-1142.

Belovsky, G.E. 1990. How important are nutrient constraints in optimal foraging models or are spatial/temporal factors more important? In *Behavioural mechanisms of food selection* (ed. R.N. Hughes), pp. 255-278. NATO ASI series, Springer-Verlag, Berlin.

Berthoud, H.R. 2000. An overview of neural pathways involved in the control of food intake and selection. In *Neural and metabolic control of macronutrient intake* (ed. H.R. Berthoud and R.J. Seeley), pp. 361-388, CRC Press LLC, Boca Raton, Florida.

Blair-West, J.R., Denton, D.A., McKinley, M.J., Radden, B.G., Ramshaw, E.H. and Wark, J.D. 1992. Behavioural and tissue responses to severe phosphorus depletion in cattle. *American Journal of Physiology*, **263:** 656-663.

Booth, A. 1985. Food conditioned eating preferences and aversions with interceptive elements-conditioned appetites and satiates. *Annals of the New York Academy of Sciences*, **443:** 22-41.

Bradley, B.L. 1978. Animal flavor types and their specific uses in compound feeds by species and age. Proceeding of the First International Symposium on Palatability and Flavor Use in Animal Feeds, pp. 110-122. Zurich.

BrunBellut, J. 1997. Urea recycling in the rumen of dairy goats: Effects of physiological stage and composition of intake. *Small Ruminant Research*, **23:** 83-90.

Burritt, E.A. and Provenza, F.D. 1991. Ability of lambs to learn with a delay between food ingestion and consequences given meals containing novel and familiar foods. *Applied Animal Behaviour Science*, **32:** 179-189

Burritt, E.A. and Provenza, F.D. 1992. Lambs form preferences for non-nutritive flavors paired with glucose. *Journal of Animal Science*, **70:** 1133-1136.

Burritt, E.A. and Provenza, F.D. 1996. Amount of experience and prior illness affect the acquisition and persistence of conditioned food aversions in lambs. *Applied Animal Behaviour Science*, **48:** 73-80.

Caldow, R.W.G., Goss-Custard, J.D., Stillman, R.A., Durell, S.E.A.L.D., Swinfen, R. and Bregnballe, T. 1999. Individual variation in the competitive ability of interference-prone foragers: the relative importance of foraging efficiency and susceptibility to interference. *Journal of Animal Ecology*, **68:** 869-878.

Campbell, D.H., Capaldi, E.D. and Myers, D.E. 1987. Conditioned flavour preferences as a function of deprivation level: Preferences or aversions? *Animal Learning and Behavior*, **15:** 193-200.

Capaldi, E.D. 1992. Conditioned food preferences. In *The Psychology of Learning and Motivation.* Vol. 28. (ed. D. Medin), pp 1-31. New York Academic Press.

Capaldi, E.D., Campbell, D.H., Sheffer, J.D. and Bradford, J.P. 1987. Conditioned flavor preferences based on delayed caloric consequences. *Journal of Experimental Psychology, Animal Behavior Processes*, **13:** 150-155.

Capaldi, E.D., Sheffer, J. and Owens, J. 1991. Food-deprivation and conditioned flavour preferences based on sweetened and unsweetened foods. *Animal Learning and Behavior,* **19:** 361-368.

Capaldi, E.D., Hunter, M.J. and Lyn, S.A. 1997. Conditioning with taste as the CS in conditioned flavor preference learning. *Animal Learning and Behaviour* **25:** 427-436.

Chapple, R.S. and Lynch, J.J. 1986. Behavioural factors modifying acceptance of supplementary foods by sheep. *Research and Development in Agriculture,* **3:** 113-120.

Chowdhury, S.A., Ørskov, E.R., Hovell, D.B, Scaife, J.R. and Mollison, G. 1997. Protein utilisation during energy undernutrition in sheep sustained by intragastric infusion: effects of protein infusion level, with or without sub-maintenance amounts of energy from volatile fatty acids, on energy and protein metabolism. *British Journal of Nutrition,* **77:** 565-576.

Concha, M.A. and Nicol, A.M. 2000. Selection by sheep and goats for perennial ryegrass and white clover offered over a range of sward height contrasts. *Grass Forage Science,* **55:** 47-58.

Cook, N.E., Kane, E., Rogers, Q.R. and Morris, J.G. 1995. Self-selection of dietary casein and soy-protein by the cat. *Physiology & Behavior,* **34:** 583-594.

Cooper, S.D.B., Kyriazakis, I. and Oldham, J.D. 1994. The effect of late pregnancy on the diet selection made by ewes. *Livestock Production Science,* **40:** 263-275.

Cooper, S.D.B., Kyriazakis, I. and Nolan, J.V. 1995. Diet selection in sheep: The role of the rumen environment on the selection of a diet from two foods that differ in their nutrient density. *British Journal of Nutrition,* **74:** 39-54.

Cropper, M.R. 1987. Growth and development of sheep in relation to feeding strategy. PhD Thesis, University of Edinburgh.

Curnow, R.N. 1973. A smooth population response curve on an abrupt threshold and plateau model for individuals. *Biometrics,* **29:** 1-10.

Davidson, T.L. Morell, J.R. and Benoit, S.C. 2000. Memory and macronutrient regulation. In *Neural and metabolic control of macronutrient intake* (ed. H.R. Berthoud and R.J. Seeley), pp. 203-217, CRC Press LLC, Florida.

Day, J.E.L., Kyriazakis, I. and Rogers, P.J. 1998. Food choice and intake: towards a unifying framework of learning and feeding motivation. *Nutrition Research Reviews,* **11:** 25-43.

Denton, D.A. 1982. *The hunger for salt.* Springer-Verslag, Berlin.

Deutsch, J.A., Moore, B.O. and Heinrichs, S.C. 1989. Unlearned specific appetite for protein. *Physiology & Behavior,* **46:** 619-624.

DiBattista, D. and Holder, M.D. 1998. Enhanced preference for a protein-containing diet in response to dietary protein restriction. *Appetite,* **30:** 237-254.

DiBattista, D. and Mercier, S. 1999. Role of learning in the selection of dietary protein in the golden hamster (*Mesocricetus auratus*). *Behavioural Neuroscience,* **113:** 574-586.

Domjan, M. 1985. Cue-consequence specificity and long-delay learning revised. *Annals of the New York Academy of Sciences,* **443:** 54-66.

Dove, W.F. 1935. A study of individuality in the nutritive instincts and the causes and effects of variations in the selection of foods. *American Naturalist,* **69:** 469-544.

Dumont, B. and Petit, M. 1998. Spatial memory of sheep at pasture. *Applied Animal Behaviour Science,* **60:** 43-53.

Dumont, B., Dutronc, A. and Petit, M. 1998. How readily will sheep walk for a preferred forage? *Journal of Animal Science,* **76:** 965-971.

Dumont, B. Maillard, J.F. and Petit, M. 2000. The effect of the spatial distribution of plant species within the sward on the searching success of sheep when grazing. *Grass Forage Science,* **55:** 138-145.

Duncan, A.J. and Gordon, I.J. 1999. Habitat selection according to the ability of animals to eat, digest and detoxify foods. *Proceeding of the Nutrition Society,* **58:** 799-805.

Duncan, A.J., Frutos, P. and Young, S.A. 2000. The effect of rumen adaptation to oxalic acid on selection of oxalic-acid-rich plants by goats. *British Journal of Nutrition,* **83:** 59-65.

duToit, J.T., Provenza, F.D. and Nastis, A. 1991. Conditioned taste-aversions- how sick must a ruminant get before it learns about toxicity in foods. *Applied Animal Behaviour Science,* **30:** 35-46.

Early, D.M. and Provenza, F.D. 1998. Food flavor and nutritional characteristics alter dynamics of food preference in lambs. *Journal of Animal Science,* **76:** 728-734.

Edwards, G.R., Newman, J.A., Parsons, A.J. and Krebs, J.R. 1997. Use of cues by grazing animals to locate food patches: an example with sheep. *Applied Animal Behaviour Science,* **51:** 59-68.

Egan, J.K. and Doyle, P.T. 1985. Effects of intraruminal infusion of urea on the response in voluntary food intake by sheep. *Australian Journal of Agricultural Research,* **36:** 483-495.

Ellis, W.C., Poppi, D.P., Matis, J.H., Lippke, H., Hill, T.M. and Rouquette, F.M. 1999. Dietary-digestive-metabolic interactions determining the nutritive potential of ruminant diets. In *Nutritional ecology of herbivores* (ed. H.-J.G. Jung and G.C. Fahey), pp. 423-481. *American Society of Animal Science,* Savoy, Illinois.

Elston, D.A., Illius, A.W. and Gordon, I.J. 1996. Assessment of preference among a range of options using log ratio analysis. *Ecology,* **77:** 2538-2548.

Emmans, G.C. 1977. The nutrient intake of laying hens given a choice of diets in relation to their production requirements. *British Poultry Science,* **18**, 227-236.

Emmans, G.C. 1981. A model of the growth and feed intake of *ad libitum* fed animals, particular poultry. In *Computers in animal Production.* Occasional Publication (ed. G.M. Hillyer, C.T. Whittemore and R.G. Gunn), pp.103-105. British Society of Animal Production, Edinburgh.

Emmans, G.C. 1991. Diet selection by animals: theory and experimental design. *Proceedings of the Nutrition Society,* **50:** 59-64.

Emmans, G.C. 1997. A method to predict the food intake of domestic animals from birth to maturity as a function of time. *Journal of Theoretical Biology*, **186:** 189-199.

Emmans, G.C. and Fisher, C. 1986. Problems in nutritional Theory. In *Nutrient Requirements of Poultry and Nutritional Research* (ed. C. Fisher and K.N. Boorman), pp. 9-39. Butterworths, London.

Emmans, G.C. and Kyriazakis, I. 1995. The idea of optimisation in animals: Uses and dangers. *Livestock Production Science,* **44:** 189-197.

Emmans, G.C. and Kyriazakis, I. 2000. Consequences of genetic change in farm animals on food intake and feeding behaviour. *Proceeding of the Nutrition Society* (in press).

Fahse, L. Wissel, C. and Grimm, V. 1998. Reconciling classical and individual-based approaches in theoretical population ecology: a protocol for extracting population parameters from individual-based models. *American Naturalist,* **152:** 838-852.

Farnsworth, K.D. and Illius, A.W. 1998. Optimal diet choice for large herbivores: an extended contingency model. *Functional Ecology*, **12:** 74-81.

Faverdin, P. 1999. The effect of nutrients on feed intake in ruminants. *Proceedings of the Nutrition Society,* **58:** 523-531.

Faverdin, P. and Bareille, N. 1999. Lipostatic regulation of feed intake in ruminants. In *Regulations of feed intake* (ed. D. Van der Heide, E.A. Huisman, E. Kanis, J.W.M. Osse and M.W.A. Vestegen), pp. 89-102. CABI, Publishing.

Ferguson, N.S., Gous, R.M. and Emmans, G.C. 1994. Preferred components for the construction of a new simulation model of growth, feed intake and nutrient requirements of growing pig. *South African Journal of Animal Science,* **24:** 10-17.

Ferguson, N.S., Gous, R.M. and Emmans, G.C. 1997. Predicting the effects of animal variation on growth and food intake in growing pigs using simulation modelling. *Animal Science,* **64:** 513-522.

Fisher, C. and Morris, T.R. 1970. The determination of the methionine requirements of laying pullets by a diet dilution technique. *British Poultry Science,* **11:** 67-82.

Fisher, C., Morris, T.R. and Jennings, R.C. 1973. A model for the description and prediction of the response of laying hens to amino acid intake. *British Poultry Science,* **14:** 469-484.

Firkins, J.L., Weiss, W.P. and Piwonka, E.J. 1992. Quantification of intraruminal recycling of microbial nitrogen using nitrogen-15. *Journal of Animal Science,* **70:** 3223-3233.

Forbes, J.M. 1995. Voluntary Food Intake and Diet Selection in Farm Animals. CAB International, Wallingford, Oxon.

Forbes, J.M. 1996. Integration of regulatory signals controlling forage intake in ruminants. *Journal of Animal Science,* **74:** 3029-3035.

Forbes, J.M. 1998. Dietary awareness *Applied Animal Behaviour Science,* **57:** 287-297.

Forbes, J.M. 1999. Natural feeding behaviour and feed selection. In *Regulations of feed intake* (ed. D. Van der Heide, E.A. Huisman, E. Kanis, J.W.M. Osse, and M.W.A. Vestegen), pp. 3-12. CABI, Publishing.

Forbes, J. M. and Kyriazakis, I. 1995. Food preferences in farm animals: why don't they always choose wisely. *Proceedings of the Nutrition Society,* **54:** 429-440.

Forbes, J.M. and Shariatmadari, F. 1996. Short-term effects of food protein content on subsequent diet selection by chickens and the consequences of alternate feeding of high- and low-protein foods. *British Poultry Science,* **37:** 597-607.

Freeland, W.J. and Janzen, D.H. 1974. Strategies in herbivory by mammals: the role of plant secondary compound. *American Naturalist,* **108:** 269-289.

Freer, M., Moore, A.D. and Donnelly, J.R. 1997. GRAZPLAN: Decision support systems for Australian grazing enterprises. 2. The animal biology model for feed intake, production and reproduction and the GrazFeed DSS. *Agricultural Systems,* **54:** 77-126.

Froetschel, M.A. 1996. Bio-active peptides in digesta that regulate gastrointestinal function and intake. *Journal of Animal Science,* **74:** 2500-2508.

Fromentin, G., Feurte, S. and Nicolaidis, S. 1998. Spatial cues are relevant for learned preference/aversion shifts due to amino-acid deficiencies. *Appetite,* **30:** 223-234.

Galef, B.G. 1991. A contrarian view of the wisdom of the body as it related to dietary self-selection. *Psychological Reviews,* **98:** 218-223.

Galef, B.G. 1996. Social Enhancement of Food Preferences in Norway Rats: A brief review. In *Social learning in animals: The root of culture* (ed. C.M. Heyes and B.F. Galef), pp. 49-64. Academic Press, New York.

Galef, B.G. 2000. Is there a specific appetite for protein? In *Neural and metabolic control of macronutrient intake* (ed. H.R. Berthoud and R.J. Seeley), pp. 19-28, CRC Press LLC, Florida.

Galef, B.G. and Stein, M. 1985. Demonstrator influence on observer diet preference: Analysis of critical social interactions and olfactory signals. *Animal Learning and Behavior,* **13:** 31-38.

Ganskopp, D. and Cruz, R. 1999. Selective differences between naive and experienced cattle foraging among eight grasses. *Applied Animal Behaviour Science,* **62:** 293-303).

Garcia, J. 1989. Food for Tolman: cognition and cathexis in concept. In *Aversion avoidance and anxiety* (ed. T. Nilson and L. Archer), pp. 45-85. Lawrence Erlbaum and Associates, Hillsdale, New Jersey.

Garcia, J. and Koelling, R. A. 1966. Relation of cue to consequence in avoidance learning. *Psychonomic Science,* **4:** 123-124.

Garcia, J., Ervin, F.R. and Koelling, R.A. 1966. Learning with prolong delay of reinforcement. *Psychonomic Science,* **5:** 121-122.

Garcia, J. and Hankins, W.G. 1977. On the origin of food aversion paradigms. In *Learning Mechanism In Food Selection* (ed. L.M. Barker, M.R. Best and M. Domjan), pp. 3-23. Baylor University Press Waco, Texas.

Garcia, J., Hankins, W.G. and Rusiniak, K.W. 1974. Behavioral regulation of the milieu interne in man and rat. *Science*, **185:** 824-831.

Garcia, J., Lasiter, P.A., Bermudez-Rattoni, F. and Deems, D.A. 1985. A general theory of aversion learning. In *Experimental assessments and clinical applications of conditioned food aversion.* (ed. N.S. Braveman and P. Bonstein), pp. 8-21. New York Academy of Science, New York.

Gibson, E.L. and Booth, D.A. 1986. Acquired protein appetite in rats. Dependence on a protein-specific state. *Experientia,* **42:** 1003-1004.

Gietzen, D.W., McAthur, L.H., Theisen, J.C. and Rogers, Q.R. 1992. Learned preference for the limiting amino acid in rats fed a threonine-deficient diet. *Physiology & Behavior,* **51:** 909-914.

Glasbey, C.A., Abdalla, I. and Simm, G. 1996. Towards automatic interpretation of sheep ultrasound scans. *Animal Science,* **62:** 309-315.

Gordon, I.J., Illius, A.W. and Milne, J.D. 1996. Sources of variation in the foraging efficiency of grazing ruminants. *Functional Ecology,* **10:** 219-226.

Goss-Custard, J.D. 1981. Feeding Behavior of Redshank, *Tringa totanus* and Optimal Foraging Theory. In *Foraging Behaviour, Ecological, Ethological and Psychological Approaches* (ed. A.C. Kamil and T.D. Sargent), pp. 115-133. Garland STMP Press, New York.

Goss-Custard, J.D., Caldow, R.W.G., Clarke, R.T. and West, A.D. 1995a. Deriving population parameters from individual variation in foraging behaviour. 1. Empirical game-theory distribution model of oystecatchers *Haematopus ostralegus* feeding on mussels mytilus-edulis. *Journal of Animal Ecology,* **64:** 265-276.

Goss-Custard, J.D., Caldow, R.W.G., Clarke, R.T., Durell, S.E.A.L.D. and Sutherland, W.J. 1995b. Deriving population parameters from individual variation in foraging behaviour. 2. Model tests and population parameters. *Journal of Animal Ecology,* **64:** 277-289.

Grovum, W.L. 1987. A new look at what is controlling intake. In *Feed intake by beef cattle* (ed. F.N. Owens), pp. 1-40. Oklahoma State University, Stillwater.

Hall, G. 1994. Pavlovian Conditioning: Laws of association. In *Animal learning and cognition* (ed. N.J. Mackintosh), pp. 15-40. Academic press, New York.

Harris, L., Clay, J. Hargreaves, F. and Ward, A. 1933. Appetite and choice of diet. The ability of Vitamin B deficient rats to discriminate between diets containing and lacking the vitamin. *Proceedings of the Royal Society (Section B),* **133:** 161-190.

Heyes, C.M. 1994. Social learning in animals: Categories and mechanisms. *Biological Reviews,* **69:** 207-231.

Hills, J., Kyriazakis, I., Nolan J.V., Hinch, G.N. and Lynch, J.J. 1999. Conditioned feeding responses in sheep to flavoured feeds associated with sulphur doses. *Animal Science,* **69:** 313-325.

Horgan, G.W. and Sword, A.M. 1995. *Statistical Methods for Repeated Measures Data.* Edinburgh: Bio-mathematics and Statistics Scotland, University of Edinburgh.

Howard, J.J., Raubenheimer, D. and Bernays, E.A. 1994. Population and individual polyphagy in the grasshopper *Taenipoda eques* during natural foraging. *Entomologia Experimentalis et Applicata*, **71:** 167-176.

Howery, L.D., Provenza, F.D., Banner, R.E. and Scott, C.B. 1996. Differences in home range and habitat use among individuals in a cattle herd. *Applied Animal Behaviour Science*, **49:** 305-320.

Howery, L.D., Bailey, D.W., Ruyle, G.B. and Renken, W.J. 2000. Cattle use visual cues to track food locations. *Applied Animal Behaviour Science*, **67:** 1-14.

Huber, J.T. and Herrera-Saldana, R. 1994. Synchrony of protein and energy supply to enhance fermentation. In *Principles of protein nutrition in ruminants*. (ed. J.M. Asplund) pp. 113-126. CRC Press, London.

Hughes, B.O. 1979. Appetites for specific nutrients. In *Food intake regulation in poultry* (ed. K.N. Boorman and B.M. Freeman), pp. 141-169. Proceedings of the fourteenth Poultry Science Symposium, British Poultry Science Ltd, Edinburgh.

Hughes, J.A. and Wood-Gush, D.G.M. 1971. A specific appetite for calcium in domestic chickens. *Animal Behaviour* **19:** 490-499.

Hume, I. D., Moir, R.J. and Somers, M. 1970. Synthesis of microbial protein in the rumen. I. Influence of the level of nitrogen intake. *Australian Journal of Agricultural Research*, **21:** 283-296.

Hunter, R.A. and Siebert, B.D. 1987. The effect of supplements of rumen-degradable protein and formaldehyde-treated casein on the intake of low-nitrogen roughage's by *Bos taurus* and *Bos indicus* steers at different stages of maturity. *Australian Journal of Agricultural Research*, **38:** 208-218.

Hutchings, M.R., Kyriazakis, I., Gordon, I.J. and Jackson, F. 1999. Trade-offs between nutrient intake and faecal avoidance in herbivore foraging decisions: the effect of animal parasitic status, level of feeding motivation and sward nitrogen content. *Journal of Animal Ecology*, **68:** 310-323.

Illius, A.W. and Gordon, I. 1993. Diet selection in mammalian herbivores, constraints and tactics. In *Diet selection, an interdisciplinary approach* (ed. R.N. Hughes), pp. 157-181. Blackwell Scientific Publications, Oxford.

Illius, A.W. and Hodgson, J. 1996. Progress in understanding the ecology and management of grazing systems. In *The ecology and management of grazing systems* (ed. A.W. Illius and J. Hodgson), pp. 429-457. CAB International, Wallingford, Oxon.

Illius, A.W. and Jessop, N.S. 1996. Metabolic constraints on voluntary intake in ruminants.

Journal of Animal Science, **74:** 3052-3062.

Illius, A.W. and Gordon, I.J. 1999. The physiological ecology of mammalian herbibory. In *Nutritional ecology of herbivores* (ed. H.-J.G. Jung and G.C. Fahey), pp. 71-96. *American Society of Animal Science,* Savoy, Illinois.

Illius, A.W., Clark, D.A. and Hodgson, J. 1992. Discrimination and patch choice by sheep grazing grass-clover swards. *Journal of Animal Ecology,* **61:** 183-194

Illius, A.W., Albon, S.D., Pemberton, J.M., Gordon, I.J. and Clutton-Brock, T.H. 1995. Selection for foraging efficiency during a population crash in Soay sheep. *Journal of Animal Ecology*, **64:** 481-492.

Ingrand, S., Agabriel, J., Lassalas, J. and Dedieu, B. 1999. How group feeding influences intake level of hay and feeding behaviour of beef cows. *Annales De Zootechnie,* **48:** 435-445.

Kacelnik, A. and Abreu, F.B. 1998. Risky choice and Weber's law. *Journal of Theoretical Biology,* **194:** 289-298.

Kacelnik, A. and Bateson, M. 1996. Risky theories - The effects of variance on foraging decisions. *American Zoologist,* **36:** 402-434.

Kalat, J.W. and Rozin, P. 1973. "Learned safety" as a mechanism in long-delay taste-aversion learning in rats. *Journal of Comparative Physiology and Psychology,* **83:** 198-207.

Kendrick, K.M. 1994. Cognition. In *Farm animals and the environment* (ed. C. Phillips and D. Piggins), pp. 209-231. CAB International, Wallingford, Oxon.

Ketelaars, J.J.M.H. and Tolkamp, B.J. 1991. Toward a new theory of feed intake regulation in ruminants. PhD Thesis, pp. 119-144. Wageningen Agricultural University, Holland.

Keverne, E.B. 1995. Olfactory learning. *Current opinion in Neurobiology,* **5:** 482-488.

Kirk, J.M. and Logue, A.W. 1997. Effects of deprivation level on humans' self-control for food reinforcers. *Appetite*, **28:** 215-226.

Krebs, J.R. and Inman, A.J. 1992. Learning and foraging: Individuals, groups and populations. *American Naturalist,* **140:** S63-S89.

Krebs, J.R., Huston, A.I. and Charnov, E.L. 1981. Some Recent developments in Optimal Foraging. In *Foraging Behaviour, Ecological, Ethological and Psychological Approaches* (ed. A.C. Kamil and T.D. Sargent), pp. 3-18. Garland STMP Press, New York.

Kronberg, S.L., Muntifering, R.B. and Ayers, E.L. 1993. Feed aversion learning in cattle with delayed negative consequences. *Journal of Animal Science,* **71:** 1767-1770.

Kronberg, S.L. and Malechek, J.C. 1997. Relationship between nutrition and foraging behaviour of free-ranging sheep and goats. *Journal of Animal Science*, **75:** 1759-1763.

Kutlu, H.R. and Forbes, J.M. 1993. Self-selection of ascorbic acid in coloured foods by heat-stressed broiler chicks. *Physiology & Behavior*, **53:** 103-110.

Kyriazakis, I. 1997. The nutritional choices of farm animals: to eat or what to eat? In *Animals choices*, Occasional Publication (ed. J.M. Forbes, T.L.J. Lawrence, R.G. Rodway and M.A. Varley), pp. 55-65. British Society of Animal Science, Edinburgh.

Kyriazakis, I. and Emmans, G.C. 1991. Diet selection in pigs: Dietary choices made by growing pigs following a period of underfeeding with protein. *Animal Production*, **52:** 337-346.

Kyriazakis, I. and Emmans G.C. 1992a. The growth of mammals following a period of nutritional limitation. *Journal of Theoretical Biology*, **156:** 485-498.

Kyriazakis, I. and Emmans, G.C. 1992b. Selection of a diet by growing pigs given choices between food differing in contents of protein and rapeseed meal. *Appetite*, **19:** 121-132.

Kyriazakis, I. and Emmans, G.C. 1993. The effect of protein-source on the diets selected by pigs given a choice between a low and a high protein food. *Physiology & Behavior* **53:** 683-688.

Kyriazakis, I. and Oldham, J.D. 1993. Diet selection in sheep: the ability of growing lambs to select a diet that meets their crude protein (nitrogen x 6.25) requirements. *British Journal of Nutrition*, **69:** 617-629.

Kyriazakis, I. and Oldham, J.D. 1997. Food intake and diet selection in sheep - the effect of manipulating the rates of digestion of carbohydrates and protein of the foods offered as a choice. *British Journal of Nutrition*, **77:** 243-254

Kyriazakis, I. and Day, J.E.L. 1998. Does the study of feeding behaviour benefit from a teleonomic framework? *Nutrition Research Reviews*, **11:** 223-229.

Kyriazakis, I., Emmans, G.C. and Whittemore, C.T. 1990. Diet selection in pigs: choices made by growing pigs given foods of different protein concentrations. *Animal Production*, **51:** 189-199.

Kyriazakis, I., Emmans G.C. and Whittemore, C.T. 1991. The ability of pigs to control their protein intake when fed in three different ways. *Physiology & Behavior*, **50:** 1197-1203.

Kyriazakis, I., Oldham, J.D., Coop R.L. and Jackson, F. 1994. The effect of subclinical intestinal nematode infection on the diet selection of growing sheep. *British Journal of Nutrition,* **72:** 665-677.

Kyriazakis, I., Papachristou, T.G., Duncan, A.J. and Gordon, I.J. 1997. Mild conditioned food aversions developed by sheep towards flavours associated with plant secondary compounds. *Journal of Chemical Ecology,* **23:** 727-745.

Kyriazakis, I., Tolkamp, B.J. and Hutchings, M.R. 1998a. Towards a functional explanation for the occurrence of anorexia during parasitic infections. *Animal Behaviour,* **56:** 265-274.

Kyriazakis, I., Anderson, D.H. and Duncan, A.J. 1998b. Conditioned flavour aversions in sheep: the relationship between the dose rate of a secondary plant compound and the acquisition and persistence of aversions. *British Journal of Nutrition,* **79:** 55-62.

Kyriazakis, I., Tolkamp, B.J. and Emmans, G.C. 1999. Diet selection and animal state: an integrative framework. *Proceedings of the Nutrition Society,* **58:** 765-772.

Laca, E.A. 1998. Spatial memory and food searching mechanisms of cattle. *Journal of Range management,* **51:** 370-378.

Lat, J. 1967. Self-selection of dietary components. In *Handbook of physiology, I: Alimentary canal* (ed. F. Code), pp. 367-386. American Physiological Society, Washington, DC.

Launchbaugh, K.L. 1996. Biochemical aspects of grazing behaviour. In *The ecology and management of grazing systems* (ed. Illius, A.W. and Hodgson, J.), pp. 159-184. CAB International, Wallingford, Oxon.

Launchbaugh, K.L. and Provenza, F.D. 1994. The effect of flavor concentration and toxin dose on the formation and generalisation of flavor aversions in lambs. *Journal of Animal Science,* **72:** 10-13.

Launchbaugh, K.L. Provenza, F.D. and Burritt, E.A. 1993. How herbivores tract variable environments: response to variability of phytotoxins. *Journal of Chemical Ecology,* **19:** 1047-1056.

Lawes Agricultural Trust 1993. GENSTAT 5 release 3.2 reference manual second edition. Clarendon Press, Oxford.

Lawrence, A.B. and Illius, A.W. 1997. Measuring preferences and the problems of identifying proximate needs. *Animal Choices*, Occasional Publication No 20 (ed. J.M. Forbes, T.L.G. Lawrence, R.G. Rodway and M.A. Varley), pp. 19-26. British Society of Animal Science, Edinburgh.

Lawson, R.E., Redfern, E.J. and Forbes, J.M. 2000. Choices by lactating cows between concentrates high or low in digestible undegraded protein. *Animal Science,* **70:** 515- 525.

Le Magnen, J. 1985. *Hunger - physiological aspects*. pp. 113-121. Cambridge University press, London.

Lemon, W.C. 1991. Fitness consequences of foraging behaviour in the zebra finch. *Nature,* **352:** 153-155.

Leng, R. A. 1993. Quantitative ruminant nutrition - A green science. *Australian Journal of Agricultural Research,* **44:** 363-80.

Leng, R.A. and Nolan, J.V. 1984. Nitrogen metabolism in the rumen. *Journal of Animal Science,* **67:** 1072-1089.

Leshem, M. 1999. The ontogeny of salt hunger in the rat. *Neuroscience and Biobehavioral Reviews,* **23:** 649-659.

Leshem, M., DelCanho, S. and Schulkin, J. 1999. Calcium hunger in the parathyroidectomized rat is specific. *Physiology & Behavior,* **67:** 555-559.

Lewis, D. and Hill, K.L. 1983. The provision of nutrients. In *Nutritional physiology of farm animals* (ed. J.A.F. Rook. and P.C. Thomas), pp. 3-40. Longman, Harlow.

Lindberg, M.A., Beggs, A.L., Chezik D.D. and Ray, D. 1982. Flavor-toxicosis associations: tests of three hypotheses of long delay learning. *Physiology & Behavior,* **29:** 439-442.

Liu, S.M., Lobley, G.E., Macleod, N.A., Kyle, D.J., Chen, X.B. and Ørskov, E.R. 1995. Effects of long-term protein excess or deficiency on whole-body protein turnover in sheep nourished by intragastric infusion of nutrients. *British Journal of Nutrition,* **73:** 829-839.

Logue, A.W. 1979. Taste aversion and the generality of the laws of learning. *Psychological Bulletin,* **86:** 276-296.

Lomas, C.A., Piggins, D. and Phillips, C.J.C. 1998. Visual awareness. *Applied Animal Behaviour Science,* **57:** 247-257.

Mackintosh, N.J. 1973. Stimulus selection: Learning to ignore stimuli that predict no change in reinforcement. In *Constraints on Learning, limitations and predispositions* (ed. R.A. Hinde and J. Stevenson-Hinde), pp. 75-101. Academic press, New York.

Mackintosh, N.J. 1994. *Animal learning and cognition* (ed. N.J. Mackintosh), pp. 1-13. Academic press, New York.

Martin, G.M. Bellingham, W.P. and Storlien, L.H. 1977. Effects of varied colour experience on chickens' formation of colour and texture aversions. *Physiology & Behavior,*

8: 415-420.

Matthews L.R. and Kilgour, R. 1980. Learning and associated factors in ruminant feeding behaviour. In *Digestive physiology and metabolism in ruminants* (ed. Y. Ruckebusch and P. Thivend), pp. 123-144. MTP Press Limited, Lancaster.

McBride, B., Berthiaume, R. and Lapierre, H. 1998. Nutrient flow in the lactating cow. *Canadian Journal of Animal Science,* **78:** 91-104.

McDonald, P., Edwards, R.A. and Greenhalgh, J.F.D. 1988. *Animal Nutrition.* 4th edition. Longman Scientific & Technical, New York.

Meissner, H.H., Smuts, M., VanNiekerk, W.A. and Acheampong-Boatend, O. 1993. Rumen ammonia concentrations, and non-ammonia nitrogen passage to and apparent absorption from the small intestine of sheep ingesting subtropical, temperate, and tannin-containing forages. *South African Journal of Animal Science,* **23:** 92-97.

Merchen, N.R. and Titgemeyer, E.C. 1992. Manipulation of amino acid supply to the growing ruminant. *Journal of Animal Science,* **70:** 3238-3247.

Minitab Inc. 1996. State College, 3081 Enterprise Drive, PA 16801-3008, USA.

Myers, K.P. and Hall, W.G. 1998. Evidence that oral and nutrient reinforcers differentially condition appetitive and consumatory responses to flavors. *Physiology & Behavior* **64:** 493-500.

Myers, K.P. and Hall, W.G. 2000. Conditioned changes in appetitive and consummatory responses to flavors paired with oral or nutrient reinforcement among adult rats. Physiology & Behavior, **68:** 603-610.

National Research Council, NRC. 1998. *Nutrient Requirements of Swine. Tenth Revised Edition,* pp. 33-45. National Academy Press, Washington.

Nicholson, J.W.G., Charmley, E. and Bush, R.S. 1992. The effect of supplemental protein source on ammonia levels in rumen fluid and blood and intake of alfalfa silage by beef cattle. *Canadian Journal of Animal Science,* **72:** 853-862.

Nicol, C.J. 1995. The social transmission of information and behaviour. *Applied Animal Behaviour Science,* **44:** 78-97.

Nielsen, B.L. 1999. On the interpretation of feeding behaviour measures and the use of feeding rate as an indicator of social constraint. *Applied Animal Behaviour Science,* **63:** 79-91.

Nolte, D.L. and Provenza, D. 1992. Food preferences in lambs after exposure to flavours in solid foods. *Applied Animal Behaviour Science,* **32:** 337-347.

Nolte, D.L., Provenza, D., Callan, R. and Panter, K.E. 1992. Garlic in the ovine fetal environment. *Physiology & Behavior*, **52:** 1091-1093.

Oddy, V.H., Edwards, S.R., Warren, H.M., Speck, P.A., Nicholls, P.J. and Neutze, S.A. 1997. Interrelationships between amino acid and glucose metabolism in lambs of different dietary history supplemented with rumen escape protein. *Journal of Agricultural Science*, **128:** 105-116.

Oldham, J.D. 1993. Recent progress towards matching feed quality to the amino-acid needs of ruminants. *Animal Feed Science and Technology*, **45:** 19-34.

Oldham, J.D., Emmans, G.C. and Kyriazakis, I. 1997. Limits and limitations to nitrogen use in farm animals. *Proceeding of the Nutrition Society*, **56:** 525-534.

Olsen, J.D., Ralphs, M.H. and Lane, M.A. 1989. Aversion to eating poisonous larkspur plants induced in cattle by intraruminal infusion with lithium chloride. *Journal of Animal Science*, **67:** 1980-1985.

Orians, G.H. 1981. Foraging behaviour and the evolution of discriminatory abilities. In *Foraging Behaviour, Ecological, Ethological and Psychological Approaches* (ed. A.C. Kamil and T.D. Sargent), pp. 409-454. Garland STMP Press, New York.

Ørskov, E.R. 1988. *Protein nutrition in ruminants*. Second edition, pp. 43-91. Academic Press Ltd. London.

Overmann, S.R. 1976. Dietary self-selection by animals. *Physiological Bulletin*, **83:** 218-235.

Owen, J.B. 1990. Weight control and appetite: nature over nurture. *Animals Breeding Abstracts*, **58:** 583-591.

Parks, J.R. 1970. Growth curves and the physiology of growth. I. Animals. *American Journal of Physiology*, **219:** 833-836.

Parsons, A.J., Thornley H.M., Newman, J. and Penning, P.D. 1994. A mechanistic model of some physical determinants of intake rate and diet selection in a two-species temperate grassland sward. *Functional ecology*, **8:** 187-204.

Pavlov, I.P. 1927. *Conditioned Reflexes; An investigation of the physiological activity of the cerebral cortex* (ed. G.V. Anrep). London: Oxford University Press.

Perez, C., Lucas, F. and Sclafani, A. 1995. Carbohydrate, fat, and protein condition similar flavour preferences in rats using an oral-delay procedure. *Physiology & Behavior*, **57:** 549-554.

Perez, C., Ackroff, K. and Sclafani, A. 1996. Carbohydrate-and protein-conditioned flavor preferences: Effects of nutrient preloads. *Physiology & Behavior* **59:** 467-474.

Perry, G. and Pianka, E.R. 1997. Animal Foraging: past, present and future. *American Naturalist,* **12:** 360-363.

Piggins, D. and Phillips, C.J.C. 1998. Awareness in domesticated animals - concepts and definitions. *Applied Animal Behaviour Science,* **57:** 181-200.

Provenza, F.D. 1994. Ontogeny and social learning transmission of food selection in domesticated ruminants. In *Behaviour aspects of feeding* (ed. B.G. Galef, J.R.G. Mainardi and P. Valsecchi), pp. 147-164. Harwood Academic Publishers, Reading.

Provenza, F.D. 1995a. Tracking variable environments. There is more than one kind of memory. *Journal of Chemical Ecology,* **21:** 911-923.

Provenza, F.D. 1995b. Role of learning in food preferences of ruminants: Greenhalgh and Reid revisited. *Ruminant Physiology: Digestion, metabolism, growth and reproduction*, (ed. W.V. Engelhardt, S. Leonhard-Marek, G. Breves and D. Giesecke), pp. 233-247. *Proceedings of the VIII international symposium on ruminant physiology*. Stuttgart, Germany.

Provenza, F.D. 1995c. Postingestive feedback as an elementary determinant of food preference and intake in ruminants. *Journal of Range Management,* **48:** 2-17.

Provenza, F.D. 1996. Acquired aversions as the basis for varied diets of ruminants foraging on rangelands. *Journal of Animal Science,* **74:** 2010-2020.

Provenza, F.D. and Balph, D.F. 1987. Diet learning by domestic ruminants: Theory, evidence and practical implications. *Applied Animal Behaviour Science,* **18:** 211-232.

Provenza, F.D. and Balph, D.F. 1990. Applicability of five diet-selection models to various foraging challenges ruminants encounter. In *Behavioural mechanisms of food selection* (ed. Hughes, R.N.), pp 423-459. NATO ASI Series G: Ecological Sciences, 20. Springer-Verslag, Heidelberg, Berlin.

Provenza, F.D. and Cincotta, P. 1993. Foraging as a self-organizational learning process: Accepting adaptability at expense of predictability. In *Diet selection, an interdisciplinary approach* (ed. R.N. Hughes), pp. 78-110. Blackwell Scientific Publications, Oxford.

Provenza, F.D., Burritt, E.A. Clausen, T.P., Bryant, J.P., Reichardt, P.B. and Distel R.A. 1990. Conditioned flavor aversion – a mechanism for goats to avoid condensed tannins in blackbrush. *American Naturalist,* **136:** 810-828.

Provenza, F.D., Lynch, J.J., Burritt, E.A. and Scott, C.D. 1994a. How goats learn to distinguish between novel foods that differ in postingestive consequences. *Journal of Chemical Ecology*, **20:** 609-624.

Provenza, F.D., Ortega-Reyes, L., Scott, C.B., Lynch, J.J. and Burritt, E.A. 1994b. Antiemetic drugs attenuate food aversions in sheep. *Journal of Animal Science*, **72:** 1989-1994.

Ralphs, M.H. 1992. Continued food aversion – training livestock to avoid eating poisonous plants. *Journal of Range Management*. **45:** 46-51.

Ralphs, M.H. and Cheney, C.D. 1993. Influence of cattle age, lithium-chloride dose level, and food type in the retention of food aversions. *Journal of Animal Science*, **71:** 373-379.

Ralphs, M.H. and Provenza, F.D. 1999. Conditioned food aversions: principles and practices, with special reference to social facilitation. *Proceeding of the Nutrition Society*, **58:** 813-820.

Ralphs, M.H., Provenza, F.D., Wiedmeier, R.D. and Bunderson, F.B. 1995. Effects of energy source and food flavor on conditioned preferences in sheep. *Journal of Animal Science*, **73:** 1651-1657.

Ramirez, I. 1996 Stimulus specificity in flavor acceptance learning. *Physiology & Behavior* **60:** 595-610.

Raubenheimer, D. and Simpson, S.J. 1997. Integrative models of nutrient balancing: application to insects and vertebrates. *Nutrition Research Reviews*, **10:** 151-179.

Raubenheimer, D. and Simpson, S.J. 1999. Integrating nutrition: a geometrical approach. *Entomologia Experimentalis et Applicata*, **91:** 67-82.

Rescorla, R. A. and Wagner, A. R. 1972. A theory of Pavlovian conditioning: Variations in the effectiveness of reinforcement and non-reinforcement. In *Classical conditioning II: Current research and theory* (ed. A.H. Black and W.F. Prokasy), pp. 64-99. Appleton-Century-Crofts, New York.

Revusky, S. H. 1977. Learning as a general process with an emphasis on data from feeding experiments. In *Food aversion learning* (ed. N.W. Milgram, L. Krames and T.M. Alloway), pp. 1-5. Academic Press, New York.

Richter, C.P. 1943. Total self-regulatory functions in animals and human beings. *Harvey Lecture Series*, **38:** 63-103.

Richter, C.P., Holt, L.E. and Barelare, B. 1938. Nutritional requirements for normal growth and reproduction in rats studied by the self-selection method. *American Journal of Physiology*, **122:** 734-744.

Rooke, J.A., Lee, N.H. and Armstrong, D.G. 1987. The effects of intraruminal infusion of urea, casein, glucose syrup and a mixture of casein and glucose syrup on nitrogen digestion in the rumen of cattle receiving grass silage diets. *British Journal of Nutrition*, **57:** 89-98.

Rozin, P. 1966. Specific hunger for thiamine: recovery from deficiency and thiamine preference. *Journal of Comparative and Physiological Psychology*, **59:** 98-101.

Rozin, P. 1976. The selection of foods by rats, humans, and other animals. In *Advances in the study of behavior* (ed. J.S. Rosenblatt, R.A. Hinde, E. Shaw and C. Beer), Vol. 6, pp. 21-76. Academic Press, New York.

Rozin, P. 1977. The significance of learning mechanisms in food selection: some biology, psychology and sociology of science. In *Learning mechanism in food selection* (ed. L.M. Barker, M.R. Best and M. Domjan), pp. 557-581. Baylor University Press Waco, Texas.

Rozin, P. and Zellner, D. 1985. The role of Pavlovian conditioning in the acquisition of food likes and dislikes. *Annals of the New York Academy of Science*, **443:** 189- 202.

Rozin, P.N. and Schulkin, J. 1990. Food selection. In *Handbook of Behavioral Neurobiology: Neurobiology of food and fluid intake* (ed. E.M. Stricker), pp. 297-328. Plenum Press, New York.

Ruel, J.J. and Ayres, M.P. 1999. Jensen's inequality predicts effects of environmental variation. *Trends in Ecology & Evolution*, **14:** 361-366.

Russell, J.B., Sniffen, C.J. and Van Soest, P.J. 1982. Effect of Carbohydrate limitation on degradation and utilisation of casein by mixed rumen bacteria. *Journal of Dairy Science*, **66:** 763-775.

Sarraseca, A., Milne, E., Metcalf, M.J. and Lobley, G.E. 1998. Urea recycling in sheep: effects of intake. *British Journal of Nutrition*, **79:** 79-88.

Schulkin, J. 1991. *Sodium hunger: The search for a salty taste*. Cambridge: Cambridge University Press.

Sclafani, A. 1995. How food preferences are learned: laboratory animal models. *Symposium on 'Basic mechanisms of food preference and linking' Proceedings of the Nutrition Society* **54:** 419-427.

Sclafani, A. 1997. Learned controls of ingestive behaviour. *Appetite*, **29:** 153-158.

Sclafani, A. 2000. Macronutrient-conditioned flavour preferences. In *Neural and metabolic control of macronutrient intake* (ed. H.R. Berthoud and R.J. Seeley), pp. 93-106, CRC Press LLC, Boca Raton, Florida.

Scott, E.M., Verney, E.L. and Morissey, P.D. 1950. Self-selection of diet, 1: Appetites for calcium, magnesium, and potassium. *Journal of Nutrition,* **21:** 187-202.

Scott, L.L. and Provenza, F.D. 1998. Variety of foods and flavors affects selection of foraging location by sheep. *Applied Animal Behaviour Science,* **61:** 113-122

Scott, L.L. and Provenza, F.D. 1999. Variation in food selection foods among lambs: Effects of basal diet and offered in a meal. *Journal of Animal Science,* **77:** 2391-2397.

Scott, L.L. and Provenza, F.D. 2000. Lambs fed protein or energy imbalanced diets forage in locations and on foods that rectify imbalances. *Applied Animal Behaviour Science,* **68:** 293-305.

Seligman, M.E.P. 1970. On the generality of the laws of learning. *Psychological Review,* **77:** 406-418.

Sherratt, T.N. and MacDougall, A.D. 1995. Some population consequences of variation in preference among individual predators. *Biological Journal of the Linnean society,* **55:** 93-107.

Shettleworth, S. J. 1998. Learning from others. In *Cognition, evolution and behavior*. (ed. S.J. Shettleworth), pp. 425-474. New York: Oxford University Press.

Siegel, P.B. 1993. Behaviour-genetic analyses and poultry husbandry. *Poultry Science,* **72:** 1-6.

Simpson, S.J. and Raubenheimer, D. 1996. Feeding behaviour, sensory physiology and nutrient feedback: A unifying model. *Entomologia Experimentalis et Applicata*, **80:** 55-64.

Sinclair, L.A., Garnsworthy, P.C., Newbold, J.R. and Buttery, P.J. 1995. Effects of synchronising the rate of dietary energy and nitrogen release in diets with similar carbohydrate-composition on rumen fermentation and protein-microbial synthesis in sheep. *Journal of Agricultural Science,* **124:** 463-472.

Skinner, B.F. 1931. The concept of the reflex in the description of behavior. *Journal of General Psychology,* **5:** 427-458.

Slater, P.J.B. 1981. Individual differences in animal behaviour. In *Perspectives in ethology: advantages of diversity.* (ed. P.P.G. Bateson and P.H. Klopfer), Vol. 4, pp. 35-49. Plenum Press, New York.

Sloan, B.K. 1996. Developments in amino acid nutrition of dairy cows. In *Recent advances in animal nutrition* (ed. P.C. Garnsworthy and J. Wiseman), pp. 167-197. Nottingham University Press.

Spector, A.C. 2000. Linking gustatory neurobiology to behaviour in vertebrates. *Neuroscience and Biobehavioral Reviews,* **24:** 391-416.

Staddon, J.E.R. 1983. *Adaptive behaviour and learning*, pp. 395-403. Cambridge University Press.

Stefferns, A.B. and Benthem, L. 1999. Central nervous control of nutrient availability and utilazation In *Regulations of feed intake* (ed. D. Van der Heide, E.A. Huisman, E. Kanis, J.W.M. Osse, and M.W.A. Vestegen), pp. 29-40. CABI, Publishing.

Stephens, D.W. and Krebs, J.R. 1986. *Foraging theory*, pp. 104-182. Princeton University Press, Princeton, New Jersey.

Sutherland, W.J. 1996. *From Individual Behaviour to Population Ecology*, pp. 130-156. Oxford University Press, New York.

Thibault, I. and Booth, D.A. 1999. Macronutrient-specific dietary selection in rodents and its neutral bases. *Neuroscience and Biobehavioral Reviews,* **23:** 457-528.

Tilley, J.H.A. and Terry, R.A. 1963. A two stage technique for the *in vitro* digestion of forage crops. *Journal of the British Grassland Society* **18:** 104-111.

Tolkamp, B.J. 1999. Limitations in the use of constraints for intake predictions. In *Regulations of feed intake* (ed. D. Van der Heide, E.A. Huisman, E. Kanis, J.W.M. Osse and M.W.A. Vestegen), pp. 151-166. CABI, Publishing.

Tolkamp, B.J. and Ketelaars, J.J.M.H. 1992. Toward a new theory of feed regulation in ruminants. 2. Costs and benefits of feed consumption: an optimisation approach. *Livestock Production Science,* **30:** 297-317.

Tolkamp, B.J. and Kyriazakis, I. 1997. Measuring diet selection in dairy cows: Effect of training on choice of dietary protein level. *Animal Science,* **64:** 197-207.

Tolkamp, B.J., Kyriazakis, I., Oldham, J.D., Lewis, M., Dewhurst, R.D. and Newbold, J.R. 1998. Diet choice in dairy cows. II. Selection for metabolizable protein or for rumen degradable protein? *Journal of Dairy Science,* **81:** 2670-2680.

Urbaniak, M. 1995. Effects of blood meal, fish meal, soybean meal or casein on rumen protein metabolism in lambs. *Small Ruminant Research,* **18:** 207-212.

Van Soest, P.J. 1994. *Nutritional ecology of the ruminant*, second edition, pp. 290-311. Cornell University Press

Van Tien, D. Lynch, J.J., Hinch, G.N. and Nolan, J.V. 1999. Grass odor and flavor overcome feed neophobia in sheep. *Small Ruminant Research,* **32:** 223-229.

Villalba, J.J. and Provenza, F.D. 1996. Preference for flavored wheat straw by lambs conditioned with intraruminal administrations of sodium propionate. *Journal of Animal Science,* **74:** 2362-2368.

Villalba, J.J. and Provenza, F.D. 1997a. Preference for flavored wheat straw by lambs conditioned with intraruminal infusions of acetate and propionate. *Journal of Animal Science,* **75:** 2905-2914.

Villalba, J.J. and Provenza, F.D. 1997b. Preference for wheat straw by lambs conditioned with intraruminal infusions of starch. *British Journal of Nutrition,* **77:** 287-297.

Villalba, J.J. and Provenza, F.D. 1997c. Preference for flavoured foods by lambs conditioned with intraruminal administration of nitrogen. *British Journal of Nutrition,* **78:** 545-561.

Villalba, J.J. and Provenza, F.D. 1999a. Nutrient-specific preferences by lambs conditioned with intraruminal infusion of starch, casein, and water. *Journal of Animal Science,* **77:** 378-387.

Villalba, J.J. and Provenza, F.D. 1999b. Effects of food structure and nutritional quality and animal nutritional state on intake behaviour and food preferences of sheep. *Applied Animal Behaviour Science,* **63:** 145-163.

Villalba, J.J. and Provenza, F.D. 2000a. Postingestive feedback from starch influences the ingestive behaviour of sheep consuming wheat straw. *Applied Animal Behaviour Science,* **66:** 49-63.

Villalba, J.J. and Provenza, F.D. 2000b. Discriminating among novel foods: effects of energy provision on preferences of lambs for poor-quality foods. *Applied Animal Behaviour Science,* **66:** 87-106.

Wallace, R.J., Atasoglu, C. and Newbold, C.J. 1999. Role of peptides in rumen microbial metabolism - Review. *Asian-Australasian Journal of Animal Science,* **12:** 139-147.

Wang, J. and Provenza, F.D. 1996. Food preference and acceptance of novel foods by lambs depend on the composition of the basal diet. *Journal of Animal Science,* **74:** 2349-2354.

Westoby, M. 1974. An analysis of diet selection by large generalist herbivores. *American Naturalist,* **108:** 290-304.

Westoby, M. 1978. What are the biological bases of varied diets? *American Naturalist,* **112:** 627-631.

Weston, R.H. 1996. Some aspects of constraint to forage consumption by ruminants *Australian Journal of Agricultural Research,* **47:** 175-197.

Wilson, G.W. 1998. Resolving discrepancies between deterministic population models and individual-based model. *American Naturalist,* **151:** 116-133.

Wood-Gush, D.G.M. and Kare, M.R. 1966. The behaviour of calcium-deficient chickens. *British Poultry Science,* **7:** 285-290.

Zach, R. and Smith, N.M. 1981. Optimal foraging in wild birds? In *Foraging Behaviour, Ecological, Ethological and Psychological Approaches* (ed. A.C. Kamil and T.D. Sargent), pp. 3-18. Garland STMP Press, New York.

Zahoric, D.M. and Houpt, K.A. 1981. Species differences in feeding strategies, food hazards, and the ability to learn food aversions. In *Foraging Behaviour, Ecological, Ethological and Psychological Approaches* (ed. A.C. Kamil and T.D. Sargent), pp. 289-310. Garland STMP Press, New York.

Zahoric, D.M., Houpt, K.A. and Swartzman-Ander, J. 1990. Taste-aversion learning in three species of ruminants. *Applied Animal Behaviour Science,* **26:** 27-39.

Appendices

Appendix I: Programs to predict diet selection in groups of animals

The programs are named as: Choice.mtb; choicey.mtb; choicex.mtb; choice1.mac; choice2.mac. The sequence is started by typing exec 'choice' in Minitab. This goes through the sequence choice.mtb to choicey.mtb to choicex.mtb to choice2.mac to choice1.mac. Choice1.mac is the program for the individual animal. Choice2.mac draws an individual from the population.

The constants that need to be specified before starting the sequence are:

K1: the protein content of the lower protein content basal feed (PC_L)
K2: the protein content of the higher protein content basal feed (PC_H)
K5: the step size in the dilution series (*z*)
K8: the CV of the requirement (see K30)
K9: the CV of the lower boundary (see K40)
K10: the CV of the discrimination ratio (see k60)
K11: the data from the first animal will be stored in C(K11+1)
K13: the number of replicate animals wanted for each experiment
K21: the parameter to be varied
K22: the first value of the parameter to be used in the 'experiment'
K23: the last value of the parameter to be used in the 'experiment'
K24: the step size for the experimental variable
K30 the mean requirement of the population, *R* (K3 for the individual)
K40 the sampling ratio, *S* (K4 for the individual)
K60 the discrimination ratio, *D* (K6 for the individual)
K70: the column for the first mean
K71: the column for the first standard deviation

The number of treatments is calculated as K25.

For the first level of the variable the mean value and the standard deviation are stored in ck70 and ck71 respectively. For each level of the variable the experiment is to always offer the lower protein basal food L, with a protein content of PC_L, and the other as M with a protein content of PC_M. Food M is made by mixing a proportion z of the higher protein food H with (1-z) of L. Its protein content is given by $PC_M = ((z.PC_H) + ((1-z).PC_L))$.

The program choice1.mac predicts the diet choice, as the proportion $PC_L/(PC_L + PC_M)$, for an individual animal. The symbol # indicates comments.

choice1.mac

```
GMACRO
CHOICE1
#This program predicts the diet choice of an individual
#k1 and k2 are the protein contents of the low (L) and
#high (H) basal feeds respectively
#c1 is z, the proportion of H in the feed M which is offered with L
#the choice is between L and M
eras c17
set c1
0.01:1/k5
END
#k12 is the number of rows i.e the number of treatments
let k12=n(c1)
#c2 is the calculated protein content of M
let c2=(k2*c1)+(k1*(1-c1))
#c3 is the ratio of the protein content of L to that of M
#it will always be less than 1
let c3=k1/c2
#c4 is the minimum content in the diet of any one food taken as k4
set c4
k12(k4)
end
#c5 is the maximum content of any one food in the diet taken as (1-k4)
LET C5=1-C4
#DOES D APPLY?
LET C6=k6-c3
#IF C7 IS 1 THEN D APPLIES
```

```
code (-1:0) 1 (0:1) 0 c6 c7
#where D applies then the proportion of L in the diet is 0.5
let c8=c7*0.5
#to test if the R is less than k1, the protein of feed L.
#if it is then k80 is negative
let k80=(k3-k1)
#the value of k80 goes into c17
set c17
k12(k80)
end
#the value in c17 becomes -1 if k80 is negative
code (-1:0) -1 (0:1) 1 c17 c17
IF C17 = -1
CODE (0.5) 0.5 (0) K81 C8 C9
RMIN C5 C9 C10
#the value of c17 becomes 1 if k80 is positive
ELSEIF C17 = 1
CODE (0.5) 0.5 (0) K4 C8 C9
RMAX C4 C9 C10
ELSE
ENDIF
#c11 is the requirement
let c11=(k3-c2)/(k1-c2)
#the next line changes the sign of c11 where k80 is negative
let c11=c11*c17
#THE FURTHER TESTS DEPEND ON WHETHER R IS < K1 OR NOT
IF C17 = -1
COPY C10 C12
LET C17=-C17
ELSEIF C17=1
RMAX C10 C11 C12
ELSE
ENDIF
```

```
#c13 is the protein content of the selected diet
let c13=(c12*k1)+((1-c12)*c2)
let k11=k11+1
copy c12 ck11
end
endmacro
```

The program, choice2.mac draws individuals from the population at random and gives them values for the requirement (k3), the sampling ratio (k4) and the discrimination ratio (k6).

choice2.mac

```
GMACRO
CHOICE2
#samples are drawn from the distributions of requirement, k3,
#sampling ratio, k4, and discrimination ratio, k6.
rand 1 c14-c16
let k3=k30+(k30*k8*c14(1))
let k4=k40+(k40*k9*c15(1))
let k6=k60+(k60*k10*c16(1))
#k3 is forced to be between 0 and 1 inclusive
IF k3 <= 0
let k3=0
ELSEIF k3 > 0
let k3=k3
ELSE
ENDIF
IF k3 > 1
let k3=1
ELSEIF k3 <= 1
let k3=k3
ELSE
ENDIF
#k4 is forced to be between 0 and 1 inclusive
IF k4 <= 0
```

```
let k4=0
ELSEIF k4 > 0
let k4=k4
ELSE
ENDIF
IF k4 > 1
let k4=1
ELSEIF k4 <= 1
let k4=k4
ELSE
ENDIF
#k6 is forced to be between 0 and 1 inclusive
IF k6 <= 0
let k6=0
ELSEIF k6 > 0
let k6=k6
ELSE
ENDIF
IF k6 >= 0.99
let k6=0.99
ELSEIF k6 < 0.99
let k6=k6
ELSE
ENDIF
%choice1
end
ENDMACRO
```

The programs choice.mtb, choicex.mtb and choicey.mtb are listed:

program choice.mtb:

```
let k25=((k23-k22)/k24)+1
exec 'choicey' k25
end
```

program choicex.mtb:

```
%choice2
end
```

program choicey.mtb:

```
let kk21=k22
exec 'choicex' k13
let k99=k11+1-k13
rmean ck99-ck11 ck70
rstdev ck99-ck11 ck71
let k70=k70+1
let k71=k71+1
let k11=k11-k13
let k22=k22+k24
let kk21=k22
end
```

The data produced are stored in the worksheet in columns c(k11+1), through to c(k11+1+k12). The means are in ck70 and the standard deviations in ck71.

Appendix II: Hypothetical Experiments

An experiment uses K13 (always 100 in these cases) animals and K12 diet choices for each experimental treatment.

The treatment is described by:

- the parameter to be varied (its K number is in K21)
- the lower and upper bounds for the treatment variable are in K22 and K23
- the step size is in K24
- the means for the first treatment are in cK70
- the standard deviations for the first treatment are in cK71

Experiment 1. The effect of varying the mean *R*. The mean requirement was varied between 0.06 and 0.24 in steps of 0.02. The other 5 parameters were at their default values as shown in Table 6.1.

Experiment 2. The effect of varying the variation in the mean *R*. The CV of the mean requirement was varied from 0.06 to 0.24 in steps of 0.03. The other 5 parameters were at their default values as shown in Table 6.1.

Experiment 3. The effect of varying the mean *D* value. The mean *D* value was varied between 0.65 and 1.00 in steps of 0.05.

Experiment 4. The effect of varying the variation in *D*. The CV of *D* was varied between 0.03 and 0.15 in steps of 0.02. The other 5 parameters were at their default values as shown in Table 6.1.

Experiment 5. The effect of varying the mean *S* value. The mean *S* value was varied between 0.05 and 0.50 in steps of 0.05. The other 5 parameters were at their default values as shown in Table 6.1.

Experiment 6. The effect of varying the variation in *S*. The variation in *S* was varied between 0.1 and 0.5 in steps of 0.05. The other 5 parameters were at their default values as shown in Table 6.1.