Nutritional sensitivity of periparturient breakdown of immunity to gastrointestinal nematode parasites in mammals

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Abstract

Mammals usually develop immunity to gastrointestinal nematode parasites. However, during late pregnancy and lactation, this immunity often breaks down, resulting in elevated levels of parasitism. This periparturient relaxation of immunity (PPRI) renders lactating hosts main sources of infection for their parasite-naïve offspring, and may have a nutritional basis. Results of studies on parasitized hosts suggest that both crude protein (CP) and metabolizable energy (ME) supply may be important in regulating the degree of PPRI by affecting the immune response towards parasites. However there is a scarcity of data supporting such a role for ME in periparturient hosts, while there is sufficient evidence to support the view that CP in general, and amino acids are potent immunonutrients in various disease states (Chapter 1). In the first experiment (Chapter 2) I seperated effects of CP and ME on PPRI by feeding parasitized lactating rats at two levels of ME supply and one of three levels of CP supply. The results show that PPRI is sensitive to CP scarcity, and not to moderate ME scarcity. Increasing CP supply improved lactational performance and reduced PPRI, as observed by reduced worm burdens. In the second experiment (Chapter 3) I examined the rate at which improved nutrition can restore immunity by feeding low protein diets to rats nursing high number of pups, and then reduced litter size in a sub-group so that host nutritional status would change from scarce to adequate. The egg production of the parasite population of the latter group reduced within days to similarly low levels as rats that had always reared low number of pups and this was associated with an increased number of musosal mast cells and increased dam weight gain. Since host responses to dietary CP are almost by

definition responses to essential amino acids, the third experiment assessed the sensitivity of PPRI to methionine and leucine deficiency (Chapter 4). The latter resulted in increased worm burdens and egg production to similar levels when low protein diets are fed and imposed penalties in lactational performance. Finally, in the fourth experiment it was investigated whether similar outcomes can be expected in periparturient ruminant hosts by supplementing sheep with field beans, which are deficient in methionine, instead of soybean meal (Chapter 5). Indeed, feeding high protein diets based on field beans was less effective in reducing the worm egg excretion and improving lactational performance. The data from this thesis (Chapter 6) provide novel information on the nutritional basis of PPRI, showing that the latter can be rapidly reduced through improved protein nutrition. This may be seen as a response to the protein quality of the diet and the supply of amino acids in optimum quantities. These results have implications for parasite control strategies in farm animals.

Author's Declaration

I hereby declare that this thesis is my own work and all research carried out within it is my own work unless otherwise stated

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Chapter 1. General Introduction

1.1. The control of gastrointestinal parasitism in livestock

Gastrointestinal nematodes (GIN) infect every species of domestic animals. They have a major effect on the efficiency of production of grazing ruminants (Sykes, 1994) and are responsible for significant economic losses (Newton & Munn, 1999), which can reach up to 50% in the small ruminant livestock sector (Sykes, 1994). GINs of greatest importance in the grazing ruminant sector are members of the order Strongylida, which contains several superfamilies of economic importance such as Trichostrongyloidea, Strongyloidea, Metastrongyloidea and Ancylostomatoidea. The superfamily Trichostrongyloidae harbours a large number of species which inhabit the gastrointestinal tract of all classes of vertebrates. Species belonging to this superfamily such as *Haemonchus contortus, Trichostrongylus* spp*,* and *Teladorsagia circumcincta*, are the main disease-causing parasites in sheep in temperate climates (Urquhart *et al.,* 1996; Roberts & Javony, 2000). Gastrointestinal parasitism attributed to species belonging to Trichostrongyloidae occurs worldwide. This is due to the fact that they exhibit a great diversity in terms of the environmental requirements for the hatching of their eggs (O'Connor *et al.,* 2006). The life cycle is similar in all Trichostrongyloid species; it is direct (i.e. no intermediate host is required), their eggs hatch in soil, water or faecal paths and develop via intermediate stages into infective L3 larvae. Some infections may infect the host by penetration of the skin but in the majority of cases infection is established following swallowing of the infective form of parasites with contaminated food (grass) or water (Schmidt & Roberts, 2000).

The control of GIN nematodes is currently based on the use of a relatively small number of anthelmintic drugs combined with grazing management strategies (Coop & Kyriazakis, 2001). However, their control with anthelmintics alone is often

considered unsustainable, mainly due to the development of multiple anthelmintic resistance towards ruminant GIN (Schmidt & Roberts, 2000; Waller, 1997) diminishing their efficacy (Kaplan, 2004). In many areas of the world, multiple resistance towards all the classes of anthelmintics is developing among the GIN to the extent that sheep farming is becoming impossible in some areas (Stepek *et al.,* 2007). The most serious problems have been with the nematodes belonging to the family of Trichostrongyloidae which infect sheep. Resistance to the three major classes of anthelmintics, which are benzimidazoles, tetrahydropyrimidines/imidazothiazoles and macrocyclic lactones, has been reported around the world (Gilleard, 2006). Recently, a new class of anthelmintics has been introduced, the amino-acetonitrile derivative class (Kaminsky *et al.,* 2008), which is expected to help towards managing parasitism in areas where resistance is a major issue. Nonetheless, as the emergence of resistance towards this class in the future cannot be excluded, additional measures are required to maintain its efficacy for years to come.

In addition to the extent of anthelmintic resistance, there are reasons that make the use of anthelmintics alone for parasite control unsustainable. The anthelmintic's high cost can prohibit their use by livestock producers in the developing countries (Knox *et al.,* 2006). Moreover, consumers are increasingly concerned about the possible existence of drug residues in animal products and their environmental impact. Finally, there is a requirement for reduced anthelmintic input in organic systems of production, whilst organic livestock should still enjoy high health status. The aforementioned reasons have created the urgent need of the development of new alternative, integrated non-chemical strategies in order to control GIN (Athanasiadou *et al.,* 2005; Coop & Kyriazakis, 2001). It is expected that in the future sustainable

parasite control will be achieved with the use of selected combination of various alternatives, rather than a single approach (Coop & Kyriazakis, 2001).

Pasture management such as reducing the stocking rate, the duration of the grazing season on the pastures, and applying mixed grazing between animal species (Ihler, 2010) is a mean to reduce parasitism in flocks of ruminants. However, research is trying to identify and exploit alternative measures for the control of GIN in grazing ruminant production systems:

- The application of targeted selective treatment and building up refugia of susceptible parasites (Kenyon *et al.,* 2009).
- The use of *Nematophagus fungi,* which colonize the faeces and have the ability to kill the developing larvae (Fontenot *et al.,* 2003).
- The use of genetic selection, which includes the identification and selection of resistant animals within breeds or the use of resistant breeds for the production of crosses with improved ability to regulate the GIN populations by expressing increased immunity towards them (Good *et al.,* 2006; Jackson & Miller, 2006).
- The development of vaccines (Jackson & Miller, 2006; Ketzis *et al.,* 2006) against GIN infections and although at the moment there is no candidate (Smith & Zarlenga 2006), there is a successful example of a vaccine for lungworms in cattle and sheep and research is aspiring to create one for GIN (Stear *et al.,* 2007).
- The manipulation of host nutrition, which is the focus of this thesis.

The manipulation of host nutrition in order to improve the host resistance and/or resilience to parasitic infections is generally considered a promising approach,

which in combination with other measures for parasite control can lead to sustainable control of GIN parasitism. Resilience can be defined as the host's ability to maintain a reasonable level of production in the face of a parasitic challenge (Albers *et al.,* 1987) and resistance is a measure of the host's ability to limit the establishment, growth rate, fecundity and/or persistence of a parasite population (Coop & Kyriazakis, 1999). Intake of dietary components by the host can contribute to regulating parasite populations in a variety of ways. Nutrition may affect pathogen fitness through the ingestion of compounds such as plant secondary metabolites, which are found in bioactive plants (Athanasiadou *et al.,* 2008). Such metabolites can influence host resistance through two mechanisms. Firstly, they can exert directly toxic effects on the parasite population (Athanasiadou & Houdijk, 2010). Secondly, they can improve the immune response of ruminant hosts by binding to dietary protein and protect it from rumen degradation, thus making it available in the lower gastrointestinal tract for absorption (Barry & McNabb, 1999). Additionally, host nutrition can indirectly affect the host's resistance and resilience to GIN, via improving the immune response through the increased influx of nutrients (Houdijk & Athanasiadou, 2003; Athanasiadou *et al.,* 2008). This form of nutritional modulation is the main focus of this thesis and is of particular relevance during certain periods of the host's life, for example when a host's acquired immunity breaks down. The next section briefly describes a host's acquired immunity to parasites.

1.2. Immune response to GIN

There seems to be a very close association in the way the organism generates an immune response towards infections by helminthes (both nematodes and platyhelminthes), in spite of the fact that species of GIN have unique features in terms of phylogeny, biochemistry, morphology and lifecycle strategies (Artis, 2006). The first line of defence the parasite has to encounter upon invasion is the host's physical barriers, which comprise of the skin and the mucosal surfaces (De Veer *et al.,* 2007). Once these are penetrated, the host's immune system must (i) recognize the parasite as foreign, (ii) activate effector mechanisms that either eliminate the parasite or severely limit the parasite's ability to induce injury, and (iii) limit the activation of effector mechanisms that can cause self-damage, particularly effector mechanisms that have little ability to damage or contain the relevant parasite (Finkelman *et al.,* 2004).

Immunity to extracellular parasites such as GIN is critically dependent on a type 2 T cell response (Th2), which is essential for their expulsion (Artis, 2006; Lawrence, 2003). Th1 and Th2 subsets are characterized by the particular set of cytokines released. Th1 responses are typically induced by intracellular microparasites (such as bacterial, protozoan and viral pathogens) and are associated with elevated levels of IFN-γ, TNFα, IL-12 and IL-2, resulting in a cell-mediated immune response. Th2-type responses are typically characterized by increases in the levels of interleukin-4 (IL-4) and other Th2-type cytokines including IL-5, IL-9, IL-13 and IL-21 (Anthony *et al.,* 2007) and production of antibodies. The production of such cytokines is accompanied by the activation and expansion of CD4+ Th2 cells and of specific effector cells, such as mast cells, eosinophils, and basophils all of

which can further produce several types of Th2-type cytokines (Maizels *et al.,* 2004, Anthony *et al.,* 2007). Each cytokine binds to a specific receptor, which in turn leads to the activation of specific signalling molecules (Lawrence, 2003). Recently, neutrophils (Padigel *et al.,* 2007), basophils (Mitre & Nutman, 2006) and macrophages (Pesce *et al.,* 2006), which have long been thought not to play a major role in the worm expulsion, have been implicated in the Th2-type response towards GIN. Additionally, a new type of effector cells named nuocytes (Neill *et al.,* 2010) has been discovered, which may hold important roles in worm expulsion.

Th2 cytokines attract progenitors of B-cells, mucosal mast cells (MMC) and eosinophils to the gut where they proliferate and mature (Koski & Scott, 2001). MMC are important effector cells in nematode infections. They contain many granules filled with bioactive compounds like histamine, heparin and proteases and can secrete cytokines such as IL-4 and IL-5, as well as leukotrienes and chemokines following activation (Lawrence, 2003; Maizels & Holland, 1998).

Both peripheral and tissue eosinophilia are characteristic features of infection with GI helminthes (Lawrence, 2003) and along with MMC, eosinophils are the most common cells to infiltrate the site of nematode infection (De Veer *et al.,* 2007). IL-5 stimulates the activation and differentiation of eosinophils. They contain granules filled with a number of cationic proteins and can release an array of pro-inflammatory cytokines, chemokines and lipid mediators (Rothenberg & Hogan, 2006). Upon stimulation they de-granulate and release highly toxic proteins, oxidizing agents and neurotoxins in proximity to the nematodes in tissue (Lawrence, 2003).

GIN infections are also characterized by an increase in the numbers of goblet cells (GC), their size and in the profile of secreted mucin proteins (De Veer *et al.,*

2007; Mahida, 2003). Mucins from GC play an important role in the trapping of worms in the mucus layer and inhibiting worm motility and feeding (Miller, 1987, Tsubokawa *et al.,* 2009). It has been suggested that various effects of both cytokines and effector cells during a Th2 response lead to the creation of an inhospitable environment for the parasite, resulting in the parasite being 'flushed out' by increased fluid and mucus secretion and by increased propulsive activity of the gut. This has been termed the 'weep and sweep' response (Shea-Donohue & Urban, 2004).

1.3. Breakdown of acquired immunity

During their life cycle mammals acquire immunity towards parasites as they are continuously exposed to parasite stimulation (Houdijk *et al.,* 2001a). However, the expression of acquired immunity can break down during their life span (Houdijk *et al.,* 2003a) as it has been shown to occur in growing, periparturient (Kyriazakis & Houdijk 2006) and ageing animals (Houdijk *et al.,* 2003a).

In the reproducing animal this phenomenon typically occurs around parturition and is better known as the periparturient relaxation of immunity (PPRI). It has been described in detail in small ruminants infected with gastrointestinal nematodes, but also in other parasite host systems, and it has been suggested that is a rather general phenomenon in mammalian hosts (Houdijk *et al.,* 2001a; Houdijk, 2008).

PPRI is of particular importance for grazing livestock, as it plays an important role in the epidemiology of GIN infections. This is because it is characterized by an increased nematode egg excretion from periparturient ewes onto the pasture, which can be a main source of infection for their immunologically naive lambs (Heath & Michel, 1969). Therefore, controlling worm egg excretion from periparturient ewes

should be included in any strategy aimed at a reduction of parasitism in young grazing lambs. There is evidence suggesting that PPRI may be sensitive to the nutritional status of the host and a nutrient partitioning framework has been introduced to account for this phenomenon (Coop & Kyriazakis, 2001; Kyriazakis & Houdijk, 2006) and will be detailed below.

1.4. Nutrient partitioning framework

Although immune responses to pathogens have traditionally been regarded as part of body maintenance and hence may be prioritised in terms of scarce nutrient allocation (Houdijk *et al.,* 2001a), there is an increasing body of evidence which suggests that under certain conditions some aspects of immunity may be sensitive to changes in nutrient supply (Coop & Kyriazakis, 1999). This is particularly evident during periods of elevated nutritional demands, such as the growing and the periparturient period. The increased nutrient requirements during these periods can lead to nutrient scarcity, which has been linked to a breakdown of acquired immunity. During such periods increased nutrient, and in particular protein intake, has been shown to greatly improve the host's ability to deal with the parasite population (Datta *et al.,* 1998; Houdijk *et al.,* 2003b; Van Houtert *et al.,* 1995b).

A nutrient partitioning framework has been put forward to account for the increased susceptibility to disease, during times of nutrient scarcity (Coop & Kyriazakis, 1999). This framework introduces the concept of prioritization for the allocation of scarce nutrients toward different bodily functions, including immune functions. Nutrients are scarce when nutrient demand outweighs nutrient supply. The suggestion is that under conditions of nutrient scarcity the higher the priority of a

bodily function, the less likely host nutrition would affect it (Table 1.1). Maintenance requirements are always given priority, as inability to satisfy those, will result into penalties on the survival of the host. The naive growing host that encounters a parasite for the first time is expected to prioritize scarce nutrient allocation to the acquisition of immunity over growth. This ensures its survival in the long term as in that way it is allowed to reach reproductive maturity and not to succumb to the adverse consequences of parasitism. However, once immunity has been acquired, hosts would be expected to prioritize growth or reproduction over expression of immunity to parasites, as the former bodily functions ensure the preservation of the host's genetic material (Coop & Kyriazakis 1999). This means that once immunity towards a pathogen has been acquired, scarce nutrients are allocated firstly to satisfy the maintenance of bodily functions, then to satisfy the growth and/or reproductive functions and finally to satisfy the expression of acquired immunity. This does not necessarily mean that the allocation strategy is absolute as experimental data indicate that it might be partial (Houdijk *et al.,* 2003b).

According to the framework, the principles of nutrient partitioning should be applicable to all scarce nutrients. However, most evidence on nutrient scarcity particularly around parturition, have been related to dietary protein. In the next section, the role of protein supplementation in periparturient animals will be detailed.

Table 1.1. Proposed order of partitioning of scarce resources to body functions. The acquisition (naïve animals) and expression (immune animals) phase of immunity are considered separately in the growing animal (Coop & Kyriazakis, 1999).

Growing animal		Reproducing animal
Acquisition phase	Expression phase	Expression phase
1. Maintenance of body protein	1. Maintenance of body protein	1. Maintenance of body protein
2. Acquisition of immunity	2. Protein gain	2. Reproductive effort (pregnancy/lactation)
3. Protein gain	3. Expression of immunity	3. Expression of immunity
4. Maintenance and gain of body lipid	4. Maintenance and gain of body lipid	4. Attainment of desired fatness

1.5. Competition for protein during the expression of immunity in periparturient mammals

Coop and Kyriazakis (1999) and Kyriazakis and Houdijk (2006) have supported the view that protein is expected to be more important in cases of parasitism than other nutrients. In the case of periparturient animals, its importance is relevant to the high demands reproductive and immune functions impose to the host and to the limited capacity of the host to meet these demands through the mobilization of labile protein reserves.

During the periparturient period, nutrient demand can be up to six times higher than that of non-reproducing animals (Houdijk, 2008). Both protein and energy demands increase dramatically during late pregnancy and during the onset of lactation. During late pregnancy up to 80% of apparently digested crude protein is partitioned to the gravid uterus of the ewe (Bell & Ehrhartd, 2000). The gut, increases in size during lactation so as to cope with the increased food intake during that time

(Adams & Liu, 2003). The increase in nutrient uptake drives the synthesis of lactose, fat and protein, which are secreted in milk. The body is able to mobilise fat which is stored during gestation, while unavailability of fat reserves results in a drop of the total output of milk (Adams & Liu, 2003). It has been shown that the lactating ewe can mobilize 80% of its fat reserves in the first 3 weeks of lactation without any penalties for the animal (Adams & Liu, 2003). Unlike fat, the reserves of labile protein are limited (Bocquier *et al.,* 2000) and are potentially depleted much quicker (Adams & Liu, 2003). The rate of depletion of body protein in the lactating host probably accounts for the fact that protein supplementation results in increased resistance, as protein becomes more available for the covering of the nutrient demands of expressing adaptive immunity.

In addition to the increased periparturient requirements for productivity, also the activated immune system imposes additional nutrient and energy demands to the host. In the presence of pathogens T-cells must actively acquire metabolic substrates from their extracellular environment so as to promote their growth and proliferation. Upon ligation of their antigen receptors nutrients such as glucose, fatty acids and amino acids are internalised and degraded into intermediates so as to provide the cell with metabolic substrates. These are either further catabolised to provide ATP, or used for the anabolic process of constructing macromolecules such as cytokines (Fox *et al.,* 2005). The immune system is expected to draw heavily on protein supply so as to activate an effective protective response (Kyriazakis & Houdijk, 2006). This is mainly because the effector mechanisms of the immune response are highly proteinaceous in nature; inflammation and immune system activation are characterised by the synthesis of specific proteins that play crucial roles in the defence

of the organism against pathogens and the modulation of immune response (Coop & Kyriazakis, 2001; Le Floc'h *et al.,* 2004). Thus, nutritional support, in the form of protein and/or individual amino acids, may alter the availability of substrates for the synthesis of proteins and peptides important in the inflammatory process and its regulation (Grimble, 1998).

When dietary protein supply is limited, then body protein reserves, if adequate, can serve as a source of essential amino-acids for the immune function. However, during the catabolic phase of gestation and the onset of lactation, the development of the foetus, the placenta and the production of milk for the nourishment of the offspring imposes excessive nutrient (protein) demands to the host (Adams & Liu, 2003). The depleted state of the labile protein tissue of the host at the same time has as a result their supply to be critically dependent on the nutrient intake of the host.

1.6. Protein supplementation in the periparturient host

Most research on overcoming nutrient scarcity in periparturient parasitized hosts has focused on the effects of protein supplementation. Especially in periparturient ewes, it has been shown that levels of metabolizable protein (MP) supply 20-30% greater than the theoretical requirements of uninfected sheep (Sykes & Kyriazakis, 2007) are required to maintain immunity, levels considerably above those at which milk production is optimized in infected animals (Adams & Liu, 2003; Houdijk *et al.,* 2003b). The improvement of immunity was demonstrated in these experiments by a reduction in FEC and in worm burdens, which are indirect measurements of immunity. A variety of protein sources was used in these studies

such as fishmeal (Donaldson *et al.,* 2001), xylose-treated soybean meal (Houdijk *et al.,* 2003b; Houdijk *et al.,* 2005b), cottonseed meal (Kahn *et al.,* 2003a) and the parasites used were *T. circumcincta, T. colubriformis* and *H. contortus.* Similar results have been obtained in goats (Chartier *et al.,* 2000; Etter *et al.,* 1999).

In addition to indirect measurements of the expression to immunity to parasites such as worm counts and nematode egg excretion, also direct measurements show an up-regulation of immune responses to GIN in response to protein supplementation. Studies in sheep have shown that protein supplementation results in an increased concentration of circulating and local inflammatory cells, mast cell proteases and circulating antibodies, especially during the phase of expression of immunity (Athanasiadou *et al.,* 2008). Some experiments have demonstrated higher IgA antibody concentrations in the plasma of infected sheep receiving higher levels of dietary protein, providing evidence that the antibody production is influenced by nutritional intake in sheep (Houdijk *et al.,* 2003b; Strain & Stear, 2001). In addition, protein supplementation increased the proportion of thymus-derived cells that are associated with expression of cellular immunity in the local immune response of *T. colubriformis*-infected sheep (Kambara & McFarlane, 1996). Finally, increased protein supplementation has consistently resulted in lower plasma pepsinogen concentrations, which is indicative of less severe abomasal damage due to parasitism, possibly due to an enhanced expression of immunity (Houdijk *et al.,* 2000; 2003b; Zaralis *et al.,* 2009).

Recently, the effects of protein supplementation on the lactating host have also been demonstrated in rodent models, in particular in lactating rats given a secondary infection with *Nippostrongylus brasiliensis*. Increased protein levels resulted in a

reduction of FEC and worm burdens (Houdijk *et al.,* 2005a; Jones *et al.,* 2009). These reductions correlated with increased mastocytosis, greater goblet cell hyperplasia and a stronger antigen specific IgG2b response earlier in infection in comparison with their LP counterparts (Jones *et al.,* 2009).

However, effects of increased protein supply on the resistance of ruminant hosts have been variable. This variability seems to be related with the protein quality of the foods offered to them as it will be detailed below.

1.7. Protein quality and PPRI

In ruminant diets, dietary protein is provided in the form of effective rumen degradable protein (ERDP) and digestible undegradable protein (DUP). ERDP, which is the main source of digestible protein for the animals, is converted mainly to ammonia in the rumen where it is the principal starting material for microbial crude protein synthesis (Lobley *et al.,* 1995). The protein which is truly available to the animal comprises part of microbial protein and part of the usually relatively smaller amount of DUP flowing from the rumen, via the abomasum to the small intestine where it is digested and absorbed (Hristov *et al.,* 2004).

The extent to which increased metabolizable protein (MP) supply reduces the degree of breakdown of immunity differs between studies in parasitised sheep. Studies where the additional MP supply derives from diets with high DUP levels (Donaldson *et al.,* 1998; Houdijk *et al.,* 2003b; Van Houtert *et al.,* 1995b) seem to result in a more pronounced reduction in parasitism compared to studies where it derives from diets with low DUP levels (Kahn *et al.,* 2003a, 2003b, Knox & Steel, 1996; Shaw *et al.,* 1995; Van Houtert *et al.,* 1995a). It is likely that this variability in

response is due to variation in MP quality, defined as its amino acid (AA) composition. The relatively unbalanced AA profile of microbial protein, compared to that of animal protein in general and immune-proteins in particular, may account for their limited effect on reducing PPRI. This is because parasitism increases specific AA requirements to synthesize proteins to maintain homeostasis (e.g. albumin) and to mount immune responses (e.g. inflammatory agents, mucins and antibodies) (Houdijk & Athanasiadou, 2003). There is currently no evidence on the effects of different protein sources with variable DUP/MP proportion in their ability to enhance the resistance and resilience of periparturient hosts.

1.8. The effects of amino-acids on expression of immunity

As effects of dietary protein supply on the resistance of parasitized hosts are responses to dietary amino acids (AAs), it may be expected that their reduced supply would penalise immunity to parasites.

Mammals require the dietary provision of a core of nine AAs (Table 1.2). This is due to their inability to synthesize *de novo* their corresponding carbon skeleton, or to synthesize them in adequate quantities relative to their demands (D'Mello, 2003; Wu, 2009). These are classified as essential amino acids (EAAs) and provision of these nutrients is mandatory to ensure their survival. In addition to EAA, there are also the conditionally essential AAs (CEAAs) which are those that can normally be synthesized in adequate amounts, but must be provided from the diet in order to meet optimal needs under conditions where rates of utilization are greater than rates of synthesis (Wu, 2009). Non-essential AAs (NEAAs) are those which

mammals are able to synthesize in adequate amounts by the body to meet optimal requirements under all conditions (D'Mello, 2003).

Table 1.2. Dietary classification of amino acids.

Essential Amino acids (EAA)	Conditionally essential amino acids (CEAA)	Non essential amino acids (NEAA)
Lysine	Cysteine	Glutamate
Histidine	Proline	Glutamine
Leucine	Tyrosine	Glysine
Isoleucine	Arginine	Serine
Valine		Alanine
Methionine		Aspartate
Threonine		Asparagine
Tryptophan		
Phenylalanine		

Adequate dietary supply of all amino acids is necessary for maintenance and productive purposes (D'Mello, 2003), for sustaining normal immunocompetence and protecting the host from a variety of diseases in all species (Li *et al.,* 2007). Both innate and acquired immune responses are dependent upon the provision of AAs for the synthesis of antigen-presenting molecules, immunoglobulins, cytokines and acute phase proteins, as well as for the provision of energy providing substrates either directly, or following their conversion to other AAs (e.g., glutamine) or to glucose (Calder, 2006; Kim *et al.,* 2007). At the same time, requirements for AA may also increase as a direct consequence of metabolic changes associated with inflammation

and infection (Le Floc'h *et al.,* 2004) and the physiological status of the animal, such as pregnancy and lactation (Clowes *et al.,* 2005). For example, a 35% increase in leucine turnover has been reported in lactating rats (Vina & Williamson, 1981). Additionally, the intestinal tract is one of the largest lymphoid organs in the body, consisting of immune cells in organized gut associated lymphoid tissues (Field *et al.,* 2002) and as such is expected to have increased demands for AAs during inflammation and disease. The fact that 30–50% of EAAs in the food may be catabolized by the small intestine (Stoll *et al.,* 1998; Wu, 1998) is indicative of their role in gut integrity, function and local immune response (Wang *et al.,* 2009, Wu, 2009).

There is a large body of evidence suggesting that most AA deficiencies can influence aspects of immunity in various host pathogen systems (Li *et al.,* 2007). However, here I will focus on those that may influence the host's resistance to GIN parasitism.

Methionine. Sulphur-containing amino acids in particular are required during infection for many of the substances produced in the presence of cytokines (Grimble & Grimble, 1998). Methionine is the only essential sulphur-containing amino acid and the precursor of the sulphur amino acids homocysteine, cystathionine, cysteine, and taurine (Grimble, 2006). An adequate methionine and cysteine intake is important for protein synthesis, critical to maintain gut functions including digestion, absorption and nutrient metabolism and for the regulation of the mucosal response to antigens (Fang *et al.,* 2010, Grimble, 2006). Cysteine is the precursor of glutathione, which is an important regulator of the intracellular redox potential and is thought to be critical for the defence of the intestinal mucosa against pathogens (Grimble, 2001;

Wu, 1998; Malmezat *et al*., 2000). Glutathione synthesis is influenced by dietary intakes of sulphur amino acids (Wu *et al.,* 2004). Glutathione also plays a role in the production of chemokines, which are utilised by the innate immune system as signalling molecules and contain two cysteine molecules (Parkin & Cohen, 2001). Furthermore, methionine plays important part in DNA and protein methylation, required for lymphocyte proliferation and differentiation and is a substrate for the synthesis of choline and thus phosphatidylcholine, essential for leucocyte metabolism (Kim *et al.,* 2007; Grimble, 2006; Flynn *et al.,* 2002; Li *et al.,* 2007). Demand for sulphur containing amino acids may be increased during GIN infections due to the loss of endogenous protein via increased sloughing of epithelial cells and mucin secretion (Poppi *et al.,* 1986). In parasitized hosts, methionine supplementation in low protein diets offered to growing rats infected with *N. brasiliensis,* resulted in a reduction of the worm burdens (Cummins *et al.,* 1986). As a result of infection of sheep with *Trichostrongylus colubriformis,* the cysteine flux declined, compared to pre-infection levels (Hoskin *et al.,* 2002) and spermidine and spermine concentrations in sheep tissues increased (Liu *et al.,* 2007).

Leucine. Branch chain amino acids (BCAA) consist of leucine, valine and isoleucine. In vitro studies have shown that BCAA are essential for protein synthesis and lymphocyte proliferation whilst dietary restriction of BCAA in mice and chickens impaired several aspects of the immune function and increased the susceptibility to pathogens (Konashi *et al.,* 2000; Calder, 2006). Additionally, lactation increases the channelling of BCAAs from the blood flow to the mammary gland (Trottier *et al.,* 1997, DeSandiago *et al.,* 1998a; 1998b). Among BCAAs, leucine is the sole branched chain AA that can activate the mTOR signaling pathway

in intestinal epithelial cells (Ban *et al.,* 2004) and it may play an important role in intestinal repair via stimulation of protein growth (Naomoto *et al*., 2005). Furthermore, leucine has been directly related to the immune response in both *in vitro* and *in vivo* studies (Calder *et al.,* 2006) and its requirements are increased during parasitism (Yu *et al.,* 2000). In parasitized hosts, increased gastrointestinal tract leucine metabolism, which renders leucine unavailable for other tissues, has been observed (Yu *et al*., 2000; Liu *et al.,* 2007).

Arginine. The arginine family of amino acids include glutamine, glutamate, proline, aspartate, asparagine, ornithine, citrulline, and arginine. A large body of evidence from animal studies and human studies shows an important role for arginine in the intestinal immune response and indicates that an adequate provision of arginine is required for lymphocyte development and that dietary arginine supplementation enhances immune function in various models of immunological challenges (Li *et al*., 2007). Although there is evidence suggesting that inadequate intake of dietary arginine affects immune responses in young rats (Wu *et al*., 1999) and chickens (Konashi *et al*., 2000) and arginine supplementation decreases mucosal injury (Sukhotnik *et al.,* 2004), there is currently no evidence implicating that arginine deficiency affects resistance to GIN.

Glutamine. Glutamine is the most abundant amino acid in the bloodstream and in the free amino acid pool of the body (Lund & Williamson, 1985). Although it has traditionally been considered as a nonessential amino acid, is now regarded as a conditionally essential nutrient under stress conditions, such as infection, injury, and weaning (Li *et al.,* 2007; Wang *et al.,* 2008) and an important nutrient for the function of the immune system (Grimble, 2001). A number of roles have been

ascribed to glutamine: a) a preferential source of energy for the immune cells, b) an important modulator of gut barrier function and c) a substrate for glutathione synthesis (Grimble, 2001). Studies have shown that dietary glutamine supplementation decreases the susceptibility of enterocytes and lymphatic cells to apoptosis of weaning piglets (Domeneghini *et al.,* 2006), while enhancing anti-oxidative function and cell proliferation in the small intestine (Wang *et al.,* 2008). There is currently no evidence implicating that glutamine deficiency affects resistance to GIN

Threonine. Another amino acid regarded as important for the immune function is Threonine, which is the most abundant essential amino acid in immunoglobulin protein (Le Floc'h *et al*., 2004). Threonine is a major component of intestinal mucin and plasma γ-globulin in animals (Li *et al*., 2007) and is regarded as crucial for mucin production and maintenance of gut function (Le Floc'h *et al*., 2004). Animal feeding studies indicate that changes in components of the immune system are sensitive to dietary threonine intake (Li *et al*., 2007). Gestating sows fed with threonine-deficient diets had as a result significant lower plasma concentration of total or specific IgG titre following bovine serum albumin injection and swine fever vaccination (Cuaron *et al*., 1984). A study with rats demonstrated that dietary threonine restriction dramatically impaired the synthesis of mucins in the small intestine (Faure *et al.,* 2005). A reduction in the synthesis of mucins as a result of reduced dietary availability can therefore lead to an impairment of gut barrier function (Wang *et al*., 2009). Currently there are no experimental data providing a link between threonine deficiency and immunity to GIN.

Although most examples of decreasing nutrient scarcity on resistance and resilience in parasitized periparturient hosts are related to protein supplementation, evidence is available on the effects of the supplementation of other nutrients which will be presented below.

1.9. Energy and other Nutrients

Like any bodily function, the immune system has also requirements for energy and micronutrients like vitamins and minerals. In theory, an increased availability of each of these resources can be expected to improve immunity to gastrointestinal nematodes provided that they are first limiting (Kyriazakis & Houdijk, 2006).

Minerals are involved in many immune functions while deficiencies in some of them have detrimental effects on the ability of the host to expel pathogens (McClure, 2008). For example, cobalt deficiency has been reported to decrease the resistance of ruminants to resist helminth infection (MacPherson *et al.,* 1987; Ferguson *et al.,* 1989), whereas molybdenum deficiency penalise the ability of sheep to clear *Haemonchus contortus* (McClure *et al.,* 1999) and *Trichostrongylus colubriformis* (Suttle *et al.,* 1992). Similarly, zinc deficiency in *H. polygyrus* infected mice impaired Th2 immune responses (Shi *et al.,* 1997). However, there is no study on periparturient hosts establishing a relationship between increased mineral supplementation and resistance to parasites.

In addition to other nutrients, infection also induces energetic demands to the host. It is known that the proliferating activated T-Cell requires glucose for the provision of ATP through glycolysis in the cytosol (Fox *et al.,* 2005). The mitogenic stimulation of thymocytes or naïve T-cells induces an almost 20-fold increase in

glucose uptake within 1 hour (Greiner *et al.,* 1994). The energetic cost of parasitism has been demonstrated by the lower amount of fat deposits in infected mice compared with their uninfected counterparts (Coltherd *et al.,* 2009; Kristan & Hammond, 2000). Nonetheless, it appears that moderate changes in the energy supply do not affect gastrointestinal parasitism in ruminant hosts (Donaldson *et al.,* 1998). In an experiment (Bown *et al.,* 1991) it was attempted to separate the effects of protein and energy supply on the resistance and resilience of growing lambs to a trickle infection of *T. colubriformis*. By using direct infusions into the abomasum of either casein, iso-energetic amounts of glucose, or saline/mineral equivalents in sheep fed chopped hay, it was shown that the intestinal infection induced a protein rather than an energy deficiency. The resilience of the lambs was improved following protein but not energy supplementation.

However, there are some researchers who suggest that protein and energy deficiencies modulate host defence mechanisms differently (Koski & Scott, 2001). A few studies have directly examined the effects of energy restriction independent of protein deficiency in growing rodent models. Lunn *et al*. (1988) fed restrictively growing rats with diets deficient in energy, but abundant in protein. The rats had a reduced growth rate, but maintained near-normal plasma albumin concentrations. However, upon infection with *N. brasiliensis* the energy deficit resulted in prolonged *N. brasiliensis* survival, which was accompanied by hypoalbuminemia that was in turn attributed to protein leakage into the intestine through parasite-induced lesions (Lunn *et al.,* 1988). Koski *et al*. (1999) showed that a 20% or 25% reduction in energy intake, independent of any alteration in protein status, had effects on survival of *H. polygyrus* in mice. The energy restricted infected mice showed impaired

lymphocyte proliferation and reduced production of Th2 cytokines and lower levels of IgE, parasite-specific IgG1, and eosinophils, which led to higher worm burdens and fecundity. Similarly, Kristan (2007) observed higher worm burdens in chronically restricted mice infected with *H. polygyrus*. The aforementioned conflicting results indicate that the effects of energy supply on the resistance of periparturient animals to parasites warrants further investigation.

1.10. Reducing nutrient demand in the periparturient host

Reducing nutrient scarcity can be achieved either through an increase in the intake of dietary nutrients, or through a reduction of nutrient demand. The latter can arise from rearing fewer offspring, which at similar level of (scarce) nutrient supply is expected to increase nutrient availability to the periparturient host for expression of immunity to parasites (Houdijk, 2008). It is well documented that rearing a larger litter increases milk production and therefore the amount of nutrients directed to reproductive functions in both sheep (Alexander & Davies, 1959; Gardner & Hogue, 1964) and rats (Morag *et al.,* 1975). Studies with lactating ruminant hosts infected with various species of gastrointestinal nematode parasites, have revealed that a lower nutritional demand arising from rearing fewer offspring significantly reduces the degree of PPRI (Romjali *et al.,* 1997; Donaldson *et al.,* 1998; Houdijk *et al.,* 2001a; Kahn *et al.,* 2003b; Baker *et al.,* 1998; Xie *et al.,* 2004). Furthermore cessation of lactation results in an even more pronounced enhancement of resistance (Houdijk *et al.,* 2005b, O'Sullivan & Donald 1973; Lloyd, 1983) and low producing goats exhibit lower FEC than their high producing counterparts (Hoste & Chartier 1993; Chartier *et al.,* 2000).

In a *N. brasiliensis* lactating rat model it has been previously established that reducing nutrient demand by reducing the number of suckling pups at the beginning of lactation (Normanton *et al.,* 2007) results in lower degree of PPRI. In addition, a reduction in the number of rearing lambs in lactating sheep infected with *T. circumcinta* during lactation resulted in a rapid reduction in the degree of PPRI expressed as lower FEC (Houdijk *et al.,* 2006). However, the latter studies failed to demonstrate an improvement in the mucosal immunity as a result of the reduction in nutrient demand.

1.11. Rodents as models to assess the effect of nutrition on GIN

Although ruminant-nematode interactions are intensively investigated (particularly in the area of immunonutrition) rodents have been used for years as model hosts of parasitic infections for the study of host-parasite interactions (Boes & Helwigh, 2000). Rodent immune responses to GIN are well defined and are used to gain insights into the underlying immune mechanisms of both human and livestock infections (Shea-Donohue & Urban, 2004). As such, most of our current detailed knowledge regarding the nematode-host interactions derives from experiments with rodent models (Dehlawi & Goyal, 2003). Species specific nematodes, such as *N. brasiliensis* and *Heligmosomoides bakeri* are used to infect the rodent hosts, as they approximate to important economical or clinical species of ruminant species (Wakelin, 1996).

Since they are monogastric, rodents are also ideal for the testing of the suggested framework, as their diets can be manipulated to a greater extent than ruminants and their nutrient requirements can rise up to tenfold during lactation in

relation to the maintenance requirements (Jessop, 1997). This allows e.g. a more detailed qualitative and quantitative approach to the protein/amino-acid demands of an efficient immune response towards GIN. At the same time it is easier to manage a larger number of rodents than ruminants and it is less complicated to perform sequential kill experiments to assess the temporal effects of nutrition on the local immune responses towards GIN.

A *Nippostrongylous brasiliensis* re-infection lactating model has been recently developed (Houdijk *et al.,* 2003). The nematode is lumen-dwelling and has been widely used as a model parasite in studies of mucosal immunity against GI nematodes (Nawa *et al*., 1994). It is a natural parasite of the rat and it has also been adapted to the mouse for experimental purposes. L4 and adult stages of *N. brasiliensis* inhabiting the small intestine induce similar pathology and immune responses to *Trichostrongylus vitrinus* and *Trichostrongylus colubriformis* intestinal nematode species of importance to the sheep farming industry (Rothwell, 1989). Rats acquire a strong immunity to the small intestinal nematode *N. brasiliensis*, and immune rats rapidly expel adult *N. brasiliensis* during a secondary infection (Jarrett *et al.,* 1968). However, during lactation, delayed expulsion and increased nematode egg excretion takes place, indicative of breakdown of previously acquired immunity (Houdijk *et al.,* 2003a). In addition, feeding secondary infected lactating rats with increased levels of protein results in reduced colon egg counts (CEC) and worm burdens (Houdijk *et al*., 2005a). Moreover, reducing the litter size, and thus protein demand, showed similar results (Normanton *et al*., 2007). A recent experiment performed by the same group (Jones *et al.,* 2009) showed that nutrient supplementation resulted in lower levels of parasitism, which also correlated with improved immune indicators. Increased dietary

protein content improved early immune responses and subsequently reduced CEC and worm burdens. Therefore, this model can be exploited in order to assess the effects nutrient interventions have on the resistance and resilience of GIN infected periparturient hosts.

1.12. PhD objectives and hypotheses

The overall objective of this PhD is to investigate the effect nutrient manipulations exert on the degree of PPRI of lactating hosts utilizing a lactating rat-*N. brasiliensis* model ***(Chapter Two, Three and Four)*** and a periparturient sheep-*T. circumcincta* model ***(Chapter Five)***. In the first experiment the effect of protein and energy nutrition on the degree of PPRI was assessed. This was achieved by the restricted feeding of diets at two levels of energy and four levels of protein supply **(Chapter Two)**. In the second experiment, the rate at which improved host nutrition can restore immunity to parasites was assessed. This was achieved by overcoming nutrient scarcity through reducing litter size, followed by assessment of immune responses and parasitism over time **(Chapter Three)**. The third experiment assessed the impact of selected amino acid deficiency on the extent of PPRI **(Chapter Four)**. In the last experiment the effects of feeding two types of protein source with variable DUP to MP ratios on the degree of PPRI was evaluated **(Chapter Five)**.

The specific objectives of this PhD project were:

1) To independently investigate the effects of protein and energy nutrition on PPRI to GIN ***(Chapter Two)***.
2) To assess the rate of improvement of immunity following a reduction of nutrient scarcity in GIN periparturient hosts ***(Chapter Three).***
3) To assess the implication of specific AA deficiencies on PPRI to GIN ***(Chapter Four).***
4) To evaluate the effects of feeding two types of protein sources with variable DUP levels on PPRI in ruminant hosts ***(Chapter Five).***

The hypotheses for these objectives are as follows:

(1) Hypothesis 1: Increasing levels of protein intake at the same level of energy intake will result in improved resistance to GIN. As protein increases, more amino-acids will be available to the immune cells for the provision of energy as well as substrates for the synthesis of proteinaceous macromolecules, which will improve resistance. On the other hand, increasing the level of energy intake at a constant level of protein intake will not result in substantially enhanced immunity. This is expected because immune cells preferentially use amino-acids as a fuel and as substrates for the synthesis of proteinaceous molecules (Yacoob and Calder 1999) and energy will be less limiting for the lactating host relative to protein.

(2) Hypothesis 2: Overcoming nutrient scarcity in lactating hosts on low nutrient (protein) diets through reduction of nutrient demand will rapidly result in an improvement in the expression of acquired immunity relative to animals that

are maintained under high nutrient demand, and thus nutrient (protein) scarcity.

(3) Hypothesis 3: Deficiency of specific amino acids with implicated roles in immune responses in general, and in (local) immunity to GIN in particular, will result in penalties in the degree of PPRI. This will be a consequence of reduced expression of immunity due to a reduction in the utilization of ingested protein and/or to a reduced availability of these specific AA for the development of a protective immune response.

(4) Hypothesis 4: The higher the DUP to MP ratio, in diets of periparturient ruminant hosts, the stronger the reduction in PPRI. This is because high MP diets with low levels of DUP will result in post ruminal supplementation of amino acids from microbial protein mainly, which greatly differ from AA demands of the host for the expression of immunity (Houdijk & Athanasiadou, 2003).

Each of the following chapters deals with a separate objective and is presented in the form of a self-contained paper including a brief introduction, and a comprehensive discussion. The chapters are then summarized in the general discussion, where the findings of all experiments are linked within the overall context of the PhD. The general discussion will also critically evaluate the research and suggest how future research using the model could be directed.

Chapter 2. Dietary protein and energy supplies differentially affect resistance to parasites in lactating mammals

2.1. Summary

Periparturient relaxation of immunity (PPRI) to parasites in mammals results in higher worm burden and worm egg excretion and may have a nutritional basis. *Nippostrongylus brasiliensis* re-infected lactating rats fed low crude protein (CP) diets show an augmented degree of PPRI compared with their high CP fed counterparts. However, such effects of CP scarcity have been confounded by metabolisable energy (ME) scarcity due to increased intake of the high CP foods. Here, we independently assessed the effects of dietary CP and ME scarcity on the degree of PPRI. Second parity rats were infected with *N. brasiliensis* larvae before mating. Upon parturition, dams were allocated to one of six feeding treatments (1–6), consisting of two levels of dietary ME supply, each with three levels of CP supply. On day 2 of lactation, dams were either re-infected with 1600 *N. brasiliensis* larvae or sham-infected with PBS, while litter size was standardised at ten pups. Dams and litters were weighed daily until either day 8 or 11 of lactation, when worm burdens were assessed as a proxy for PPRI. Increased CP and ME supply independently improved lactational performance. While ME supply did not affect parasitism, increasing CP supply reduced worm burden and the percentage of female worms in the small intestine; the latter was especially pronounced at the lower level of ME supply. The present results support the view that PPRI to parasites may be sensitive to CP scarcity, but not to moderate ME scarcity.

Keywords: energy intake, lactation, nematodes, protein nutrition, rats

2.2. Introduction

During their life cycle, mammals acquire immunity towards gastrointestinal nematode (GIN) parasites, as they are continuously exposed to their infective forms. However, the effective expression of this acquired immunity can break down during certain points in time, e.g. during the periparturient period (Houdijk *et al.,* 2001a). Such a periparturient relaxation of immunity (PPRI) plays an important role in the epidemiology of GIN infections. PPRI is associated with a higher GIN burden and nematode egg excretion (Barger, 1993; Beasley *et al.,* 2010) and as such the periparturient host is a major source of infection for its parasite-naïve offspring.

A nutrient partitioning framework has identified the hypothesis that PPRI has a nutritional basis: scarce nutrients may be preferentially used to meet the elevated nutritional demands of the reproductive effort at the expense of the immune response during the periparturient period (Coop & Kyriazakis, 1999). Consequently, at times of nutrient scarcity, increased nutrient supply would be expected to reduce the degree of PPRI to parasites and lead to reduced worm burden and worm egg excretion. There is indeed a significant body of evidence to support the view that protein supplementation in mammals, such as small ruminants, reduces the degree of PPRI (Houdijk & Athanasiadou, 2003; Kyriazakis & Houdijk, 2006). Similarly, feeding foods with higher crude protein content to lactating rodents exposed to the GIN *Nippostrongylus brasiliensis*, has also resulted consistently in reduced worm burdens (Normanton *et al.,* 2005; Houdijk *et al.,* 2005a; Jones *et al.,* 2009). However, in the above studies, the animals were fed *ad libitum* and the increased dietary protein contents resulted in increased feed intake *per se*, which confounded the effects of protein supply with other dietary factors such as energy supply. It has been suggested

that dietary protein and energy deficiency may have different effects on host defence mechanisms (Koski & Scott, 2001), whilst evidence from ruminant studies suggests that moderate protein, but not energy deficiency may increase the degree of periparturient parasitism (Donaldson *et al.,* 1998). In ruminants, however, effects of energy and protein nutrition may be difficult to assess separately due to the modifying role of the rumen (Houdijk *et al.,* 2001). Since such confounding effects can be readily avoided in non-ruminants, our objective was to dissect, for the first time in a monogastric model, the effects of moderate energy and protein restriction on PPRI to GIN parasites. We hypothesized that at times of dietary protein and energy scarcity, increasing protein supply would be more effective in reducing the degree of PPRI than increasing energy supply.

2.3. Materials and Methods

2.3.1. Animals, housing and feeding strategy during gestation

The experiment described below was approved by the Scottish Agricultural College Ethics Review Committee (EDAE 24/2007) and carried out under Home Office authorisation (PPL 60/3626). One hundred and sixteen second-parity female rats were housed in a room, where ambient temperature was maintained at 21^0C, relative humidity ranged from 45 to 65% and artificial lighting was provided between 08:00 and 18:00 hours. The rats were individually housed in solidbottomed cages, with fresh sawdust provided weekly. Shredded plastic bubble wrapping for nesting material was provided 3 d before the expected parturition date. Wirebottomed cages were used during mating and for faeces collection during the primary infection, as described previously (Houdijk *et al.,* 2003a). For mating,

female rats were placed with a proven male breeder and mating was confirmed through the presence of a vaginal plug. Until mating was confirmed, the rats were given ad libitum access to standard rat chow (Rat and Mouse no. 3; Special Diet Services, Witham, Essex, UK). After mating was confirmed, the rats were given ad libitum access to a high-protein food, with 210 g digestible CP and 16.4 MJ metabolisable energy (ME)/kg DM until 10 d into gestation to allow for establishment of pregnancy and placental development. The rats were then fed with a low-protein food, containing 60 g CP and 17.3 MJ ME/kg DM, which was continued until parturition. This feeding protocol was used to reduce body protein reserves during the second half of gestation in order to maximise the degree of protein scarcity during lactation when the rats would be fed low protein foods (Pine *et al.,* 1994; Houdijk *et al.,* 2005a).

2.3.2. Feeding treatments

Six feeding treatments (1-6) were designed, consisting of restrictedly feeding one of two levels of predetermined ME supply at one of three levels of predetermined CP supply (Figure 2.1). Feeding treatments 1 to 3 and 4 to 6 were calculated to supply 1.05 and 1.40 MJ ME per kg parturition body weight (PBW) per day, respectively. These planes of ME nutrition were 90% of previously observed mean ME intakes on low and high protein foods respectively (Jones *et al.,* 2009). In addition, CP supply was calculated to increase incrementally from scarce to more than adequate at each level of ME supply. This was 6.6, 13.3 and 19.9 g per kg PBW per day for the low ME level, and 13.3, 19.9 and 26.5 g per kg PBW per day for the high ME level treatments. These CP allowances were chosen to reflect the range of

achieved mean daily CP intake on similar foods in previous experiments under *ad libitum* feeding (Normanton *et al.,* 2005; Houdijk *et al.,* 2005a; Jones *et al.,* 2009) using dam PBW as a scaling factor. Variation in dietary CP content was achieved through the isoenergetic exchange of casein with digestible carbohydrates (starch/sucrose). The resulting composition of the experimental foods is presented in Table 2.1.

Foods were offered on a daily basis in increasing amounts during lactation, reflecting the natural increase in food intake observed in previous experiments with this animal-parasite model system (Normanton *et al.,* 2005; Houdijk *et al.,* 2005a; Jones *et al.,* 2009). We omitted *a priori* feeding treatments with very low or high CP to ME ratios that would jeopardise food intake, as earlier work has shown that voluntary intake on such foods was unacceptably low in lactating animals (Oldham & Friggens, 1989; Friggens *et al.,* 1993). Thus, our experimental design can be described as having an incomplete 4 x 2 factorial arrangement (Figure 2.1).

Table 2.1. Composition and analysis of the experimental food used during lactation

Experimental foods	1	2	3	4	5	6
Ingredients (g/kg fresh)						
Casein	102	204	306	153	229	306
Methionine	1	2	3	2	2	3
Starch	343	275	206	309	257	206
Sucrose	172	137	103	154	129	103
Corn oil	141	141	141	141	141	141
Vit	47	47	47	47	47	47
Min	47	47	47	47	47	47
Cornflour	46	46	46	46	46	46
Choline	7	7	7	7	7	7
Lecithin	2	2	2	2	2	2
Alphacel	94	94	94	94	94	94
Analysed chemical composition						
DM (g/kg fresh matter)	715.2	656.5	604.0	696.6	644.4	612.5
ME (MJ/kg DM)*	15.9	15.9	15.9	15.9	15.9	15.9
CP (g/kg DM)	99.0	191.0	285.0	142.0	213.0	285.0
Ash (g/kg DM)	38.3	38.4	40.6	38.5	39.0	40.6
Acid detergent fibre (g/kg DM)	74.7	78.7	91.8	74.8	78.7	91.8
Diethyl ether extract (g/kg DM)	162	158	154	154	153	154

* Food ME contents was calculated by multiplying its contents of protein (casein), digestible carbohydrates (starch, sucrose, corn flour) and fat (corn oil) with the ME contents of protein (17 MJ/kg), carbohydrates (17 MJ/kg) and fat (38 MJ/kg) (Astrup & Tremblay, 2009)

2.3.3. Infection protocol

All rats received a primary infection of 1600 third-stage infective larvae (L_3) of *N. brasiliensis* on day –37 (with day 0 as mean achieved parturition date), which were suspended in 0.5 ml sterile phosphate buffered saline (PBS) that was subcutaneously injected in the hind leg. A secondary infection of 1600 L_3 *N. brasiliensis* was administered on day 2 to a sub-group of rats. At the same time, control rats (primary infected only) were sham-infected through subcutaneous injection of 0.5 ml sterile PBS only.

2.3.4. Experimental design

The effects of the six feeding treatments used were assessed on lactational performance and parasitological variables on day 8 and 11 of lactation (corresponding to days 6 and 9 post secondary infection, respectively). The two sampling time points post secondary infection were included, as previous data (Jones *et al.,* 2009) suggested that the nutritional sensitivity of host resistance may differ over time. All rats in these 12 feeding treatment-endpoint combinations (6 feeding treatments x 2 endpoints) received a secondary infection. In addition, control rats that did not receive a secondary infection were included until day 8 of lactation for each feeding treatment to assess the effect of re-infection on lactational performance (dam and litter weight gain), i.e. six additional control treatments. This resulted in a total of 18 treatments. Rats blocked for PBW, were randomly allocated to these 18 treatments on the morning parturition was observed. Total sample size aimed for was n=7 for infected rats and n=6 for control rats. However, the minimum realized sample size for each of the 18 resulting treatments was n=5.

2.3.5. Measurements

Body weight and food intake. Rats were weighed daily throughout the experiment, and daily weights taken post parturition were used to calculate lactational dam weight gain. The pups were counted and the whole litter weighed daily from day 0 of lactation. Litter size was standardized at 12 pups on day 1 and this was further reduced to 10 pups on day 2 of lactation to ensure pup survival and to have equal initial nutrient demands. Litter weights from day 2 onwards were used to calculate litter weight gain during lactation. Because of the restricted feeding regime, refusals were not expected during lactation. However, food intake was measured daily and any refusals observed during the lactation period were weighed in order to calculate mean achieved CP and ME intake. Foods offered during lactation were sampled during their preparation for the analysis of DM, CP (Kjeldahl-N x 6.38), diethyl ether extract, ash and acid detergent fibre (Table 2.1).

Faecal egg counts. A faecal egg count (FEC, in eggs per gram (epg) of fresh faeces)) was performed 7 days after the primary infection (day -30) to confirm the presence of infection. A second FEC was performed on day -23 to confirm that the expected parasite expulsion had taken place. To this effect, faeces were collected through overnight housing on bottom-wired cages (Houdijk *et al.,* 2003a) and FEC were performed using a modified saturated salt flotation method (Christie & Jackson, 1982).

Worm burden and nematode eggs in colon. Rats were killed humanely through gradually increasing ambient CO_2 concentration followed by CO_2 asphyxiation for parasitological assessment (see below) and immune responses (Jones *et al.,* 2011). The small intestine was removed and stored in formaldehyde for subsequent worm

burden assessment (number, sex and stage of maturity according to worm morphology). The contents of the large intestine were weighed and assessed for worm eggs as described for FEC above. This was then multiplied by the weight of large intestinal contents to obtain the total number of nematode eggs in the colon (EIC). The latter measure is preferred over the FEC equivalent of the colon contents, as feeding treatments used could have biased colon contents volume, and thus the concentration of eggs in the colon contents.

2.3.6. Calculations and statistical analysis

Data were analysed using Restricted Maximum Likelihood (REML), to account for the incomplete 4 x 2 factorial design of the CP-ME combinations used. Effects of CP supply, ME supply, endpoint and their 2 and 3-way interactions were assessed on dam body weight gain, litter weight gain, worm burdens, percentage of female worms and EIC. In order to investigate for the effect of infection *per se,* a separate REML analysis was used to assess effects of CP supply, ME supply, infection status and their 2- and 3-way interactions on dam body weight gain and litter growth until day 8 of lactation. For both statistical assessments, interactions that did not approach significance at $P<0.05$ were omitted from the final models used.

Because of their skewed nature, the EIC and worm burdens were transformed according to log (n + 1) to normalize data before statistical analysis. These data are reported as back-transformed means with their 95% CI as described previously (Houdijk *et al.,* 2005a). All statistical analyses were performed using Genstat 11 for Windows release 11.1, 2008 (VSN international, Hemel Hempstead, UK).

2.4. Results

2.4.1. Faecal egg counts during the primary infection and performance until parturition

FEC taken on day -37 and day -30 averaged 3563 (CI: 351-319) epg and 0 (CI: 0-0) epg, respectively. Initial mean body weight was 345 (SE 4.0) g. During the first 10 days of gestation, rats grew from 355 (SE 3.6) to 383 (SE 4.3) g and had an average DM intake of 20.6 (SE 0.40) g/d. From then onwards and until parturition, the pregnant rats continued to gain weight to a mean of 451 (SE 5.1) g and had an average DM intake of 18.7 (SE 0.51) g/d, which dropped to an average of 7.1 (SE 0.62) g/d just before parturition. Dam mean PBW averaged 360 (SE 4.6) g, mean litter size averaged 14.2 pups (SE 0.27) g and mean litter weight averaged 74.1 g (SE 0.99) g.

2.4.2. Achieved ME and CP intake

Figure 2.1 shows the expected and achieved least squares mean intake of ME and CP over the 11 day lactating period per kg PBW per day. Small amounts of feed refusals were observed for each feeding treatment. Consequently, target CP and ME supply were not exactly met. However, as intended, feeding treatment did not significantly affect achieved ME intake, within the low (P=0.130) and the high level of ME supply (P=0.897). Infection did not affect mean achieved ME intake (P=0.290) and CP intake (P=0.738).

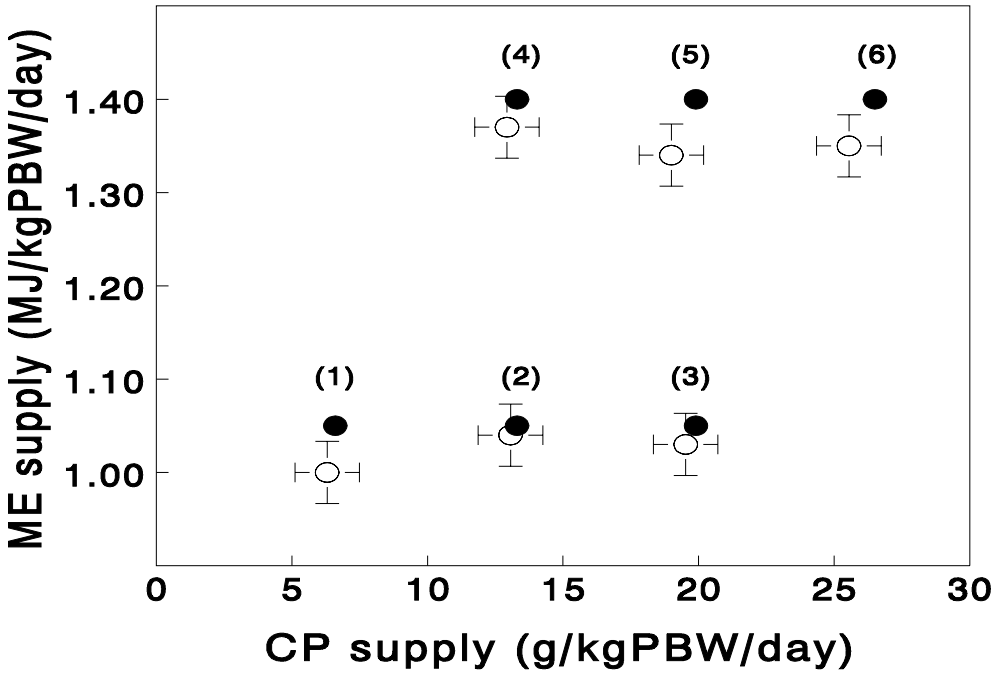


Figure 2.1. Planned (closed circles) and achieved (open circles, with s.e.) dietary supply of metabolisable energy (ME, MJ/kg parturition body weight/day) and crude protein (CP, g/kg parturition body weight/day) from 6 experimental feeding treatments over the first 11 days of lactation in rats, re-infected with *Nippostrongylus brasiliensis* (PBW: parturition body weight).

2.4.3. Dam weight gain

Figure 2.2 shows the effects of feeding treatments (Figure 2.2a) and infection for each feeding treatment (Figure 2.2b) on daily dam weight gain. The dam weight gain was significantly affected by dietary CP supply ($P<0.001$) and ME supply ($P<0.001$), but not by endpoint ($P=0.401$; data not shown). Dams at the low level of ME supply lost more weight than dams at the high level of ME supply. Increasing level of CP supply resulted in increased dam weight gain at both levels of ME supply (Figure 2.2a). Infection did not affect dam weight gain (Figure 2.2b; $P=0.494$), whilst no significant interactions between any of the factors involved were observed ($P>0.10$).

2.4.4. Litter weight gain

Figure 2.3 shows the effects of feeding treatments (Figure 2.3a) and infection for each feeding treatment (Figure 2.3b) on daily litter weight gain. Litter weight gain was affected by CP supply ($P<0.001$) and ME supply ($P=0.011$), but not by endpoint ($P=0.247$; data not shown). Litters from low ME gained less weight than litters from high ME. Increasing level of CP supply resulted in higher litter weight gain at both levels of ME supply (Figure 2.3a). Infection status (Figure 2.3b) significantly affected litter weight gain ($P=0.015$). Although the interaction between CP supply, ME supply and infection status was not significant ($P=0.113$), litters of infected dams allocated to feeding treatments 1 and 2 showing lower weight gain than their non infected counterparts.

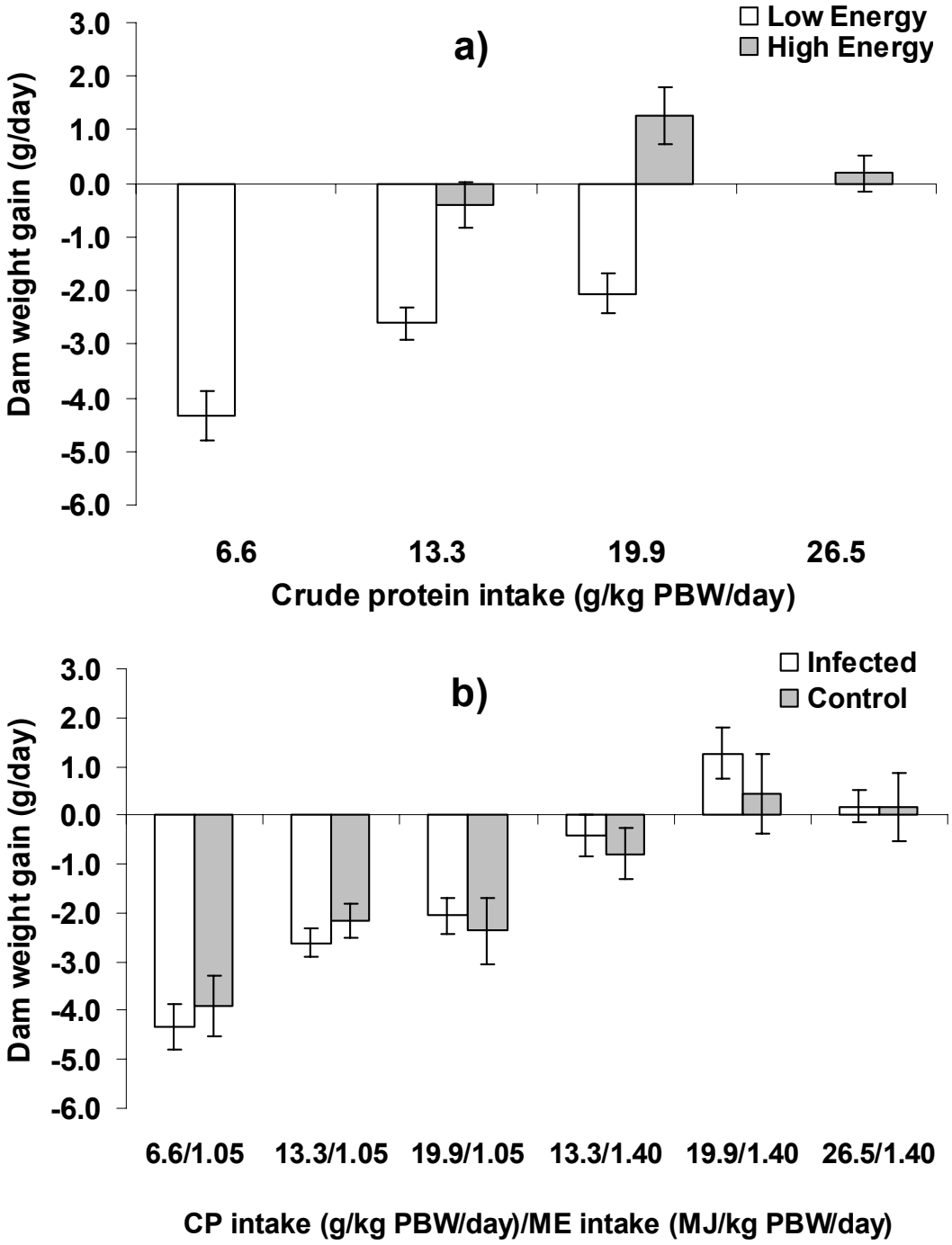


Figure 2.2. Dam weight gain (with s.e.) across four levels of dietary supply of crude protein (CP) and two levels of metabolizable energy (ME, 1.05 and 1.40 MJ/kg parturition body weight/day) over the first 11 days of lactation in rats, re-infected with *Nippostrongylus brasiliensis* (a), and across these feeding treatments for infected and non infected control lactating rats (b).

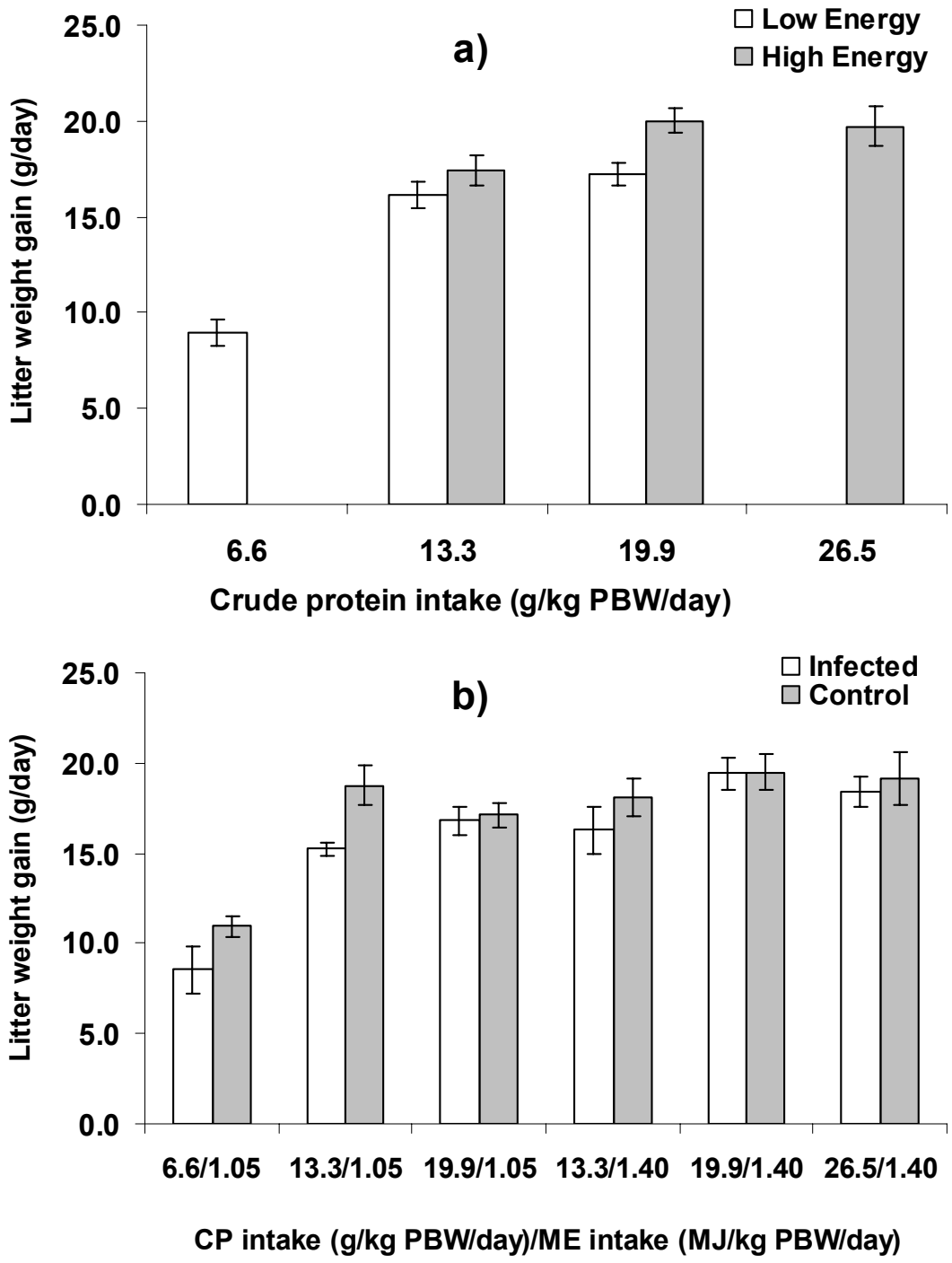


Figure 2.3. Dam weight gain (with s.e.) across four levels of dietary supply of crude protein (CP) and two levels of metabolizable energy (ME, 1.05 and 1.40 MJ/kg parturition body weight/day) over the first 11 days of lactation in rats, re-infected with *Nippostrongylus brasiliensis* (a), and across these feeding treatments for infected and non infected control lactating rats (b).

2.4.5. Total worm burden, eggs in colon and worm burden composition

Figure 2.4 shows the effects of feeding treatments on total worm burden for day 8 (Figure 2.4a) and day 11 of lactation (Figure 2.4b), whilst Figure 2.5 shows such effects for EIC on the same days. Total worm burdens were significantly affected by CP supply (P=0.023) and endpoint (P=0.013), but were not influenced by ME supply (P=0.155). Worm burdens decreased with increasing level of CP supply at both levels of ME supply and were lower on day 11 than day 8 of lactation (Figures 2.4b and 2.4a). Although Figure 2.4 suggests that these effects of CP supply were more pronounced on day 8 than on day 11, this interaction was not significant (P=0.248). EIC were not affected by dietary CP (P=0.204) and ME (P=0.720) supply, which were significantly higher on day 11 than on day 8 (Figures 2.5a and 2.5b; P=0.004). There were no significant interactions between any of the above factors for EIC (P>0.10). Figure 2.6 shows the percentage of female worms in the worm burdens. ME supply (P=0.470) and endpoint (P=0.161) did not affect worm burden composition. However, increasing CP supply reduced the percentage of female worms (P=0.049), whilst the magnitude of this effect was greater at the lower level of ME supply (P=0.022; Figure 2.6).

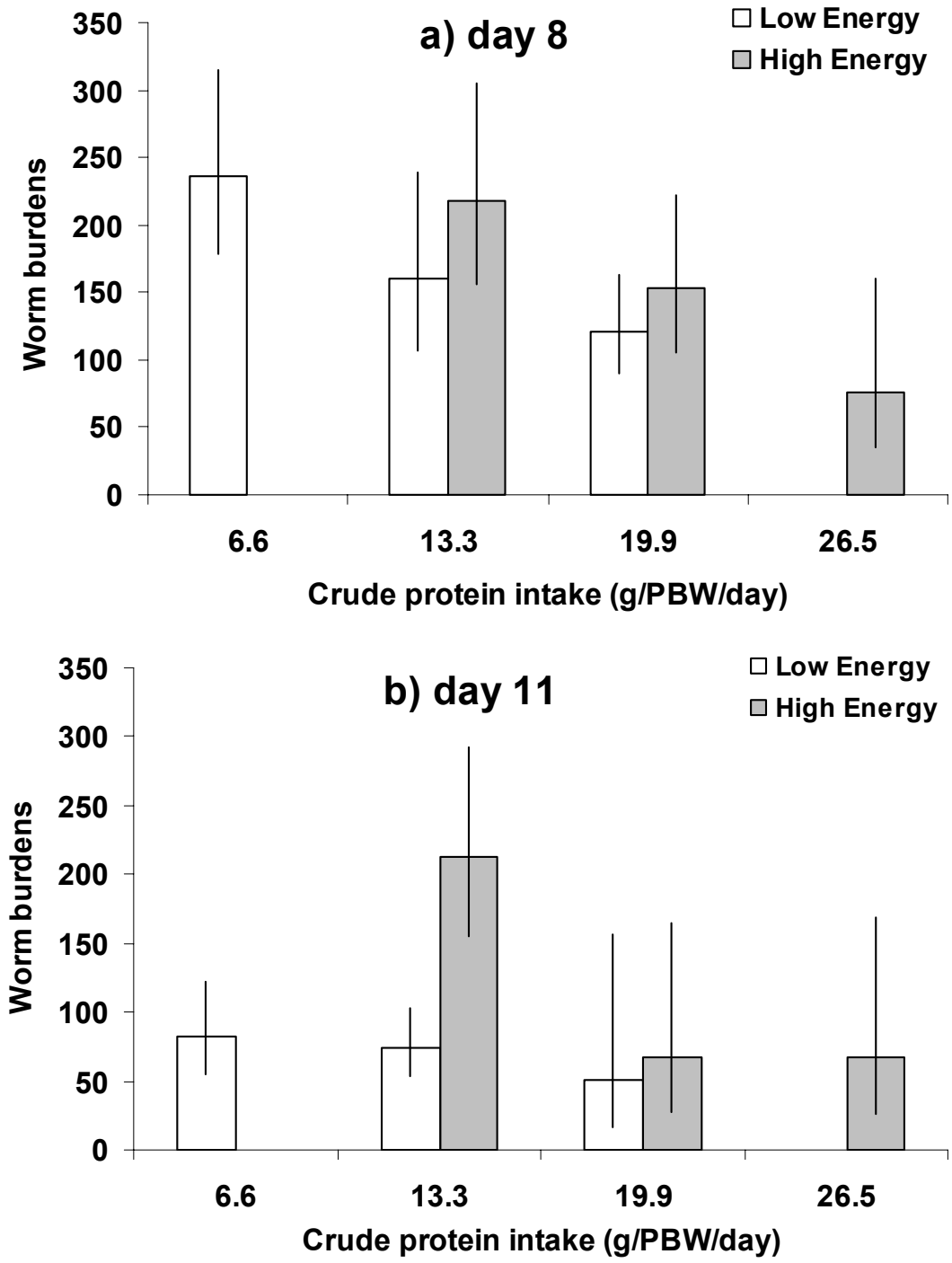


Figure 2.4. Backtransformed mean worm burdens, with backtransformed lower and upper limits of transformed error bars as 95% CI, taken on day 8 (4a) and 11 (4b) following reinfection with *Nippostrongylus brasiliensis* on day 2 of lactation in rats fed four levels of dietary crude protein (CP) and two levels of metabolizable energy (ME) supply (1.05 and 1.40 MJ/kg parturition body weight/day).

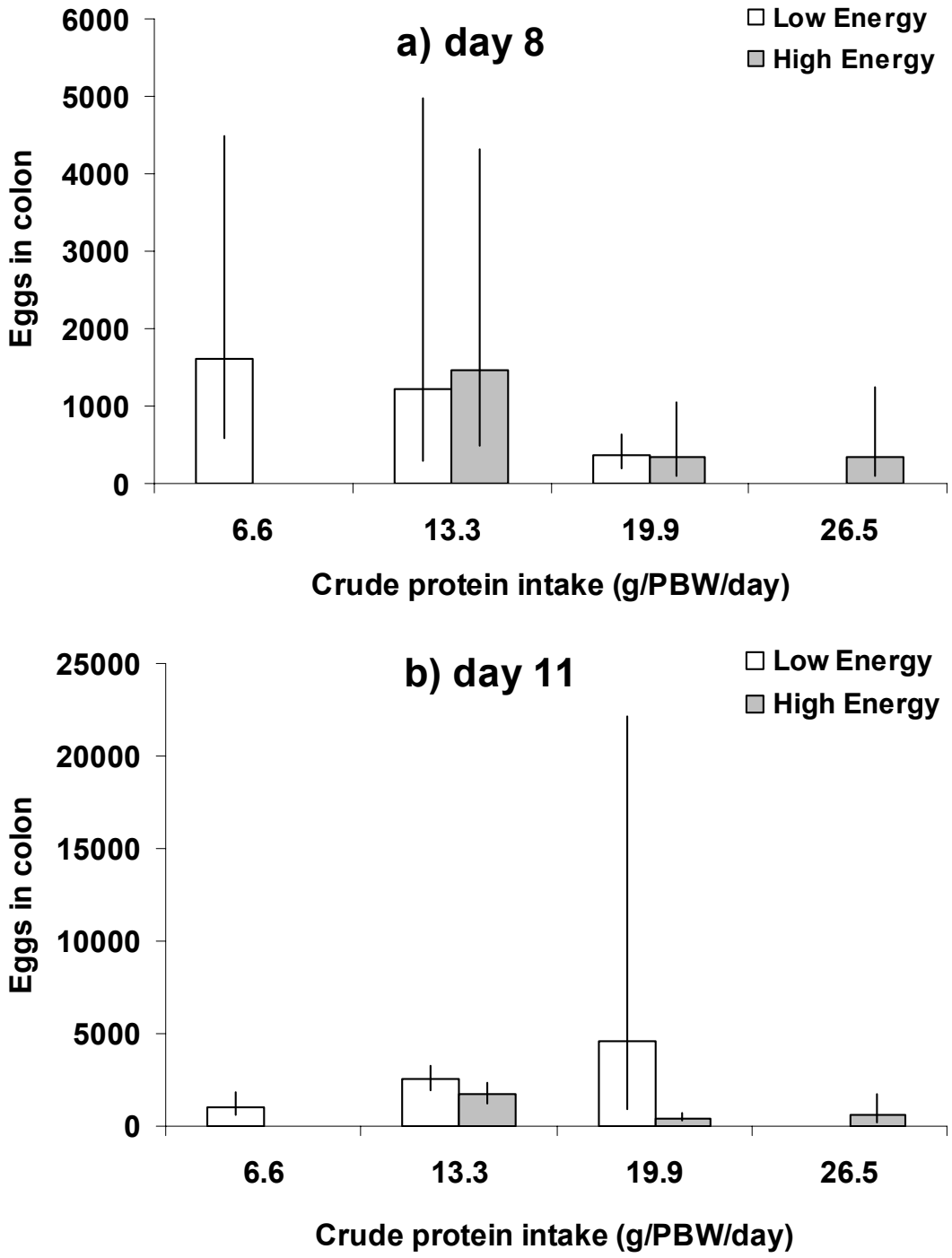


Figure 2.5. Backtransformed mean total eggs in colon, with backtransformed lower and upper limits of transformed error bars as 95% CI, taken on day 8 (4a) and 11 (4b) following reinfection with *Nippostrongylus brasiliensis* on day 2 of lactation in rats fed four levels of dietary crude protein (CP) and two levels of metabolizable energy (ME) supply (1.05 and 1.40 MJ/kg parturition body weight/day).

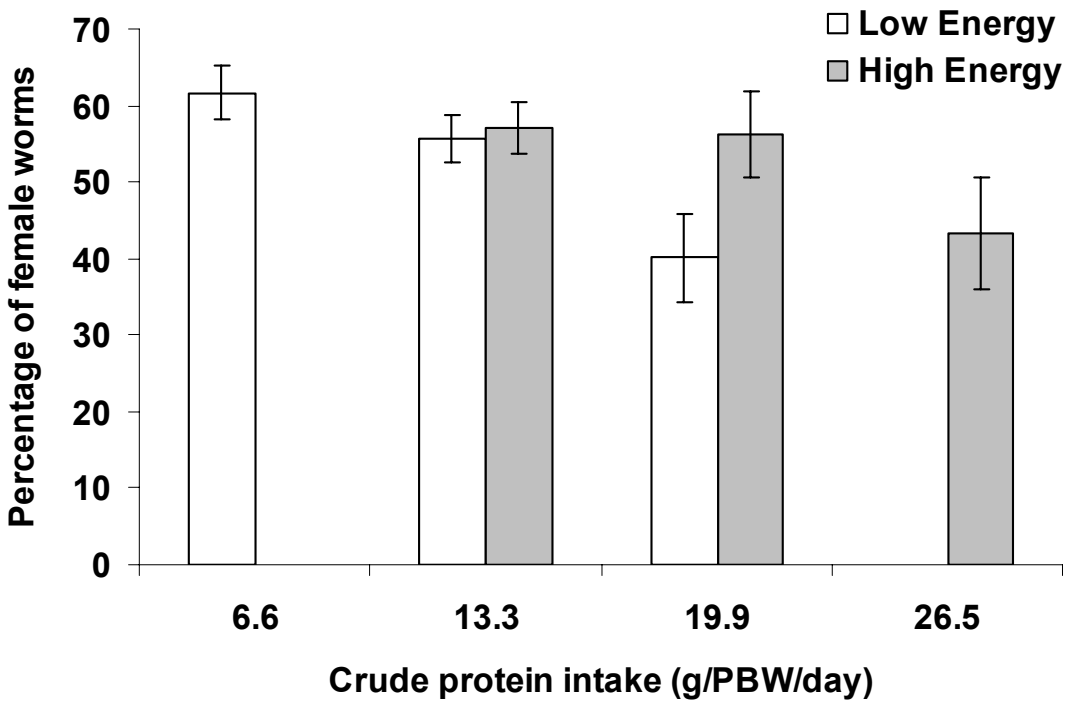


Figure 2.6. Percentage of female worms (with s.e.) in worm burdens arising from reinfection with *Nippostrongylus brasiliensis* in lactating rats at four levels of dietary crude protein (CP) and two levels of metabolizable energy (ME) supply (1.05 and 1.40 MJ/kg parturition body weight/day).

2.5. Discussion

Previous studies carried out with this rodent-nematode parasite model have consistently shown that reducing nutrient scarcity through increasing dietary CP content during lactation results in a reduced degree of parasitism (Normanton *et al.,* 2005; Houdijk *et al.,* 2005a; Jones *et al.,* 2009). In these studies, *ad libitum* feeding of high CP foods resulted in a reduced worm burden, but because food intake on the high CP foods increased, improved resistance could have arisen from the increased intake of any nutrient and/or energy. The present experiment aimed to account for this uncertainty by testing the hypothesis that parasitism during lactation is sensitive to CP supply *per se* by increasing levels of dietary CP supply at constant levels of ME supply. At the same time, the design allowed us to assess whether a moderate dietary ME restriction compromises lactating host resistance to parasites. We discuss our result that CP supply, but not ME supply, affect lactational resistance to *N. brasiliensis*. It is unlikely that the incomplete 4 x 2 factorial design used (Figure 2.1) biased our conclusions; re-analysis of the data omitting those arising from feeding treatment 1 and 6, thus addressing our hypothesis with a balanced 2 x 2 factorial design, confirmed that worm burdens were affected by increased CP supply (P=0.035) and not by ME supply (P=0.174), and although effects of CP supply on worm burdens were again more pronounced on day 8 than on day 11, as in the incomplete factorial design there was no formally significant interaction between CP supply and time (p=0.063).

The experimental design created the required conditions in order to test our hypotheses. In the present experiment, restricted feeding at 90% of the achieved feed intake observed in previous experiments, avoided the increased feed intake, which

has been observed when animals are offered foods with higher CP content. The success of the experimental design is further evidenced by the small amount of food refusals that occurred across feeding treatments and the consequently similar level of ME intake at increasing levels of CP intake, within each of the two different planes of ME supply (Figure 2.1). With regards to the performance data, the lower level of ME supply resulted in higher dam weight loss and lower litter gain. This is consistent with previous findings on the effects of energy nutrition on lactational performance of rats (Kliewer & Rasmussen, 1987; Passos *et al.,* 2000). At the same time, increased CP supply resulted in smaller dam weight loss and larger litter weight gain. Since the last increment of CP supply did not further increase lactational performance, the range of CP supply achieved ranged from scarce to more than adequate. However, it can not be discounted that the ME supply was still limiting at the highest level of ME supply used, as only two levels of ME were used.

Lactation imposes significant protein demands on mammals (Friggens *et al.,* 1993). At times of dietary protein scarcity, additional protein may be derived from labile body protein. However, the amount of labile body that can be mobilised is limited (Pine *et al.,* 1994), and this limitation was further exacerbated by feeding low protein food during late gestation. At the same time, parasitized hosts would be expected to have additional protein requirements to activate or maintain an effective protective response to parasites (Kyriazakis & Houdijk, 2006). This is because the effector mechanisms of the immune response are highly proteinaceous in nature and inflammation and immune system activation are characterised by the synthesis of specific proteins that play crucial roles in the defence of the host against pathogens and the modulation of the immune response (Coop & Kyriazakis, 2001; Le Floc'h *et*

al., 2004). Thus, feeding foods with a sufficiently low CP content to lactating parasitized hosts, as in the present experiment, would be expected to result in protein scarcity, with penalties on both lactational performance and expression of immunity to parasites. We considered changes in the number of adult nematodes in the small intestine and EIC to be the result of changes in the degree of expression of immunity to *N. brasiliensis*, as they provide the ultimate measure on how the host copes with the challenge (Normanton *et al.,* 2007). Increasing dietary CP supply resulted in a significant reduction in worm burdens. This agrees with results from studies with periparturient mammalian hosts including rats (Houdijk *et al.,* 2005a; Jones *et al.,* 2009), ewes (Donaldson *et al.,* 2001; Kahn *et al.,* 2003a; Houdijk *et al.,* 2003b; Houdijk *et al.,* 2005b) and dairy goats (Etter *et al.,* 1999; Chartier *et al.,* 2000). However, to our knowledge, the current experiment is the first to have assessed independently the effects of CP and ME supply on resistance to parasites in a non-ruminant periparturient model.

Increased CP supply did not affect EIC, although there was a substantial decrease at the higher levels of CP supply. The absence of a significant nutritional sensitivity of EIC in the presence of such effects on total worm burdens concurred with substantially larger variation (see Figure 2.5). Whilst it can not be excluded that this may have arisen to some extent from the lower than expected number of replicates, and variation in fecundity in response to dietary treatments, we have discussed previously that this may arise from the relatively large numbers of steps involved in the nematode egg counting technique used (Houdijk *et al.,* 2003a). Perhaps, to some extent, this variation could have been reduced through repeated measures of FECs and assessment of daily nematode egg excretion during secondary

infection. However, earlier attempts suggest that the highly digestible nature of the semi-synthetic foods used would not have allowed consistent production of relatively large volumes of faeces required for such purpose (Normanton *et al.,* 2007).

In addition to reducing worm burdens, the increasing level of CP supply resulted in a reduced percentage of female worms in the worm burdens, especially at the lower level of ME supply. Increased CP supply reduced the percentage of female worms not only in earlier studies using the same host-parasite system (Normanton *et al.,* 2007), but also following protein supplementation of sheep infected with *Haemonchus contortu*s (Wallace *et al.,* 1995). It has been shown that female adult nematodes are usually expelled at a higher rate than male adult nematodes (Africa, 1931; Jarrett *et al.,* 1968) and that rats infected solely with female adult *N. brasiliensis* develop a stronger immunity than rats infected with male worms (Ogilvie, 1965). Taken together, these observations suggest that expression of immunity may be more effective towards female than towards male *N. brasiliensi*s (Houdijk *et al.,* 2003a). Since female worms are longer and heavier than male worms (Kassai, 1982), it would be reasonable to assume that the mean worm length and weight of the worm burdens in the dams receiving lower protein was larger than in the dams fed higher levels of protein. For abomasal infections in sheep, it has been suggested that hosts control worm mass rather than worm number, since longer and heavier worms may cause more damage to the host (Stear *et al.,* 1999). If such arguments hold for small intestinal infections as well, then having a worm burden with shorter and lighter worms would have been beneficial to the rats fed higher levels of protein.

In the present experiment, moderate ME restriction imposed during lactation did not affect worm burdens, the worm sex ratio or the number of nematode EIC. Studies in ruminants have likewise shown that gastrointestinal parasitism is not sensitive to moderate changes in ME supply (Bown *et al.,* 1991; Donaldson *et al.,* 2001). Activation and maintenance of an immune response are expected to be energetically demanding processes (Colditz, 2008) and immune cells require high levels of glucose (Fox *et al.,* 2005). This has been evidenced by the lower amount of fat deposits in infected mice compared to their uninfected counterparts (Kristan & Hammond, 2000; Coltherd *et al.,* 2009). It should be noted that the high energetic demands for milk synthesis in lactating mammals can, at least to some degree, be met through mobilization of maternal adipose tissue (Oftedal, 2000). An energy restriction during lactation results in increased body fat mobilization in many species of mammals, including rats (Cowan *et al.,* 1980; Brendemuhl *et al.,* 1987; Brendemuhl *et al.,* 1989; Parmley *et al.,* 1996; del Rosario Ayala *et al.,* 2006). It cannot be excluded that the effects of dietary ME supply on worm burden were not observed because the likely higher rate of body fat mobilisation in the low-ME dams may have provided sufficient ME to overcome ME scarcity for the expression of immunity. The effects of energy (caloric) restriction on resistance to parasites have also been assessed in growing rodents (Lunn *et al.,* 1988; Koski *et al.,* 1999; Kristan, 2007). In contrast to our findings, energy restriction was consistently associated in these studies with higher worm burden. Careful consideration of the extent and the duration of the energy restriction imposed in these experiments suggests that these were substantially greater and longer than those used in the present study. For example, Koski *et al*. (1999) applied an energy restriction of up to 25% for 6 weeks;

Kristan (2007) imposed an energy restriction of 40% for almost 7 months, and Lunn *et al*. (1988) used a caloric restriction of 40% for 3 weeks. In contrast, we imposed a moderate ME restriction of 25% for no longer than 11 d. Growing animals, when nutritionally restricted, mobilise fat while growing protein or retaining their protein reserves (Kyriazakis & Emmans, 1992a; Kyriazakis & Emmans, 1992b) while severe and prolonged feed restriction leads to combined fat and protein losses (Hornick *et al.,* 2000). Thus, we cannot exclude the possibility that a more severe ME restriction could also have affected host resistance to parasites in our model system.

Re-infection with *N. brasiliensis* did not affect achieved DM intake and maternal body weight gain but reduced litter body weight gain (a proxy for milk production) during lactation with the consequence of infection on litter body weight being more pronounced for feeding treatments 1 and 2 (Figure 2.3b). This suggests that infection can reduce lactational performance provided that the level of protein-energy malnutrition is sufficiently low. Indeed, at higher levels of ME intake**,** re-infection with *N. brasiliensis* did not affect litter weight gain (Houdijk *et al.,* 2005a; Jones *et al.,* 2009). Anorexia has not been observed in the aforementioned studies using the same host parasite model, which is consistent with the view that it mainly characterizes primary infections (Kyriazakis *et al.,* 1998; Kyriazakis, 2010). The absence of a systematic effect of infection on litter weight in the rats during lactation supports the view that scarce nutrient allocation to milk production takes priority over expression of immunity in parasite-immunised periparturient mammals (Coop & Kyriazakis, 2001). This is because in the converse situation, infection would have been expected to penalise litter growth in any nutritional environment, and not only when both CP and ME supply are sufficiently low (treatments 1 and 2) to reduce

resilience (Houdijk *et al.,* 2005a). Variable effects of nematode infection on food intake and/or dam and pup performance have been observed in other host parasite systems (Willis & Poulin, 1999; Kristan, 2002; Odiere *et al.,* 2010). However, these experiments are not considered comparable to the present experiment, as they involved parasite-naıve animals. Such hosts are expected to prioritise resource allocation to cover the nutritional requirements of expression of immunity towards parasites over those of maintenance and growth (Kyriazakis & Houdijk, 2006).

In conclusion, the present results support the view that the degree of PPRI to *N. brasiliensis* is sensitive to changes in protein but not moderate energy supply. This is shown by a decrease in the worm burden, which was achieved in the present experiment with increasing levels of protein intake. Future work may focus on the involvement of specific, essential amino acids in these responses, as there is an increasing body of evidence for amino acid-specific effects on host immune responses (Li *et al.,* 2007). Increasing CP intake resulted in a reduction of the percentage of female worms in the remaining worm burden, which could suggest a more effective expression of immunity towards female worms. This could be beneficial to hosts, as female worms are larger and heavier than male worms and may cause more damage to parasitised hosts. The performance data suggest that secondary parasitic infections may have significant effects on host lactational performance, especially at times of protein-energy malnutrition.

Chapter 3. Effects of reducing nutrient demand on the restoration of immunity to parasites in lactating rats

3.1. Summary

Resistance to *Nippostrongylus brasiliensis* in re-infected lactating rats is sensitive to litter size and therefore nutrient demand. Here, we use the latter observation to assess the rate at which improved host nutritional status can improve periparturient resistance and immunity. Second-parity rats were infected with *N. brasiliensis* larvae prior to mating. Upon parturition, dams were fed *ad libitum* a low protein food (10% CP) and were either nursing 12 pups (LS12) or 3 pups (LS3) throughout lactation, or nursed 12 pups until day 5 when their litter was adjusted to 3 pups (LS12-3). On day 2 of lactation, dams were re-infected with 1600 *N. brasiliensis* larvae. Food intake, and dam and litter weight were assessed daily until either day 5 (for LS12 only), or day 8 and day 11 (all treatments) of lactation when worm burdens, worm eggs in the colon contents (EIC) and small intestinal mucosal inflammatory cells per villus-crypt unit (vcu) were assessed. LS12 dams showed lower dam weight gain and higher litter weight gain, higher worm burdens and EIC and lower number of mucosal mast cells and eosinophils than both LS3 and LS12-3 dams from day 8 of lactation. LS12-3 animals had similar EIC, worm burdens, mast and eosinophil cell counts, as LS3 animals from day 8 of lactation onwards and increased dam weight gain as compared with LS12 dams by day 8 of lactation. The present results show that decreasing nutrient scarcity rapidly enhances host resistance and expression of mucosal immunity.

Keywords: nutrient scarcity, litter size, lactation, nematodes, rats

3.2. Introduction

During their life cycle mammals acquire immunity towards gastrointestinal nematode (GIN) parasites as they are continuously exposed to their infective forms. However, the expression of acquired immunity can break down, e.g. during the periparturient period (Houdijk *et al.,* 2001a). This periparturient relaxation of immunity (PPRI) plays an important role in the epidemiology of GIN infections. It is observed as an increased gastrointestinal nematode burden and nematode egg excretion into the environment, and as such the periparturient host is a major source of infection to their parasite-naïve off-spring (Barger, 1993; Beasley *et al.,* 2010). A nutrient partitioning framework has put forward the hypothesis that PPRI has a nutritional basis, as scarce nutrients may be preferentially used to satisfy the elevated nutritional demands of the reproductive effort at the expense of functions associated with expression of immunity during the periparturient period (Coop & Kyriazakis, 1999). Consequently, reducing and eventually overcoming nutrient scarcity would be expected to reduce the degree of PPRI to parasites, illustrated by reduced worm burdens and worm egg excretion.

Reducing nutrient scarcity can be achieved either through an increase in the intake of dietary nutrients, or through a reduction of nutrient demand. The latter can arise from e.g. rearing fewer offspring, which at similar level of (scarce) nutrient supply is expected to increase nutrient availability to the periparturient host for expression of immunity to parasites (Houdijk, 2008). It is well documented that rearing a larger litter increases milk production and therefore the amount of nutrients directed to reproductive functions in both sheep (Alexander & Davies, 1959; Gardner and Hogue, 1964) and rats (Morag *et al.,* 1975). Indeed, rearing one instead of two

lambs has been shown to reduce parasitism in sheep infected with *Teladorsagia circumcincta* and *Trichostrongylous colubriformis* (Donaldson *et al.,* 1998; Houdijk *et al* 2001; Xie *et al.,* 2004). Likewise, rearing smaller litters in rats infected with *Nippostrongylus brasiliensis* is associated with reduction of worm burdens and egg excretion (Normanton *et al.,* 2007). Also, naturally infected high producing goats experience an augmented degree of PPRI as compared to low producing ones in grazing conditions (Chartier *et al.,* 2000).

In our *N. brasiliensis* re-infection lactating rat model, increased crude protein (CP) supply has resulted in reduced parasitism and increased local immune responses (Houdijk *et al.,* 2005; Jones *et al.,* 2009, Sakkas *et al.,* 2011 [Chapter 2], Jones *et al.,* 2011). Our hypothesis here is that a reduction in nutrient demand on low protein diets, as a consequence of reducing the number of rearing offspring, will result in similar parasitological and immunological responses. Based on previously published data (Houdijk *et al.,* 2005a; Normanton *et al.,* 2007), it was hypothesised that a nursing rat fed a low-protein food could support normal growth of three pups, resulting in nutrient abundance. When the same dam is nursing twelve pups, the achieved intake of the low-protein food would limit the dam's lactational performance, thus resulting in nutrient scarcity.

We additionally aimed to investigate the rate of improvement in resistance, i.e. the rate at which resistance and immunity increase following overcoming nutrient scarcity through litter size reduction. This was evaluated by assessing temporal effects of reducing nutrient demand through litter size reduction on worm burdens and mucosal inflammatory responses. Similar methodology has been used in sheep to demonstrate rapid improvement of host resistance upon litter size reduction (Houdijk

et al., 2006). However, the latter study did not allow for temporal assessment of associated improvement of immune responses.

3.3. Materials and Methods

3.3.1. Animals, housing and feeding strategy during gestation

The experiment described below was approved by SAC's Ethical Review Committee (ED AE 19/2008) and carried out under Home Office authorization (PPL 60/3626). Female rats were housed in a room where ambient temperature was maintained at 21°C, relative humidity ranged from 45 to 65%, and artificial lighting was provided between 08.00-18.00 hours. Rats were individually housed in solid-bottomed cages with fresh sawdust provided weekly. Shredded plastic bubble wrapping for nesting material was provided 3 days before the expected parturition date. Wire-bottomed cages were used during mating and for faeces collection during the primary infection as described previously (Houdijk *et al.,* 2003a). For mating, female rats were placed with a proven male breeder and mating was confirmed through the presence of a vaginal plug. Until mating was confirmed rats were given *ad libitum* access to standard rat chow (Rat and Mouse No 3, Special Diet Services, Witham, UK). After mating was confirmed, rats were given *ad libitum* access to a high protein food, with 210g digestible crude protein (CP) and 16.4 MJ metabolizable energy (ME) per kg dry matter (DM) until ten days into gestation to allow for establishment of pregnancy and placental development. Rats were then transferred to a low protein food containing 60g CP and 17.3 MJ ME per kg DM, which was given until parturition. This feeding protocol was used to reduce body

protein reserves during the second half of gestation to maximize the degree of protein scarcity during lactation (Pine *et al.,* 1994; Houdijk *et al.,* 2005a).

3.3.2. Treatments

Upon parturition all dams had *ad libitum* access to a semi-synthetic low CP diet (100g/kg, Table 3.1), were blocked for parturition body weight (PBW) and then randomly allocated to one of 3 treatments on the morning parturition was observed to be complete. These treatments consisted of dams, which were either nursing 12 pups (LS12) or 3 pups (LS3) throughout, or nursed 12 pups until day 5 when their litter was adjusted to 3 pups (LS12-3). Animals were killed at one of three different endpoints; day 5 (for LS12 only), day 8 or day 11 of lactation (n=6 for each of the 7 treatment groups). The day 5 endpoint was included to establish worm burden and immune responses prior to litter size change. The day 8 and 11 endpoints, i.e. 3 and 6 days post litter reduction, were selected in order to assess the rate of improvement of immunity, as nutritional improvement of immunity to parasites in this model can be observed as soon as three to six days post secondary infection (Jones *et al.,* 2009).

3.3.3 Infection protocol

Rats received a primary injection of 1600 third-stage infective larvae (L_3) of *N. brasiliensis* on day –37 (with day 0 as mean achieved parturition date), which were suspended in 0.5 ml sterile phosphate buffered saline (PBS) that was subcutaneously injected in the hind leg. A secondary infection of 1600 L_3 *N. brasiliensis* was administered on day 2 of lactation.

3.3.4. Measurements

Body weight and food intake. The dams were weighed daily throughout the experiment, and their pups were counted and total litter weighed daily from parturition. Weights taken post parturition were used to calculate lactational dam, litter weight gain, absolute and relative individual pup growth until day 5 of lactation, when pup removal took place in LS12-3 group, and from day 5 of lactation until either day 8 or day 11 of lactation. Feed intake was measured daily throughout gestation and lactation. The food offered during lactation was sampled during its preparation for the analysis of DM, CP (Kjeldahl-N x 6.38), diethyl ether extract, ash and acid detergent fibre (Table 3.1)

Faecal egg counts. A faecal egg count (FEC, in eggs per gram (epg) of fresh faeces) was performed seven days after the primary infection (day -30) to confirm the presence of infection. A second FEC was performed on day -23 to confirm that the expected parasite expulsion had taken place. To this effect, faeces were collected through overnight housing on bottom-wired cages (Houdijk *et al*., 2003a) and FEC were performed using a modified saturated salt flotation method (Christie & Jackson, 1982).

Histology, worm burden and nematode eggs in colon.

[Histology and mucosal inflammatory cell enumeration carried out by Leigh Jones]

Rats were killed humanely at the allocated endpoints through gradually increasing ambient CO_2 concentration followed by CO_2 asphyxiation for parasitological and immunological assessment (see below). The small intestine was removed and first sampled for histology as follows. A 2cm sample at a 25cm distance from the pylorus was washed with PBS to collect worms for total worm counts and then fixed in 4%

paraformaldehyde for 6 hours. The fixative was then replaced by 70% ethanol. Resulting intestinal samples were paraffin embedded and sections taken and stained using standard histological techniques. Sections were stained for counting eosinophils (carbol chromatrope 2R), goblet cells (alcian blue and counterstaining with periodic acid-schiff) and mast cells (toluidine blue). Cells were enumerated by counting 10 complete, well orientated, villus crypt units (VCU) per section and results were expressed as number of cells per VCU (Jones *et al*, 2009). Worms collected from the tissue sampled for histology were added to the remainder of the small intestine and stored in formaldehyde for subsequent worm burden assessment (number and sex according to worm morphology).

Lastly, the contents of the large intestine were weighed and assessed for worm eggs as described for FEC above (epg). This number was then multiplied by the weight of large intestinal contents to obtain the total number of nematode eggs in the colon (EIC).

Table 3.1. Composition and analysis of the experimental food used during lactation.

Experimental food	
Ingredients (g/kg fresh)	
Casein	102
Methionine	1
Starch	343
Sucrose	172
Corn oil	141
Vitamins	47
Minerals	47
Cornflour	46
Choline	7
Lecithin	2
Alphacel	94
Analysed chemical composition	
DM (g/kg fresh matter)	715.2
ME (MJ/kg DM)*	15.9
CP (g/kg DM)	99.0
Ash (g/kg DM)	38.3
Acid detergent fibre (g/kg DM)	74.7
Diethyl ether extract (g/kg DM)	162

* Food ME contents was calculated by multiplying its contents of protein (casein), digestible carbohydrates (starch, sucrose, corn flour) and fat (corn oil) with the ME contents of protein (17 MJ/kg), carbohydrates (17 MJ/kg) and fat (38 MJ/kg) (Astrup & Tremblay, 2009)

3.3.5. Calculations and statistical analysis

In order to assess the effects of litter size (12 or 3 pups) on dam, litter and pup performance until day 5 of lactation, Restricted Maximum Likelihood (REML) was used to account for the unbalanced number of replicates between dams having a litter size of 12 (LS12 and LS12-3; n=30) and those having a litter size of 3 pups (n=12).

General Linear Modelling (GLM) was applied to assess the effect of our treatments (LS12, LS12-3, LS3), endpoint (day 8 or 11) and their interaction on dam, litter and pup performance from day 5 of lactation onwards, but also for worm burdens, EIC and number of inflammatory cells at these endpoints. Whilst LS12 vs LS3 informs on the impact of rearing low litters throughout, the expectation was that over time LS12-3 data would start to deviate from LS12 data and become similar to LS3 data. As such, the rate of improvement of immunity to parasites following litter size reduction in LS12-3 can be informed by the presence or absence of treatment x end point (time) interactions. The presence of interaction could suggest that effects are more pronounced on day 11 than on day 8, and can be interpreted that it may take up to 6 days before improved host nutrition restores immunity. In contrast, the absence of interaction, in the presence of main effects, could suggest that effects are already in place by day 8, and would suggest that improved host nutrition could restore immunity to parasites within 3 days in this model.

A separate GLM was applied on LS12 and LS12-3 dams to assess the effect of endpoint (day 5, day 8 or day 11) on the worm burden expulsion and mucosal inflammatory cell recruitment. This approach, which shares LS12 data collected on day 5 for LS12 and LS12-3 dams, can further inform whether litter size reduction

affects the rate of worm expulsion and inflammatory cell recruitment. However, this could not be adopted for EIC, as the latter was 0 on day 5, which is in agreement with Jones *et al*. (2009).

Because of their skewed nature, the EIC, worm burdens, eosinophils, mucosal mast cells and goblet cells were transformed according to log (n + 1) to normalize data before statistical analysis. These data are reported as back-transformed means with their 95% CI as described previously (Houdijk *et al.,* 2005a). All statistical analyses were performed using Genstat 11 for Windows release 11.1, 2008 (VSN international, Hemel Hempstead, UK).

3.4. Results

3.4.1. Faecal egg counts and performance until parturition

FEC taken on day -37 and day -30 averaged 7580 (CI: 1142-993) epg and 0 (CI: 0-0) epg, respectively. Mean body weight on arrival was 336 (SE 6.0) g. During the first 10 days of gestation, pregnant rats grew from 351 (SE 5.7) to 393 (SE 6.5) g and had an average DM intake of 21.69 (SE 0.50) g/d. From then onwards and until parturition, the pregnant rats continued to grow to a mean weight of 467 (SE 8.1) g and had an average DM intake of 17.74 (SE 0.47) g/d, which dropped to an average of 4.42 (SE 0.50) g/d just before parturition. Dam mean PBW averaged 368 (SE 7.2) g, mean litter size averaged 15.5 pups (SE 0.58) g and mean litter weight averaged 78.6 g (SE 2.4) g. Natal litter size did not differ between the subsequent LS12, LS12-3 and LS3 treatments (16.22 vs 15.31 vs 14.50 pups; SED=1.545; P= 0.474)

3.4.2. Achieved food intake during lactation

Figure 3.1 shows the mean daily DM intake/kg PBW for the three treatments over the lactation period. Treatment (P=0.293) and its interaction with endpoint (P=0.715) did not affect achieved mean DM intake, which was greater for rats allocated for dissection on day 11 compared to those allocated for dissection on day 8 of lactation (70.4 vs 59.4 g/d; SED 3.93 g/d; P=0.008).

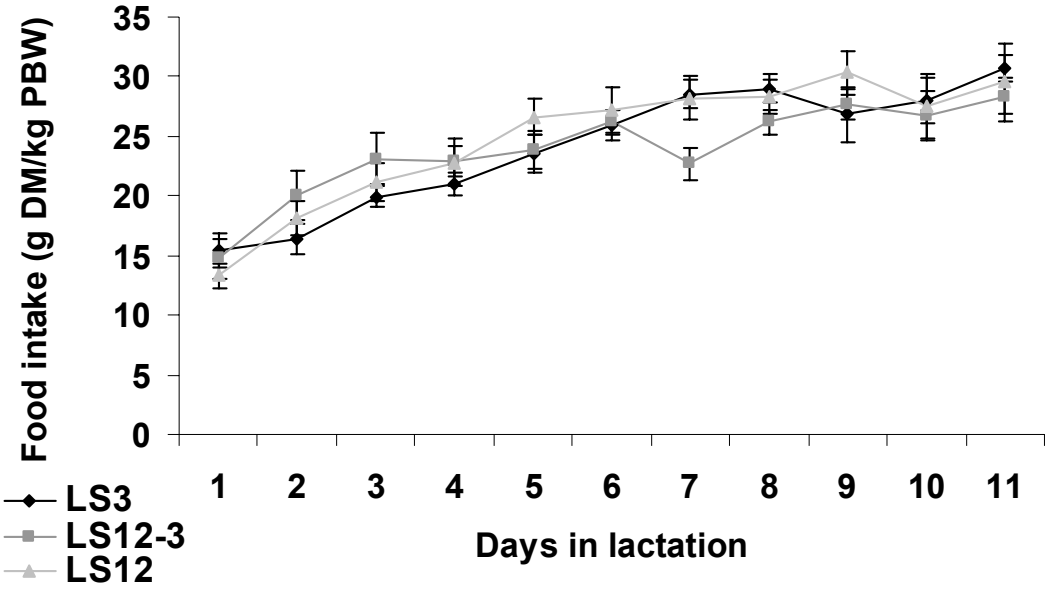


Figure 3.1. Average daily food intake (g dry matter/kg parturition body weight/day) across three experimental treatments (LS3 animals that had a litter size of 3 throughout the lactation period, LS12 animals that had a litter size of 12 throughout the lactation period and LS12-3 animals that had a litter size of 12, which was reduced to a litter size of 3 pups on day 5 of lactation for the remainder of it) over the first 11 days of lactation in rats, re-infected with *Nippostrongylus brasiliensis*.

3.4.3. Dam weight gain

Figure 3.2 shows the dam's daily weight for the three treatments over the lactation period. Litter size affected dam weight gain until day 5 of lactation, which averaged 0.70 and -3.65 g/d for LS3 and LS12 rats, respectively (SED 1.09 g/d; P<0.001). Our treatments continued to affect dam weight gain from day 5 until either day 8 or 11 of lactation, and averaged 1.91, 4.10 and -3.80 g/d for LS3, LS12-3 and LS12 dams, respectively (SED 1.336 g/d; P<0.001). Dam weight gain tended to be affected by endpoint (P=0.095) but was not affected by the interaction between treatment and endpoint (P=0.975).

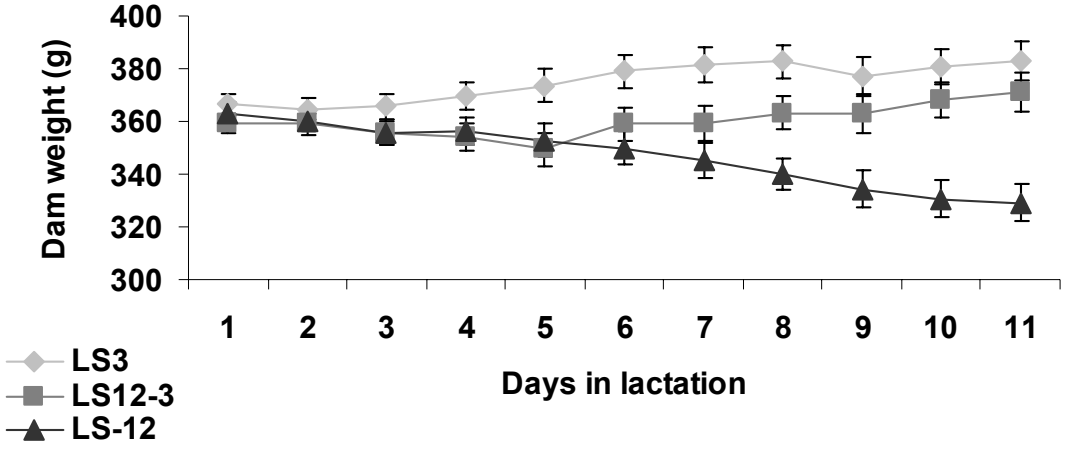


Figure 3.2. Average dam daily weight (g, with s.e.) across three experimental treatments (LS3 animals that had a litter size of 3 throughout the lactation period, LS12 animals that had a litter size of 12 throughout the lactation period and LS12-3 animals that had a litter size of 12, which was reduced to a litter size of 3 pups on day 5 of lactation for the remainder of it) over the first 11 days of lactation in rats, re-infected with *Nippostrongylus brasiliensis*.

3.4.4. Litter weight gain

Figure 3.3 shows the litter daily weight for the three treatments over the lactation period. As expected, litter size affected litter weight gain until day 5 of lactation, which averaged 4.65 and 9.47 for LS3 and LS12 rats, respectively (SED 0.78 g/d; P<0.001). Our treatments continued to affect litter weight gain from day 5 onwards, and averaged 7.48, 5.31 and 11.91 g/d for LS3, LS12-3 and LS12 rats, respectively (SED 0.94 g/d; P<0.001). Litter weight gain was not affected by endpoint (P=0.467) or the treatment and time interaction (P=0.914).

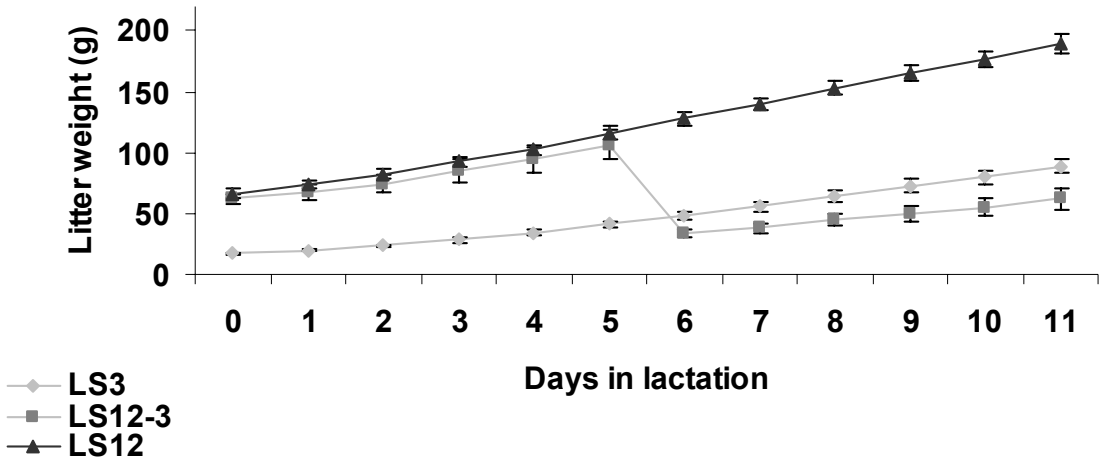


Figure 3.3. Average litter daily weight (g, with s.e) across three experimental treatments (LS3 animals that had a litter size of 3 throughout the lactation period, LS12 animals that had a litter size of 12 throughout the lactation period and LS12-3 animals that had a litter size of 12, which was reduced to a litter size of 3 pups on day 5 of lactation for the remainder of it) over the first 11 days of lactation in rats, re-infected with *Nippostrongylus brasiliensis*.

3.4.5. Individual pup and pup relative weight gain

Figure 3.4 shows the individual daily pup weight for the three treatments over the lactation period. Litter size affected individual pup weight gain until day 5 of lactation, which averaged 1.55 and 0.83 g/d for LS3 and LS12 rats, respectively (SED 0.09 g/d; $P<0.001$). Our treatments continued to affect individual pup weight gain from day 5 onwards, which averaged 2.51, 1.86 and 0.99 g/d for LS3, LS12-3 and LS12 rats, respectively (SED 0.168 g/d; $P<0.001$). Individual pup weight gain was not affected by endpoint ($P=0.124$) or the treatment and time interaction ($P=0.624$). Individual relative pup weight gain (g/d/g) was calculated from day 5 onwards to assess whether differences in body weight gain could be accounted for by differences in body weight on day 5 arising from LS3 vs LS12 treatments prior to day 5. Our treatments affected relative pup weight gain, which averaged 0.105, 0.191 and 0.189 g/d/g for LS12, LS12-3 and LS3 pups, respectively (SED 0.0178 g/d/g; $P<0.001$). Relative pup weight gain was not affected by endpoint ($P<0.869$) or the treatment endpoint interaction ($P<0.651$).

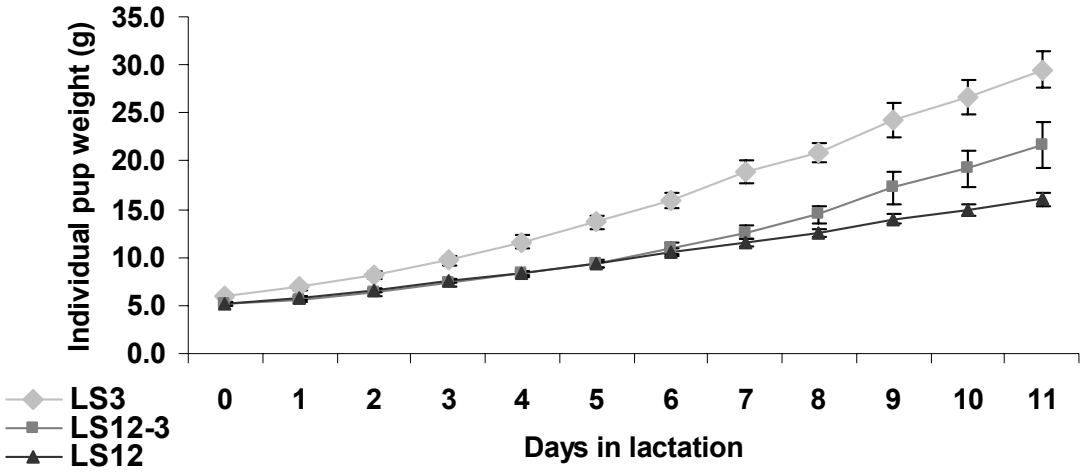


Figure 3.4. Average individual pup daily weight (g, with s.e) across three experimental treatments (LS3 animals that had a litter size of 3 throughout the lactation period, LS12 animals that had a litter size of 12 throughout the lactation period and LS12-3 animals that had a litter size of 12, which was reduced to a litter size of 3 pups on day 5 of lactation for the remainder of it) over the first 11 days of lactation in rats, re-infected with *Nippostrongylus brasiliensis*.

3.4.6. Total worm burden, eggs in colon and worm burden composition

Figure 3.5a and Figure 3.5b shows the total worm burdens and EIC, respectively for day 5 (LS12 only), day 8 and 11 of lactation. Treatment tended to affect total worm burdens (P=0.084) as LS3 and LS12-3 rats harboured fewer worms than LS12 rats. Worm burdens were not affected by endpoint (P=0.871), or treatment endpoint interaction (P=0.630). Treatment affected EIC (P<0.001), as LS3 and LS12-3 rats had lower EIC than LS12 rats. EIC were not affected by endpoint (P=0.618) or treatment endpoint interaction (P=0.798). Worm burden composition of LS12, LS12-3 and LS3 dams was not affected by treatment; the percentage of female worms averaged 54.2, 56.5 and 57.1 %, respectively (SED=6.88 %; P=0.901). Worm burden composition was also not affected by endpoint (P=0.694) or the treatment endpoint interaction (P=0.665).

Worm burdens decreased over time for both LS12 and LS12-3 rats from day 5 to day 8 but did not decrease further until day 11; log worm burdens on days 5, 8 and 11 averaged 2.60, 2.14 and 1.89, respectively, for LS12 rats (SED=0.20; P=0.011), and 2.60, 1.39 and 1.58 (SED=0.36; P=0.008) respectively, for LS12-3 rats.

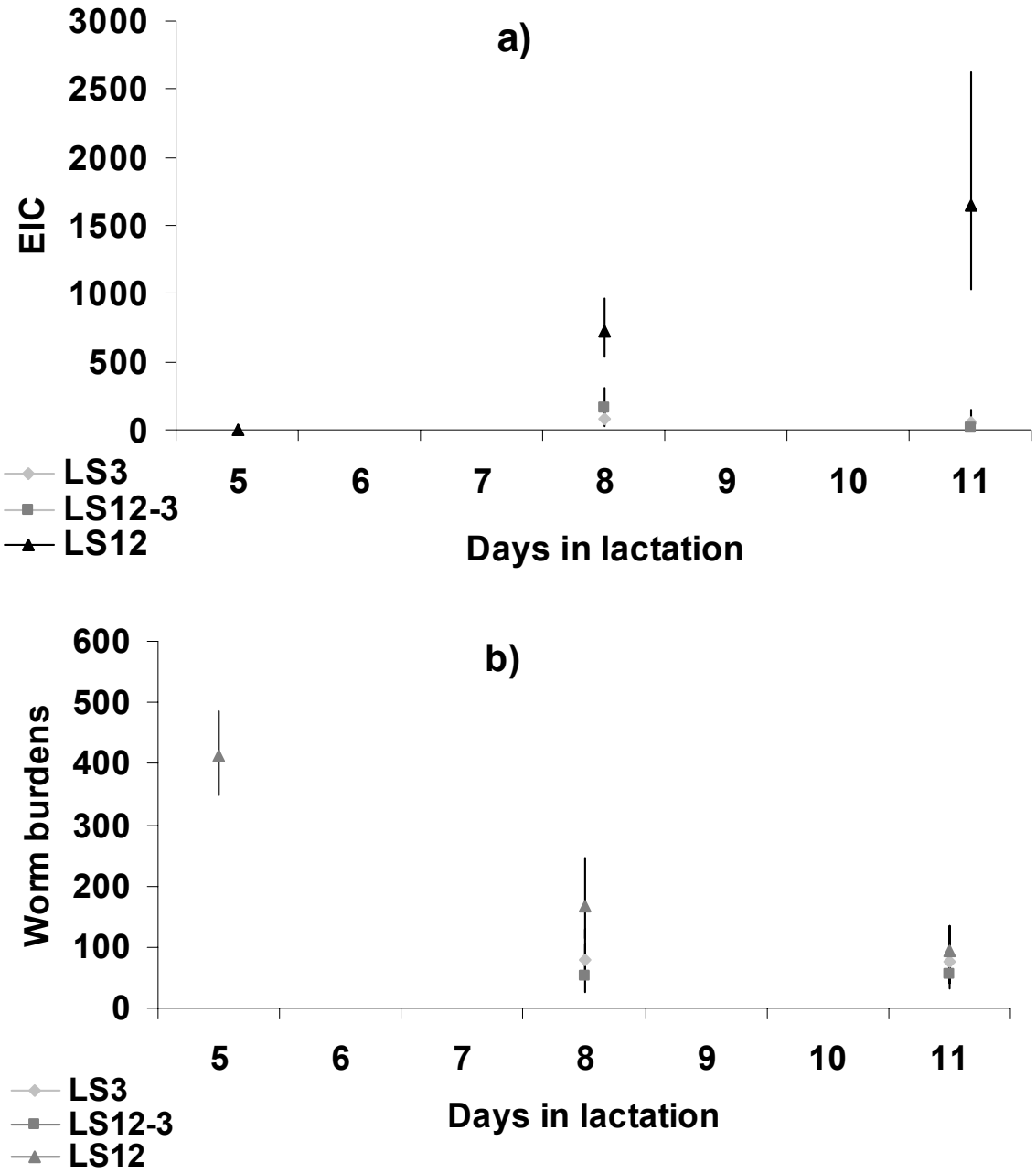


Figure 3.5. Backtransformed mean worm burdens a) and backtransformed mean total eggs in colon b) with backtransformed lower and upper limits of transformed error bars as 95% CI, across three experimental treatments (LS3 animals that had a litter size of 3 throughout the lactation period, LS12 animals that had a litter size of 12 throughout the lactation period and LS12-3 animals that had a litter size of 12, which reduced to a litter size of 3 on day 5 of lactation for the remainder of it) taken on day 5, 8 and 11 following reinfection with *Nippostrongylus brasiliensis*.

3.4.7. Gut histopathology

Figure 3.6 shows the mucosal mast cell (6a), eosinophil (6b) and goblet cell (6c) numbers per VCU for day 5 (LS12 only), day 8 and 11 of lactation. Their concentrations were all higher on day 11 than day 8 ($P<0.001$), whilst there was no interaction between time and treatment for any type of cell assessed ($P>0.30$). However, treatment affected log mucosal mast cell numbers, which averaged 1.36, 1.56 and 1.59 for LS12, LS3 and LS12-3, respectively (SED 0.069; $P=0.005$). Treatment tended to affect log eosinophil numbers, which averaged 1.48, 1.57 and 1.64, respectively (SED 0.069; $P<0.077$). Treatment did not affect log goblet cell numbers ($P=0.312$).

Log eosinophils numbers significantly increased over each time period for both LS12 and LS12-3 rats, i.e. from 1.12 on day 5 to 1.31 on day 8 and 1.65 on day 11 for LS12 rats (SED 0.094; $P<0.001$), and to 1.46 on day 8 and 1.71 on day 11 for LS12-3 rats (SED 0.097; $P<0.001$). However, log mucosal mast cell numbers of LS12 rats did not increased over time, and were 1.19 on day 5 and 1.24 on day 8 but then significantly increased to 1.49 on day 11 for LS12 rats (SED 0.076; $P=0.003$). In contrast, log mucosal mast cell numbers of LS12-3 rats significantly increased over each time period from 1.19 on day 5 to 1.46 on day 8 and 1.68 on day 11 for LS12-3 rats (SED 0.11; $P=0.003$). Log goblet cell numbers increased over time from 1.48 on day 5 to 1.59 on day 8 and 1.67 on day 11 for LS12 rats (SED 0.061; $P=0.0026$, and to 1.54 on day 8 and 1.76 on day 11 for LS12-3 rats (SED 0.061; $P<0.001$). For both LS12 and LS12-3 rats, goblet cell numbers differed significantly between day 5 and 11 only.

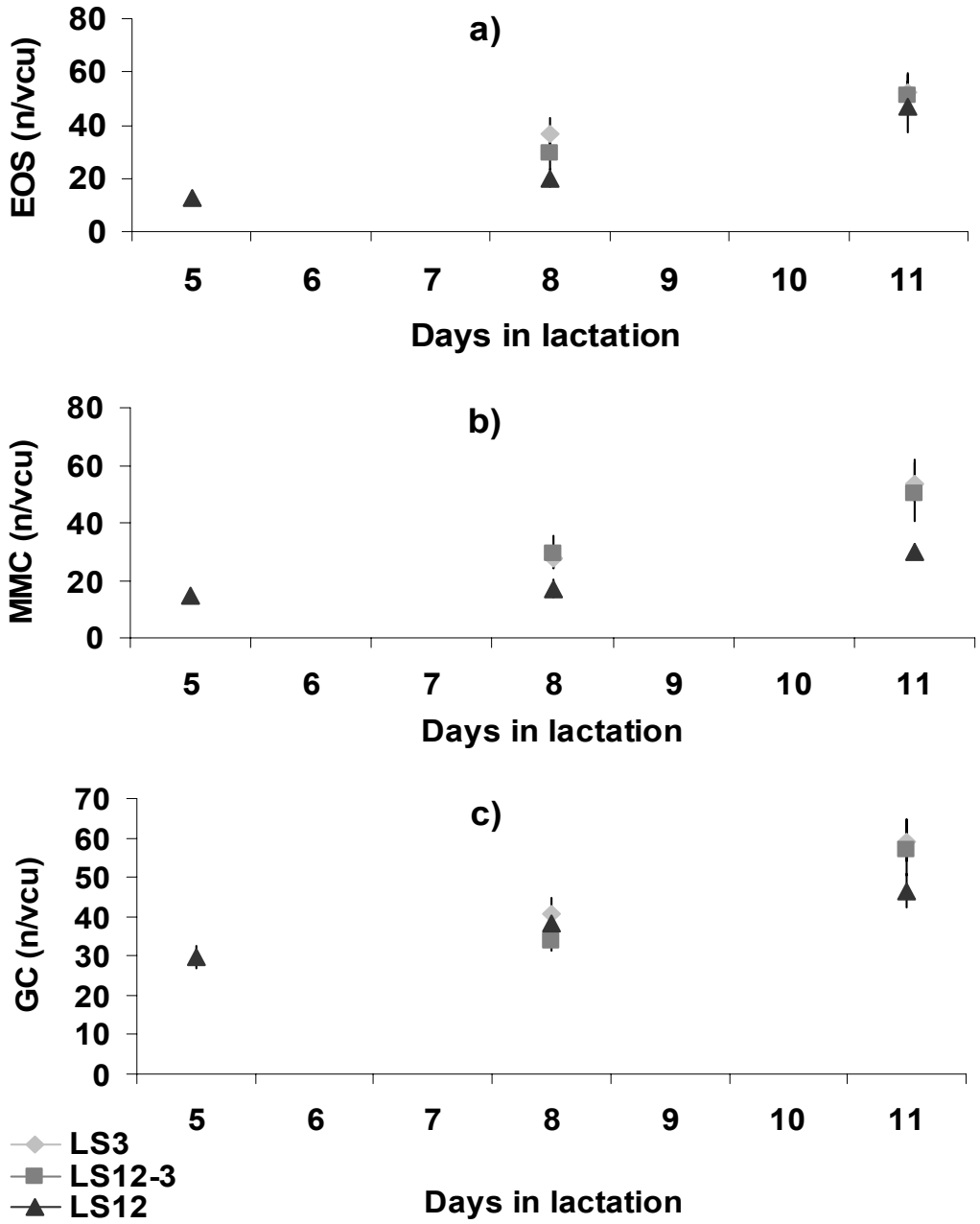


Figure 3.6. Backtransformed mean eosinophil number a), mucosal mast cell number b), goblet cell number c) in the small intestine with backtransformed lower and upper limits of transformed error bars as 95% CI, across three experimental treatments (LS3 animals that had a litter size of 3 throughout the lactation period, LS12 animals that had a litter size of 12 throughout the lactation period and LS12-3 animals that had a litter size of 12, which reduced to a litter size of 3 on day 5 of lactation for the remainder of it) taken on day 5, 8 and 11 following reinfection with *Nippostrongylus brasiliensis*.

3.5. Discussion

Previous studies carried out with this rodent - nematode parasite model have consistently shown that reducing CP scarcity through increasing dietary CP content during lactation results in a reduced degree of parasitism (Normanton *et al.,* 2005; Houdijk *et al.,* 2005a; Jones *et al.,* 2009; Sakkas *et al.,* 2011 [Chapter 2]. In the present experiment we aimed to elucidate whether reducing nutrient scarcity through reducing nutrient demand would likewise improve host resistance to GIN parasites. We hypothesized that overcoming nutrient scarcity as a result of rearing fewer pups would be expressed as reduced parasitism and that it would be associated with increased inflammatory cell counts, in much the same way as shown for increased CP supply (Jones *et al.,* 2011). Our results support this hypothesis; rearing 3 instead of 12 pups throughout, as well as a reduction of the litter size from 12 to 3 pups on day 5 of lactation, resulted in a reduction in the number of EIC and worm burden, and this was associated with an increased dam body weight gain, increased pup weight gain and an increase in the numbers of mucosal mast cells.

In order to achieve our experimental objective we could have increased the level of CP offered to lactating parasitized dams from low to high at a later time point during lactation while maintaining another group at a low level of CP supply and another group at a high level of CP supply throughout lactation. However, increasing the amount of CP supply at a later stage during lactation would have lead to an increased intake of the high CP diets. This would result to higher intake of fibre in the form of cellulose, which can potentially alter the gut environment and therefore affect gastrointestinal parasitism (Petkevicius *et al.,* 1999) and bias our results. Whilst variation in feed intake *per se* could have been controlled by restricted

feeding of low and high CP foods (Sakkas *et al.,* 2011, [Chapter 2]), a gut environment modulating effect of higher levels of CP can not be excluded.

Studies with lactating ruminant hosts infected with various species of gastrointestinal nematode parasites, have revealed that a lower nutritional demand arising from rearing fewer offspring and/or lower level of milk production significantly reduces the degree of PPRI (Romjali *et al.,* 1997; Donaldson *et al.,* 1998; Houdijk *et al.,* 2001c; Kahn *et al.,* 2003b; Baker *et al.,* 1998; Hoste & Chartier 1993; Chartier *et al.,* 2000). Furthermore, cessation of lactation results in an even more pronounced enhancement of resistance (Houdijk *et al.,* 2005b, O'Sullivan & Donald 1973; Lloyd, 1983; Beasley *et al.,* 2010). It has been previously established in this host parasite system that reducing nutrient demand by reducing the number of suckling pups at the beginning of lactation reduces the degree of PPRI (Normanton *et al.,* 2007). In addition, a reduction in the number of rearing lambs in lactating sheep infected with *T. circumcincta* at a later time point during lactation resulted in a rapid reduction in the degree of PPRI expressed in lower FEC and worm burdens (Houdijk *et al.,* 2006). However, the latter study did not observe a correlated response in mucosal immunity. To our knowledge, the current study is the first one employing the litter reduction strategy to assess rate of immunity restoration in a parasitized monogastric host.

It is important to establish whether the current experiment achieved the appropriate nutritional conditions under which the hypotheses could be tested, as variation in achieved food intake may interfere, arising from interactions between supply and demand on effects of reproductive effort on PPRI (Houdijk, 2008). As expected, and in agreement with earlier studies in sheep fed pelleted LP foods

(Houdijk *et al.,* 2001c) and lactating rats with different litter sizes on LP foods (Normanton *et al.,* 2007), we did not observe a treatment effect on the achieved *ad libitum* intake of our LP diet. One might have expected that higher litter size would result in increased food intake as dams with a higher litter size could have tried to consume more food in order to compensate for the protein shortage relative to the extra demand placed on them. However, the low protein to energy ratio of the food used may have prevented such increased food, because of the metabolic consequences that an excess energy intake imposes to the dam (Friggens *et al.,* 1993). An elevated intake of the LP diet would have resulted in even higher excess levels of energy intake relative to energy requirements, which cannot be utilized for milk production. As a result, it would have to be disposed as heat. However, there are limitations in the amount of heat lactating rats can dissipate and that limited the intake of the LP food (Friggens *et al.,* 1993).

The hypothesis tested in our study was that PPRI would be sensitive to changes in nutrient demand. It is evident from the data obtained that these litter size treatments affected dam performance, i.e. body weight change and litter gain (proxy for milk production). The higher dam weight gain of LS3 dams relative to LS12 dams over the first 5 days of lactation ($P<0.001$; Figure 3.2) shows that the former were in a less scarce nutrient environment than the latter. The effect on dam weight gain was caused by the reduction in milk production that accompanies a smaller litter size (Morag *et al.,* 1975). Litter size reduction from 12 to 3 pups on day 5 of lactation resulted in LS12-3 dams achieving higher weight gain than LS12 dams and similar to that of LS3 dams ($P<0.001$; Figure 3.2) by day 8 of lactation. Over the same period litter weight gain of LS12-3 rats was lower than that of LS3 rats. This

was likely caused by the smaller pup size at day 5 as their relative weight gain was similar to that of LS3 pups and higher than that of LS12 pups (Figure 3.5). This suggest that compensatory growth was not observed over the time course of the experiment, but it can not be excluded that the latter could have been observed had lactation been extended.

Litter size is a major determinant of net mammary amino acid uptake and milk production increases with increasing litter size in pigs and rats (Nielsen *et al.,* 2002; Morag *et al.,* 1975). Increasing litter size from 6 to 12 piglets induces a greater increase in the total mass of the mammary gland tissue and in the litter weight (Kim *et al.,* 1999). On the same diets, the aforementioned increased demands arising from nursing a larger litter result in increased protein mobilization from the carcass, the gastrointestinal tract, the liver and the reproductive tract (Kim & Easter, 2001). Changes in dam body weight gain in our treatments are expected to arise from combined protein and fat losses, in order to satisfy the increased amino acid requirements (Kim & Easter, 2001) and the energetic demands for milk synthesis (Oftedal, 2000). One could argue that potential host benefits arising from a reduced nutrient demand could be attributed to any resource and not just protein. However, there are several arguments to support the view that the responses observed are related to increased protein availability. Firstly, in this host parasite system a ME restriction in the order of 25% has not been shown to influence the degree of PPRI (Sakkas *et al.,* 2011 [Chapter 2]). Similarly, there is an absence of an effect of energy supply on the degree of PPRI in lactating ewes arising from higher dietary ME levels (Donaldson *et al.,* 1998) and/or higher levels of fat reserves (Houdijk *et al.,* 2001). Secondly, rats were not constrained in their ME intake as opposed to their CP intake

during gestation. As a result, it is expected that they accumulated adequate fat reserves as opposed to labile protein reserves, which are depleted in response to our gestational feeding protocol (Pine *et al.,* 1994). Thirdly, protein was limiting on the LP food offered during lactation. This suggests that at the level of production achieved, all other nutrients and resources, including energy, were already surplus to requirements. A further reduction in nutrient and energy requirement would have increased this surplus, rather than changing host nutritional status from scarce to more than adequate. Lastly, protein scarcity has been shown to strongly influence the degree of the lactational breakdown of immunity to parasites in a variety of periparturient mammalian hosts including rats (Houdijk *et al.,* 2005a; Jones *et al.,* 2009), ewes (Donaldson *et al.,* 2001; Kahn *et al.,* 2003a; Houdijk *et al.,* 2003b; Houdijk *et al.,* 2005b) and dairy goats (Etter *et al.,* 1999; Chartier *et al.,* 2000). The larger litter size of LS12 dams induces higher amino acid requirements to the host, which compromises their availability for the expression of immunity to parasites as the latter function is expected to take a lower priority than lactation (Coop & Kyriazakis, 1999). Parasitic infections increase AAs requirements of the host so as to activate, or maintain, an effective protective response to parasites (Kyriazakis & Houdijk, 2006). This is likely because effector molecules of the immune response are highly proteinaceous; inflammation and immune response are characterised by the synthesis of specific proteins that play crucial roles in the defence of the host against pathogens and the modulation of the immune response (Coop & Kyriazakis, 2001; Le Floc'h *et al.,* 2004). Therefore, it is strongly believed that the results arising from litter size reduction on LP foods are responses to increased protein availability.

We considered changes in the number of worm burdens and EIC to be the result of changes in the degree of expression of immunity to *N. brasiliensis*, as they provide the ultimate measure on how the host copes with the challenge (Normanton *et al.,* 2007). Additionally, an increase in the accumulation of mast cells, goblet cells and eosinophils accompany GIN infections, contributing to the expulsion of the parasite population (Shea-Donohue & Urban, 2004) and have been shown to be variably affected by CP supply in this rodent-parasite model (Jones *et al.,* 2009; Jones *et al.,* 2011). Although not formally significant, there was a strong trend for LS12 rats to carry more worms than LS3 and LS12-3 rats ($P<0.084$; Figure 3.5a) present from endpoint at day 8 of lactation, and the effect size agreed with results of studies where increased CP supply resulted in lower worm burdens (Houdijk *et al.,* 2005a; Jones *et al.,* 2009; Sakkas *et al.,* 2011 [Chapter 2]). These results indicate that enhanced worm expulsion was achieved in LS12-3 dams within 3 days from the litter size reduction. In agreement with Normanton *et al*. (2007), the reduced litter size from birth resulted in reduced EIC. However, the magnitude of this response in our study was higher, which may be related to a higher degree of nutrient scarcity imposed (12 pups in our experiment vs 9 pups in the aforementioned study). As in the case of the worm burdens, the effect of our treatments took place on EIC by day 8 of lactation and persisted on day 11 of lactation. This shows that LS12-3 dams showed reduced fecundity within 3 days from the litter size reduction, and that fecundity may be more sensitive to nutrient scarcity than worm expulsion, as observed in lactating sheep (Houdijk *et al.,* 2003). The results observed in EIC and worm burdens of LS12-3 dams are in agreement with those of Houdijk *et al.,* (2006) where the removal of one lamb from unsupplemented, twin rearing ewes resulted in

reduced FEC and worm burdens within one week by more than 50% compared to their twin rearing counterparts. Thus, both ewe and rat experiments support the view that overcoming nutrient scarcity can rapidly improve resistance to parasites.

The aforementioned effects of reduced litter size throughout and reducing litter size after day 5 of lactation on PPRI were associated with significant higher number of mast cells on both day 8 and 11 of lactation. In LS12-3 animals this was associated with an enhanced recruitment which took place within 3 days from the litter size reduction in contrast to LS12 dams where delayed mast cell recruitment was observed. Increased CP supply has been previously shown to increase the accumulation of small intestinal mast cells in this rodent-parasite model (Jones *et al.,* 2009; Jones *et al.,* 2011). These inflammatory responses are considered to be important components of a protective immune response to *N. brasiliensis* infection (Miller, 1980; Crowle & Reed, 1981; Khan *et al.,* 1995). Mast cells degranulate during worm infection to release a number of mediators including mast cell proteases, histamine and prostaglandins (Kalesnikoff & Galli, 2008) while they are a potent source of preformed cytokines such as IL-4 and IL-13 (Gessner *et al.,* 2005). Therefore, reduced mast cell accumulation could have penalised parasite expulsion from the LS12 dams.

Both peripheral and tissue eosinophilia are characteristic features of infection with GIN infections (Lawrence, 2003). Whilst increasing CP supply and rearing smaller litters have to date not affected eosinophil numbers in this rodent parasite system (Normanton *et al.,* 2007; Jones *et al.,* 2009; Jones *et al.,* 2011), in the current experiment reduction in litter size tended to increase eosinophil numbers (P=0.077). In line with greater effects on EIC, this apparent discrepancy may have resulted from

a higher degree of nutrient scarcity arising from nursing 12 pups in contrast to the 10 or 9 used in aforementioned studies.

Nutritional sensitivity of goblet cell numbers seems to vary between studies; whilst Jones *et al*. (2009) observed higher number of goblet cells due to increased dietary CP contents, in agreement with our results here, Jones *et al*. (2011) and Normanton *et al*. (2007) did not observe effects on goblet cell numbers following increased CP supply and rearing smaller litters, respectively. GIN infections are characterized by an increase in the numbers of goblet cells, their size and in the profile of the mucin proteins which are secreted by them (Mahida, 2003; De Veer *et al.,* 2007). Their mucins may play an important role in the trapping of worms in the mucus layer and inhibiting worm motility and feeding (Miller, 1987, Yamauchi *et al.,* 2006). Thus, although goblet cell numbers were not affected by litter size reduction in our study, differential expression of mucins and/or levels of mucus production cannot be excluded.

In conclusion, consistent with our hypothesis, relative to LS3 dams, the reduced dam weight gain of LS12 dams, which was imposed by the increased milk production reflected in the higher litter weight gain, penalised their expression of immunity to *N. brasiliensis*. This was observed as increased number of EIC, worm burdens and reduced number of mucosal mast cells and eosinophils. On the other hand, LS12-3 animals had similar EIC, worm burdens, mast and eosinophil cell counts, as LS3 animals from day 8 of lactation onwards as a result of a reduction of their litter size from 12 to 3 pups on day 5 of lactation. This was effectively related to an increased dam body weight gain as compared with LS12 dams and to a reduced

reproductive effort compared with both LS12 and LS3 dams, which were also present by day 8 of lactation.

These data imply that overcoming of nutrient scarcity in our host parasite system is associated with a restoration in the mucosal immune response and to a reduction of PPRI within 3 days. Additionally, they verify the fact that lactational resistance to parasites is sensitive to litter size. These findings support the implementation of strategic protein supplementation of multiple rearing, or high producing, ruminant hosts in sustainable worm control programmes with the aim to lower the dependency on chemoprophylaxis.

Chapter 4. Effects of leucine and methionine deficiency on lactational performance and immunity to N*ippostrongylus brasiliensis* re-infection in rats

4.1. Summary

Nippostrongylus brasiliensis re-infected lactating rats fed low crude protein (CP) foods show an augmented degree of PPRI compared to their high CP fed counterparts. This can be attributed to a reduced availability of amino acids for the expression of immunity during the lactational period when amino acid demands are increased. We therefore hypothesized that consumption of high CP foods deficient in individual essential amino acids would penalise lactational performance and immunity to parasites. Second parity rats were infected with *N. brasiliensis* larvae prior to mating. Upon parturition, dams were allocated to either a high protein food (HP), a low protein food (LP) or a HP food deficient in methionine (HP-Met) or leucine (HP-Leu). On day 2 of lactation, dams were either re-infected with 1600 *N. brasiliensis* larvae or sham infected with PBS, whilst litter size was standardized at 12 pups. Dams and litters were weighed daily until either day 8 or day 11 of lactation when worm burdens, number of eggs in colon, mucosal eosinophil, mast cells and goblet cell numbers, and systemic levels of parasite-specific serum levels of *N. brasiliensis* specific IgA, IgE, IgG1, IgG2a, IgG2b and total IgG were assessed. Leucine deficiency, although numerically, it did not significantly affect dam and litter weight gain, while methionine deficiency induced lower dam and litter weight gain to a varying degree and reduced numbers of eosinophils in animals that showed food refusals. Both diets resulted in increased worm burdens and number of eggs in colon compared to HP and similar to LP dams. Our results support the view that PPRI to parasites may be sensitive to specific amino acid scarcity.

Keywords: amino acid, leucine, methionine, lactation, nematodes, rats

4.2. Introduction

It has been repeatedly shown that dietary protein supplementation at times of protein scarcity results in improved resistance and expression of immunity towards gastrointestinal nematodes in periparturient mammal hosts (Donaldson *et al.,* 1998; Houdijk *et al.,* 2003, Chartier *et al.,* 2000), including laboratory rodents (Houdijk *et al.,* 2005a; Jones *et al.,* 2009; Jones *et al.,* 2011; Sakkas *et al.,* 2011 [Chapter 2]). This may be related to the increased protein demands that the periparturient period imposes on the parasitized host, i.e. for milk production, as it has been suggested that scarce protein supply may be preferentially allocated to reproductive rather than to immune functions (Coop & Kyriazakis, 1999; Kyriazakis & Houdijk, 2006). As host responses to dietary protein would be responses to dietary essential and conditionally essential amino acids (AA), it may be expected that their reduced supply can lead to reduced expression of immunity. Both innate and acquired immune responses are dependent upon adequate provision of AAs for the synthesis of antigen-presenting molecules, immunoglobulins, cytokines and acute phase proteins as well as for the provision of energy providing substrates either directly, or following their conversion to other AAs (e.g., glutamine) or to glucose (Calder, 2006; Kim *et al.,* 2007). At the same time, requirements for protein and/or AA may also increase as a direct consequence of metabolic changes associated with inflammation and infection (Le Floc'h *et al.,* 2004) and the physiological status of the animal, such as pregnancy and lactation (Clowes *et al.,* 2005). The fact that 30–50% of essential AA in the food may be catabolized by the small intestine (Stoll *et al.,* 1998; Wu, 1998) is indicative of their role in gut integrity, function and local immune response (Wang *et al.,* 2008; Wu, 2009).

Herein we used a laboratory model to investigate the effect of reduced supply of two essential AAs on host resistance and lactational performance (dam and litter weight gain). Our expectation was that single AA scarcity would impair protein synthesis in the host, with penalties on both its resistance and resilience to parasitic infection. Although there is an abundance of literature on the effects of individual AA deficiency on food intake (Gietzen *et al.,* 2007), performance (D'Mello, 2003) and immune responses (Li *et al.,* 2007) of growing animals, to our knowledge, this the first study on AA deficiency in periparturient parasitized hosts.

In the present study we focused on leucine and methionine, which have both been implicated in host responses to parasitism. Leucine has been directly related to the immune response in both *in vitro* and *in vivo* studies (Calder, 2006), and its requirements are increased during parasitism. Parasitized hosts show an increased intestinal metabolism of leucine and consequently reduced availability of leucine for other tissues (Yu *et al.,* 2000). In addition, lactation also increases leucine requirements, as an increased channelling of leucine from the blood flow to the mammary gland has been observed (Trottier *et al.,* 1997; DeSandiago *et al.,* 1998a). In lactating rats, this increase has been calculated to be up to 35% compared to non-lactating rats (Vina & Williamson, 1981).

Methionine is the only essential sulphur-containing amino acid (SAA) and as a consequence a precursor to a number of other SAA (Grimble, 2006). It plays pivotal roles in protein synthesis, maintenance of gut functions and regulation of the mucosal response to antigens (Fang *et al.,* 2010; Grimble, 2006). There is evidence suggesting that demand for SAA may be increased during GIN infections (Hoskin *et al.,* 2002; Liu *et al.,* 2007), probably due to the loss of SAA-rich endogenous protein

via increased sloughing of epithelial cells and especially mucin secretion (Poppi *et al.,* 1986). Importantly, methionine supplementation of growing rats infected with *Nippostrongylous brasiliensis* resulted in a reduction of worm burdens (Cummins *et al.,* 1986).

In this experiment we hypothesized that the reduced provision of either leucine or methionine, at high levels of other AA intake, will penalise lactational performance and resistance to *N. brasiliensis* as a consequence of reduced AA availability to maintain the protective immune response.

4.3. Materials and methods

4.3.1. Animals, housing and feeding strategy during gestation

The experiment described below was approved by SAC's Ethical Review Committee (ED AE 24/2007) and carried out under Home Office authorization (PPL 60/3626). Seventy two second parity female rats were housed in a room where ambient temperature was maintained at 21°C, relative humidity ranged from 45 to 65%, and artificial lighting was provided between 08.00-18.00 hours. Rats were individually housed in solid-bottomed cages with fresh sawdust provided weekly. Shredded plastic bubble wrapping for nesting material was provided 3 days before the expected parturition date. Wire-bottomed cages were used during mating and for faeces collection during the primary infection as described previously (Houdijk *et al.,* 2003a). For mating, female rats were placed with a proven male breeder and mating was confirmed through the presence of a vaginal plug. Until mating was confirmed rats were given *ad libitum* access to standard rat chow (Rat and Mouse No 3, Special Diet Services, Witham, UK). After mating was confirmed, rats were given *ad libitum*

access to a high protein food, with 210g digestible crude protein (CP) and 16.4 MJ metabolizable energy (ME) per kg dry matter (DM) until ten days into gestation to allow for establishment of pregnancy and placental development. Rats were then transferred to a low protein food containing 60g CP and 17.3 MJ ME per kg DM which was given until parturition. This feeding protocol was used to reduce body protein reserves during the second half of gestation in order to maximize the degree of protein AA scarcity during lactation when rats would be fed low protein foods (Pine *et al.,* 1994; Houdijk *et al.,* 2005a).

4.3.2. Feeding treatments

Upon parturition (day 0), dams were allocated to one of four feeding treatments, i.e. LP, HP, HP-Leu and HP-Met. Foods LP and HP were formulated to supply 150 and 250 g CP/kg, respectively. These CP levels were chosen on the basis of previous observations where lactating rats fed foods supplying 150 CP g/kg have shown significantly reduced lactational performance, elevated worm burdens and penalized expression of immunity compared to those receiving 250 g CP/kg (Jones *et al*., 2011; Sakkas *et al*., 2011 [Chapter 2]). The protein source used for the LP food was methionine-enriched casein, and the LP food was the basis for all other foods. Casein was methionine-enriched, to overcome its natural deficiency in SAA content, as our aim was for LP foods to be balanced in their amino acid profile (DeMan, 1999). The HP food was prepared by adding a purified AAs mixture to the LP food at the expense of starch and sucrose (Table 4.1) to mimic the 250 g CP/kg food (Jones *et al*., 2011; Sakkas *et al*., 2011 [Chapter 2]). For HP-Leu and HP-Met foods, leucine and methionine were likewise replaced in the added AA mixture with starch

and sucrose. The resulting composition of the four experimental foods is presented in Table 4.1. Daily allowances were offered at 90% of previously observed dry matter (DM) intake (Jones *et al*., 2009), using dam parturition body weight (PBW) as a scaling factor. Foods were offered in increasing amounts during lactation, reflecting the natural increase in food intake observed in previous experiments using this animal-parasite model system (Houdijk *et al*., 2005a; Normanton *et al*., 2005; Jones *et al*., 2009).

Table 4.1. Composition and analysis of the experimental food used during lactation

Experimental foods	HP	HP-Leu	HP-Met	LP
Ingredients (g/kg fresh)				
Casein*	150	150	150	150
Amino acid mixture **	100	91	96	0
Starch	232	238	235	299
Surcose	116	119	117	149
Corn oil	150	150	150	150
Vitamins	50	50	50	50
Minerals	50	50	50	50
Cornflour	43	43	43	43
Choline	7	7	7	7
Lecithin	2	2	2	2
Alphacel	100	100	100	100
Analysed chemical composition (g/kg DM unless otherwise stated)				
DM (g/kg fresh matter)	678.6	688.7	689.3	672.1
ME (MJ/kg DM)*	15.9	15.9	15.9	15.9
CP	237.3	227.1	235.3	149.8
Ash	39.0	38.6	38.2	38.3
Acid detergent fibre	66.1	61	63.6	62.7
Diethyl ether extract	150	148	149	150
Total sugars	177	185	178	209
Starch	253	248	252	313

Table 4.1. –Continue–

AA contents after hydrolysis of protein (%) DM				
Experimental foods	HP	HP-Leu	HP-Met	LP
Methionine	0.91	0.89	0.55	0.52
Cysteine	0.11	0.11	0.11	0.08
Methionine+Cysteine	1.02	0.99	0.66	0.59
Lysine	1.96	1.93	1.99	1.15
Threonine	1.03	1.02	1.03	0.61
Tryptophan	0.31	0.32	0.32	0.19
Arginine	0.92	0.90	0.92	0.51
Isoleucine	1.31	1.24	1.28	0.72
Leucine	2.36	1.39	2.43	1.36
Valine	1.61	1.59	1.65	0.93
Histidine	0.70	0.71	0.73	0.42
Phenylanine	1.22	1.21	1.25	0.72
Glycine	0.48	0.47	0.48	0.27
Serine	1.30	1.28	1.28	0.79
Proline	2.56	2.75	2.84	1.44
Alanine	0.75	0.74	0.76	0.45
Aspartic acid	1.74	1.72	1.76	1.03
Glutamic acid	5.40	5.27	5.39	3.06

* Food ME contents was calculated by multiplying its contents of protein (casein), digestible carbohydrates (starch, sucrose, corn flour) and fat (corn oil) with the ME contents of protein (17 MJ/kg), carbohydrates (17 MJ/kg) and fat (38 MJ/kg) (Astrup & Tremblay, 2009).

*Casein was enriched with methionine (casein:methionine ratio was 99:1).

**The amino acid mixture included in the experiment foods HP, HP-Leu and HP-Met represented methionine-enriched casein, or that without leucine, or that without methionine, respectively.

4.3.3. Infection protocol

All rats received a primary infection of 1600 third-stage infective larvae (L_3) of *N. brasiliensis* on day –37 (with day 0 as mean achieved parturition date), which were suspended in 0.5 ml sterile phosphate buffered saline (PBS) that was subcutaneously injected in the hind leg. A secondary infection of 1600 L_3 *N. brasiliensis* was administered on day 2 to a sub-group of rats. At the same time, control rats (primary infected only) were sham-infected through subcutaneous injection of 0.5 ml sterile PBS only.

4.3.4. Experimental design

The effects of the four feeding treatments were assessed on lactational performance and on parasitological and immunological variables on day 8 and 11 (corresponding to days 6 and 9 post secondary infection, respectively). These two sampling time points post secondary infection were included, as previous studies with the same model suggested that the nutritional sensitivity of host resistance and expression of immunity in this model may vary over time (Jones *et al.,* 2009; Jones *et al*., 2011; Sakkas *et al*., 2011 [Chapter 2]). All rats in these 8 factorial feeding treatment-endpoint combinations (4 feeding treatments x 2 endpoints) received the secondary infection. Sham-infected control rats were also included until day 8 only (first time point) for each feeding treatment to assess the effect of re-infection on lactational performance and expression of immunity. This resulted in a total of 12 experimental treatments. Rats were blocked for PBW and were randomly allocated to these 12 treatments on the morning parturition was observed to be complete. Total sample size aimed for was n=6 for infected rats and n=5 for control rats. However, the

minimum realized sample size was n=5 for infected rats and n=4 for control rats due to infertility, miscarriage or the occurence of pup deaths.

4.3.5. Performance and Parasitology

Body weight and food intake. Rats were weighed daily throughout the experiment, and daily body weights taken post parturition were used to calculate lactational dam weight gain. The pups were counted and the whole litter weighed daily from day 0. Litter size was standardized at 12 pups on day 1 to have equal initial nutrient demands. Litter weights from day 1 onwards were used to calculate litter weight gain. Any refusals observed during the lactation period were weighed to assess observed DM intake. Foods offered during lactation were sampled during their preparation for the analysis of DM, CP (Kjeldahl-N x 6.38), diethyl ether extract, ash, acid detergent fibre, starch, sucrose, estimated metabolizable energy content (ME) and amino acid profile (Table 4.1).

Faecal egg counts. A faecal egg count (FEC, in eggs per gram (epg) of fresh faeces) was performed seven days after the primary infection (day -30) to confirm establishment of infection. A second FEC was performed on day -23 to confirm parasite expulsion. To this effect, faeces were collected through overnight housing on bottom-wired cages (Houdijk *et al*., 2003a) and FEC were performed using a modified saturated salt flotation method (Christie & Jackson, 1982).

Worm burden and nematode eggs in colon. Rats were killed humanely through gradually increasing ambient CO_2 concentration for parasitological assessment and immune responses (see below). Nematodes were harvested from the small intestine using a baermanization technique as outlined previously (Houdijk *et al.,* 2003a). The

resulting worms were then stored in formaldehyde for subsequent counting and assessment of sex and maturation status according to morphology. The contents of the large intestine were weighed and assessed for worm eggs as described for FEC above. This was then multiplied by the large intestinal contents weight to obtain the total number of nematode eggs in the colon (EIC).

4.3.6. Immunology

Work carried out by Leigh Jones with the excemption of goblet cell enumeration.

Histology. A 2cm sample of small intestine at a 25cm distance from the pylorus was washed with PBS to collect worms for total worm counts (see above) and then fixed in 4% paraformaldehyde for 6 hours. The fixative was then replaced by 70% ethanol. Resulting intestinal samples were wax embedded and sections taken and stained using standard histological techniques. Sections were stained for counting three types of inflammatory cells, i.e. eosinophils (carbol chromatrope 2R), goblet cells (alcian blue and counterstaining with periodic acid-schiff) and mast cells (toluidine blue). Cells were enumerated by counting 10 complete, well orientated, villus crypt units (VCU) per section and results were expressed as number of cells per VCU.

Enzyme linked immunosorbent assays (ELISA). Terminal blood samples taken from the chest cavity following heart puncture were centrifuged at 200g for 10 minutes and serum supernatants stored at -70ºC for determination of antibody levels. The concentration of anti-*N.brasiliensis* antibodies (IgA, IgG, IgG1, IgG2a, IgG2b and IgE) were measured by ELISA. Briefly, ELISA plates were coated with 1µg/ml adult somatic *N. brasiliensis* antigen (Ball *et al*., 2007) then blocked overnight at 4°C with 10% dried milk powder in PBS. 1:1000 diluted serum samples were added to

the plate and incubated at room temperature for 1 hour. Antibody isotypes were detected by addition of rat specific secondary antibodies at 1 in 5000 dilution for anti-rat IgE, IgG, IgG1, IgG2a and IgG2b and 1 in 1000 dilution for anti-rat IgA. Anti-rat IgG, IgG2a and IgG2b were obtained from Sigma (Gillingham, UK) and anti-rat IgG1, IgA and IgE were obtained from Serotec (Kidlington, UK). Horse radish peroxidase (HRP) conjugated anti-rat IgG was quantified using Sigma Fast-OPD (Sigma) substrate with the reaction being stopped by addition of 2.5M H_2SO_4. Bound anti-rat IgE, IgG1, IgG2a and IgG2b was detected by the addition of a horse-radish peroxidase (HRP) conjugated anti-mouse Ig antibody (Dako, Ely, UK) at a dilution of 1 in 1000 before being quantified using Sigma Fast OPD as previously described. Antibody levels were expressed as the backtransformed means of OD readings at 492nm.

4.3.7. Calculations and statistical analysis

Data were analysed using Restricted Maximum Likelihood (REML), to account for differences in the achieved number of replicates per group. The factors used were feeding treatment (LP, HP, HP-Leu, HP-Met), infection pressure (secondary infection and sham control) and their 2-way interaction on achieved feed intake, dam weight gain and litter weight gain including data from all animals until day 8. The same factors were used on inflammatory cells and antibody responses for animals that were terminated at day 8 only (infected and control animals killed on day 8). REML was also used to assess effects of feeding treatment, endpoint (days 8 and 11) and their 2 way interaction on dam weight gain, litter weight gain, worm burdens, percentage of female worms, EIC, inflammatory cells and antibody responses.

Performance data are presented as arithmetic means, associated with standard errors (SE) or standard error of the difference (SED). However, because of their skewed nature, EIC, worm burdens, inflammatory cells and antibody responses were log-transformed (n + 1) to normalize variance before statistical analysis. These data are reported as back-transformed means with their 95% CI (Jones *et al*., 2009). All statistical analyses were performed using Genstat 11 for Windows release 11.1, 2008 (VSN international, Hemel Hempstead, UK).

4.4. Results

4.4.1. Faecal egg counts and performance until parturition

FEC taken on day -37 and day -30 averaged 9462 (CI: 1010-913) epg and 0 (CI: 0-0) epg, respectively. Mean body weight on arrival was 374 (SE 6.8) g. During the first 10 days of gestation, pregnant rats grew from 387 (SE 6.4) to 425 (SE 6.3) g and had an average DM intake of 21.2 (SE 0.40) g/day. From then onwards and until parturition, the pregnant rats continued to grow to a mean weight of 512 (SE 5.7) g and had an average DM intake of 19.9 (SE 0.46) g/day, which dropped to an average of 7.3 (SE 2.0) g/day just before parturition. Dam mean PBW averaged 406 (SE 7.7) g, mean litter size averaged 14.4 pups (SE 0.39) and mean litter body weight averaged 76.6 g (SE 1.8) g.

4.4.2. Achieved DM intake

Figure 4.1a shows the effects of feeding treatments on achieved DM intake/kg PBW/day during lactation in infected and sham infected control rats up until day 8. Achieved DM intake was significantly affected by feeding treatment ($p<0.002$) but

not by infection (P=0.418) or their interaction (P=0.413). This effect of food is attributed to the lower achieved DM intake in HP-Met animals due to the occurrence of refusals in this feeding treatment (Figure 4.1a). Achieved DM intake was not affected by endpoint (P=0.157) or the interaction between feeding treatment and endpoint (P=0.365).

4.4.3. Dam weight gain

Figure 4.1b shows the effects of feeding treatments on dam weight gain during lactation in infected and sham infected control rats up until day 8. Dam weight gain was significantly affected by feeding treatment (P<0.001) but not by infection (P=0.227) or their interaction (P=0.105). HP dams gained more weight than dams on the other feeding treatments, with HP-Met dams showing the lowest weight gain (Figure 4.1b). Dam weight gain was not affected by endpoint (P=0.153) but it was affected by the interaction between feeding treatment and endpoint; HP-Met animals allocated for dissection on day 8 showed lower weight gain than HP-Met dams allocated for dissection on day 11 of lactation (-6.2 vs -0.7 g/day; s.e.d. 2.91 g/day; P=0.015).

4.4.4. Litter weight gain

Figure 4.1c shows the effects of feeding treatments on litter weight gain during lactation in infected and sham infected control rats up until day 8. Litter weight gain was significantly affected by feeding treatment (P<0.001) but not by infection (P=0.088), whilst it was affected by their interaction (P=0.007). HP-met litters gained less weight than litters on the other feeding treatments with infected HP-Met litters

showing lower weight gain than their sham infected counterparts (Figure 4.1c). As expected litter weight gain was affected by endpoint as litters of dams allocated for dissection on day 8 gained more weight per day than their day 11 counterparts (15.63 vs 18.85; s.e.d 2.79; P=0.023), while it was not affected by the feeding treatment and endpoint interaction (P=0.296).

4.4.5. Total worm burden, eggs in colon and worm burden composition

Figure 4.2 shows the effects of feeding treatments on total worm burdens (a), on EIC (b) and on worm burden composition (c) for days 8 and 11 of lactation. Total worm burdens were significantly affected by feeding treatment (P=0.005) while they were not affected by endpoint (P=0.156), and there was no interaction (P=0.721). Across end points, HP rats harboured fewer worms than rats on the other diets, which showed similar worm burdens (Figure 4.2a). Likewise, EIC were significantly affected by feeding treatment (P<0.001) while they were not affected by endpoint (P=0.618) and their interaction (P=0.798). HP-met, HP-leu and LP rats had higher and similar EIC than HP rats (Figure 4.2b). Worm burden composition (Figure 4.2c) was significantly affected by endpoint (P=0.028) but not by feeding treatment (P=0.634) or their interaction (P=0.274); day 8 rats harboured fewer female worms then their day 11 counterparts.

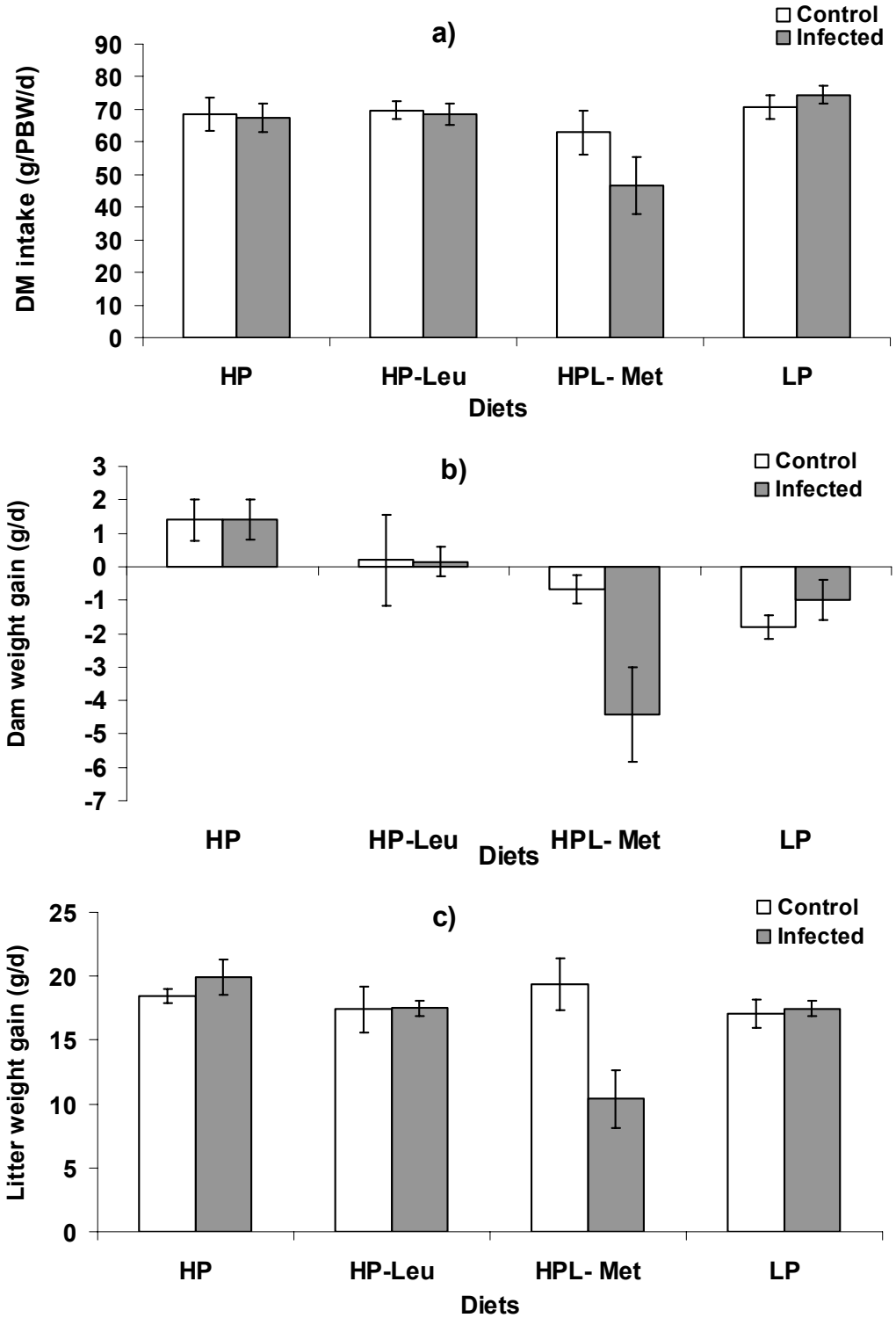


Figure 4.1. Average food intake (a; with s.e.), dam weight gain (b; with s.e.) and litter weight.gain (c; with s.e.) across the four feeding treatments for rats either re-infected with *Nippostrongylus brasiliensis* or sham-infected on day 2 of lactation and control lactating rats.

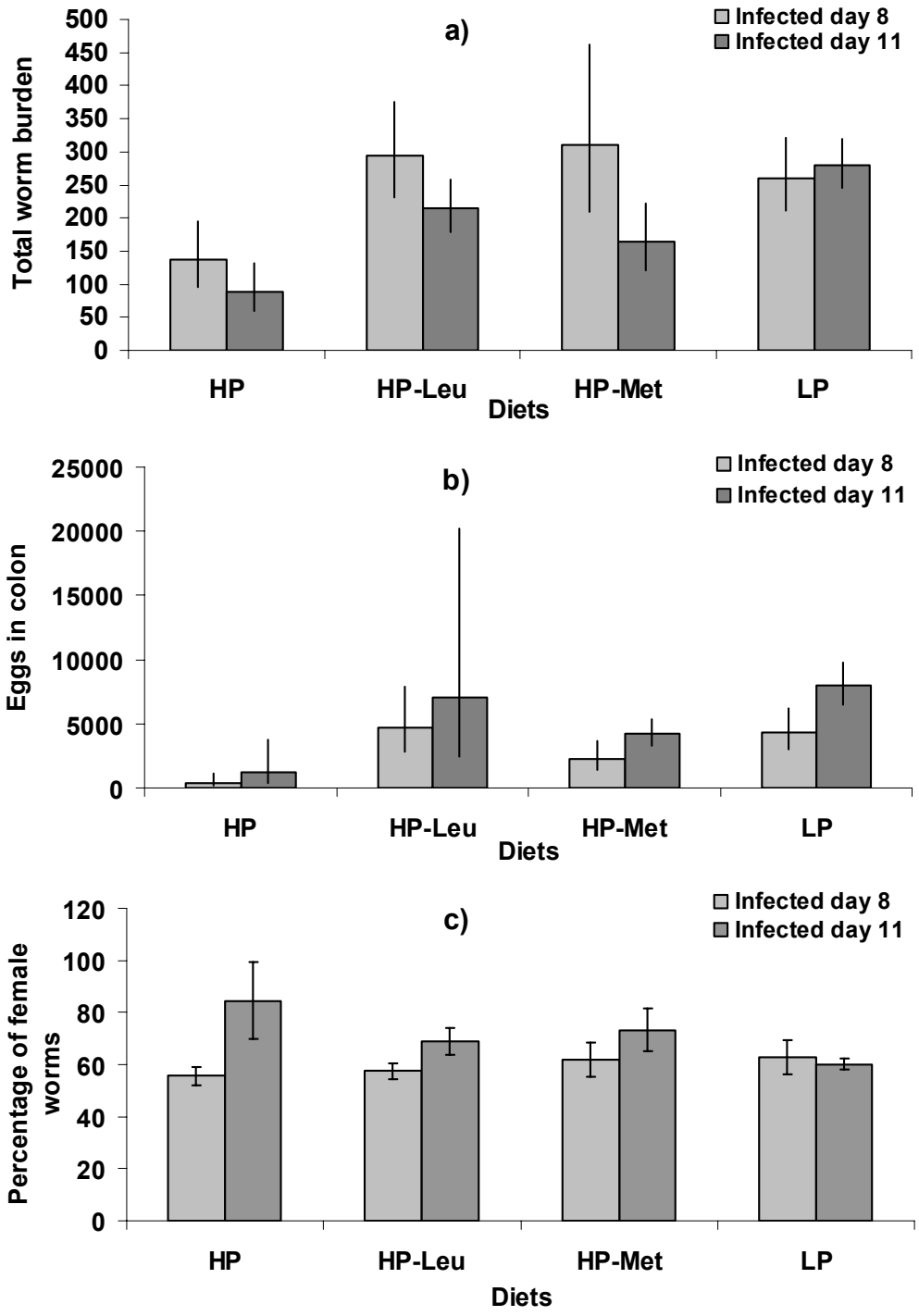


Figure 4.2. Backtransformed mean worm burdens (4.2a), backtransformed mean total eggs in colon (4.2b) with backtransformed lower and upper limits of transformed error bars and percentage of female worms (with s.e.) taken on day 8 and 11 following reinfection with *Nippostrongylus brasiliensis* on day 2 of lactation in rats in the four feeding treatments.

4.4.6. Gut histopathology

Figure 4.3a, 4.3b and 4.3c shows the effects of feeding treatments on small intestinal eosinophil, goblet cell and mucosal mast cell counts, respectively, in sham-infected control rats (day 8) and infected rats (days 8 and 11). Feeding treatment and infection interacted for eosinophil counts ($P=0.029$); infection increased eosinophil counts for HP rats only ($P<0.001$). Feeding treatment and endpoint interacted for eosinophil counts in the infected rats ($P=0.029$); Feeding treatment had a significant effect on day 8 only ($P=0.004$), where HP rats had higher eosinophil counts than HP-Met rats ($P<0.01$), whilst those of HP-Leu and LP rats were intermediate. In addition, eosinophil counts were higher on day 11 than on day 8 ($P<0.001$). Feeding treatment, infection status or endpoint did not affect goblet cell counts ($P>0.20$). Likewise, feeding treatments did not affect mucosal mast cell numbers, which did increase upon infection ($P=0.016$) and over time ($P<0.001$).

4.4.7. Systemic antibody levels

Figure 4.4 shows the effects of feeding treatments on serum levels of *N. brasiliensis* total IgG (4.4a) and specific IgA (4.4b), IgE (4.4c), IgG1, IgG2a (4.4d), IgG2b (4.4e) and total IgG (4f) in sham-infected control rats (day 8) and infected rats (days 8 and 11). Infection significantly increased the serum levels of all antibodies assessed ($P<0.001$). Feeding treatment and endpoint interacted for IgE ($P=0.023$) and total IgG ($P=0.044$) on day 11 only, where IgE levels were lower in HP-Met rats and total IgG levels were higher for LP rats compared to the other feeding treatment groups. Feeding treatment, end point and their interaction did not significantly affect IgA, IgG1, IgG2a and IgG2b levels ($P>0.10$ for all).

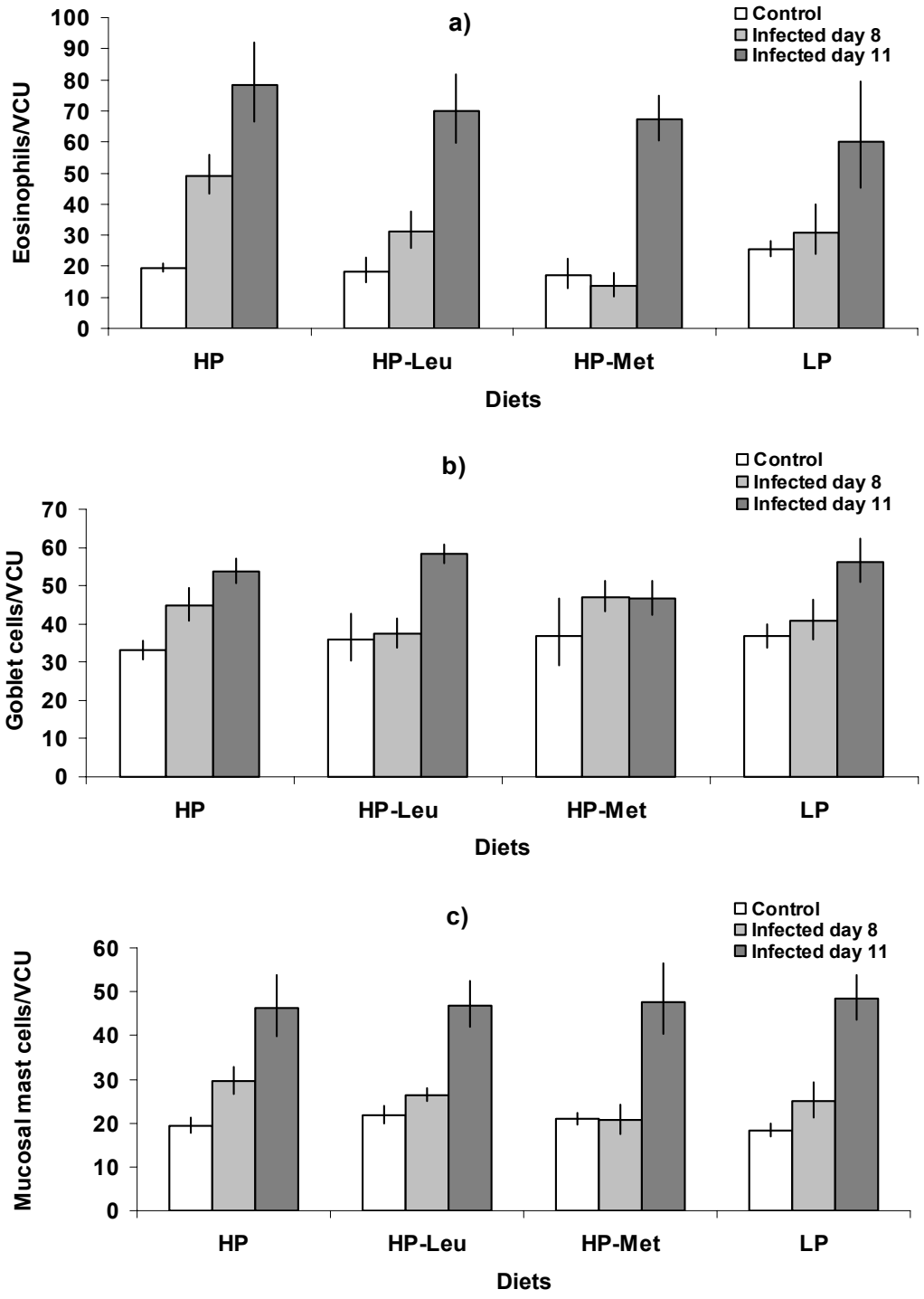
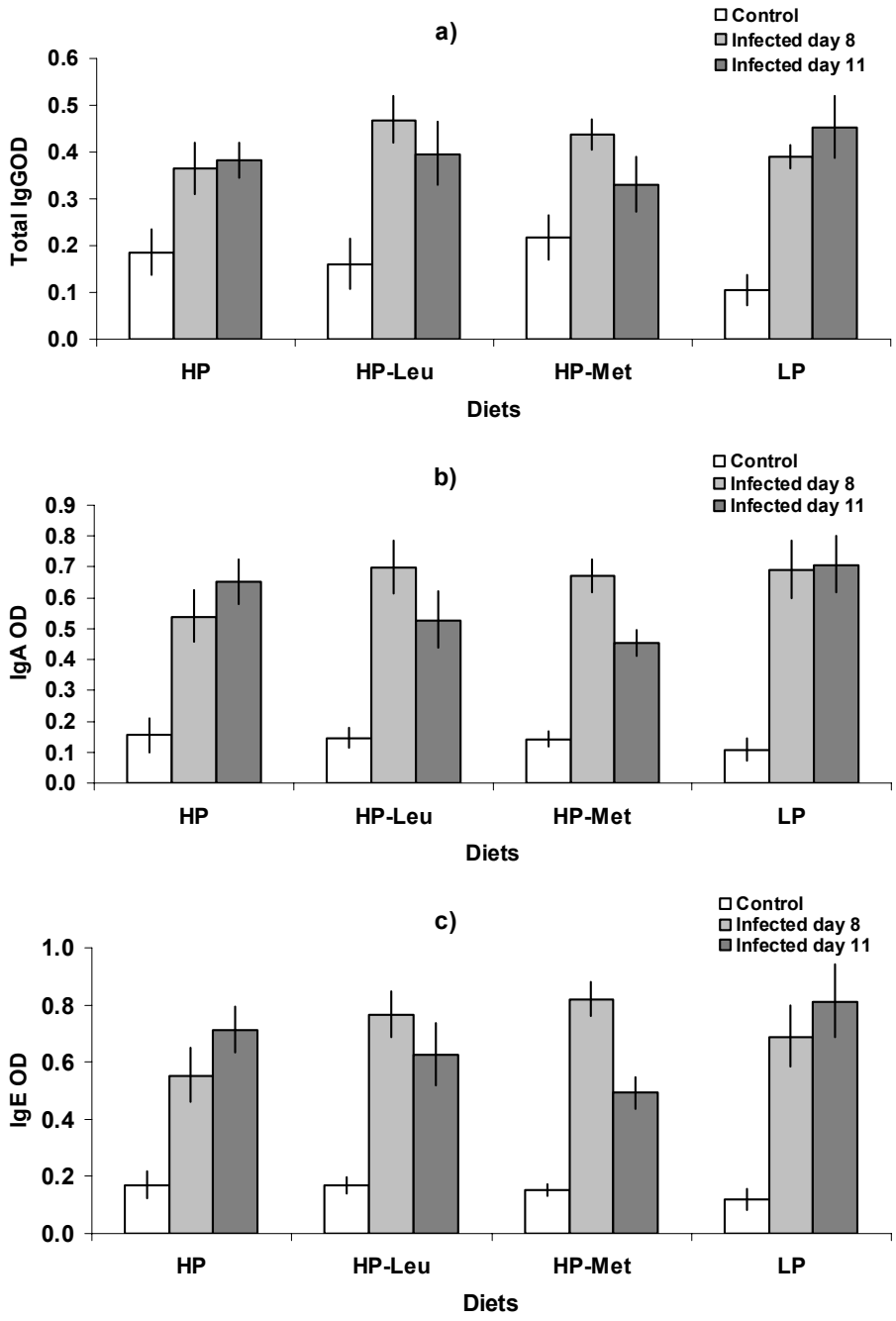


Figure 4.3. Backtransformed mean eosinophil (4.3a), mucosal mast cells (4.3b) and goblet cell numbers (4.3c) with backtransformed lower and upper limits of transformed error bars,taken on day 8 and 11 following reinfection with *Nippostrongylus brasiliensis* on day 2 of lactation or taken on day 8 following sham-infection on day 2 of lactation in rats in the four feeding treatments.


a)
Control
Infected day 8
Infected day 11
Total IgGOD
0.6
0.5
0.4
0.3
0.2
0.1
0.0
HP
HP-Leu
HP-Met
LP
Diets
b)
Control
Infected day 8
Infected day 11
IgA OD
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
HP
HP-Leu
HP-Met
LP
Diets
c)
Control
Infected day 8
Infected day 11
IgE OD
1.0
0.8
0.6
0.4
0.2
0.0
HP
HP-Leu
HP-Met
LP
Diets

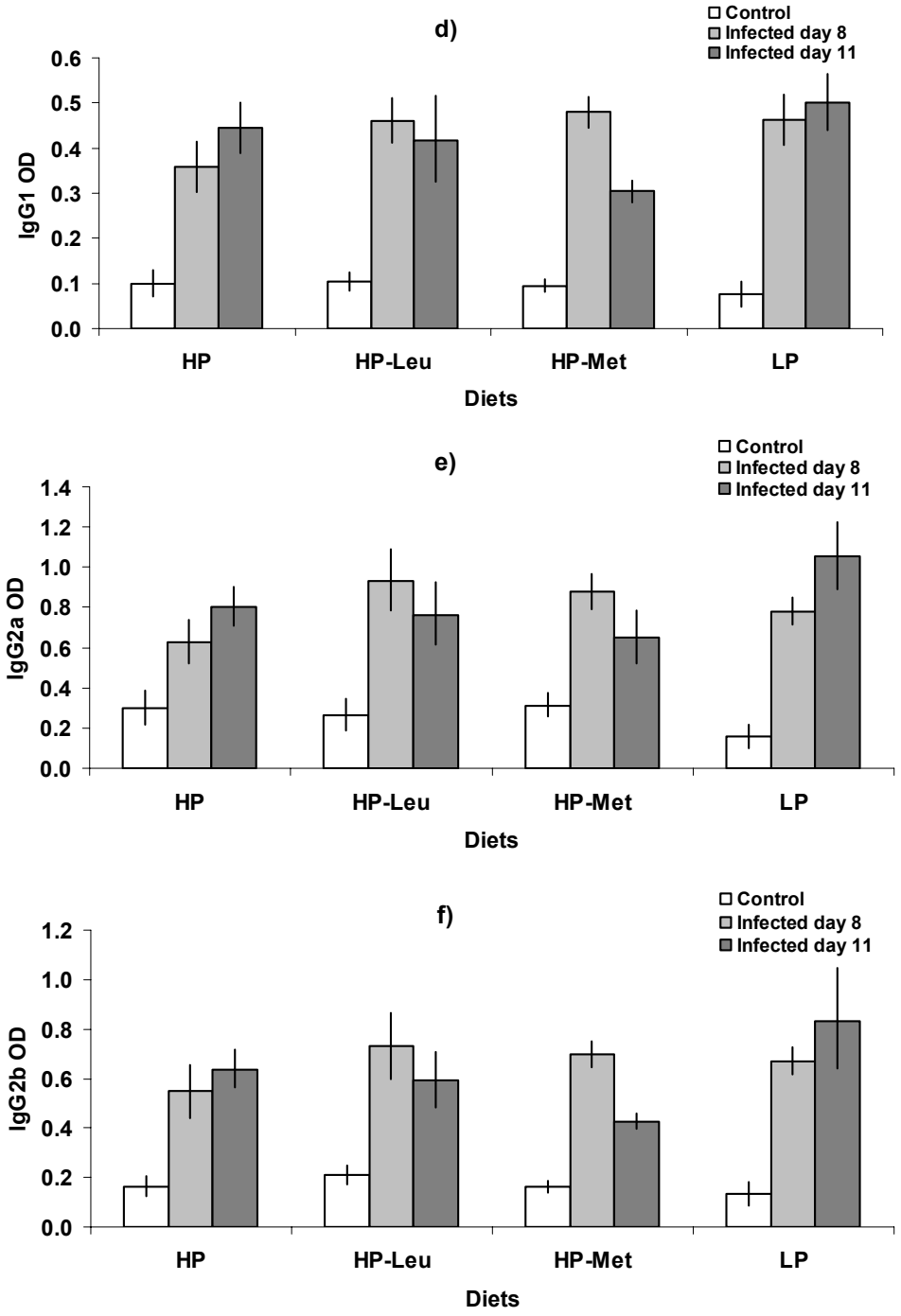


Figure 4.4. Backtransformed mean serum levels of *Nippostrongylus brasiliensis* specific IgA (4.4a), IgE (4.4b), IgG1 (4.4c), IgG2a (4.4d), IgG2b (4.4e) and total IgG (4.4f) with backtransformed lower and upper limits of transformed error bars, taken on day 8 and 11 following reinfection with *Nippostrongylus brasiliensis* on day 2 of lactation or taken on day 8 following sham-infection on day 2 of lactation in rats in the four feeding treatments.

4.5. Discussion

Previous studies carried out with this rodent – nematode parasite model have shown that feeding low CP foods results in higher worm burdens, egg excretion, increased proportion of female worms and reduced immune responses compared to feeding high CP foods (Houdijk *et al*., 2005a; Jones *et al*., 2009; Jones *et al*., 2011; Sakkas *et al*., 2011 [Chapter 2]). It is reasonable to assume that responses to CP scarcity, including during GIN infections, are responses to essential AA scarcity and it was expected that reduced availability of individual AAs would penalise host's immune response. Here we hypothesised that a reduced availability of either all AAs (LP) or of the essential AAs leucine (HP-Leu) or methionine (HP-Met) would result in penalties on both the lactational performance and the expression of immunity to parasites, thus resulting in higher worm burdens. We demonstrated that dietary scarcity of crude protein (LP) or of either methionine or leucine resulted in differences in the dam and litter weight gain, and the level of parasitism in the infected host. However, in contrast to our earlier findings (Jones *et al*., 2011), these observed effects did not appear to be related to changes in mucosal immune cell populations or serum antibody levels, which were largely unaffected by our feeding treatments. The effects observed of our EAA deficient foods are discussed below in relation to their reduced supply for the activation and maintenance of host's bodily functions associated with the expression of immunity to *N. brasiliensis* and lactation.

Parasitic infection leads to increased protein requirements of the host in order to activate, or maintain, an effective protective response (Kyriazakis & Houdijk, 2006). Effector molecules of the immune response are highly proteinaceous, and inflammation and immune system activation are characterised by

the synthesis of specific proteins that play crucial roles in the defence of the host against pathogens and modulation of the ongoing immune response (Coop & Kyriazakis 2001; Le Floc'h *et al*., 2004). In addition, lactation also increases protein requirements, resulting in increased AA uptake into the mammary gland (Trottier *et al*., 1997; Vina & Williamson, 1981). In our experimental design these effects were expected to be even more pronounced due to the depleted state of the labile protein reserves as a result of the consumption of a low CP food during the second half of gestation (Pine *et al*., 1994).

As in our previous experiment (Sakkas *et al*., 2011 [Chapter 2]), restricted feeding of LP foods to lactating parasitized hosts resulted in protein scarcity. The penalties of the protein scarcity were evident from a reduced lactational performance (lower dam weight gain and to a lesser extent a reduced litter weight gain) and higher levels of parasitism (worm burdens and EIC) in LP rats compared with HP rats. In earlier studies, effects of CP supply on these parasitological measurements have concurred with nutritional sensitivity of the number of inflammatory cells in the intestinal epithelium, such as mucosal mast cells, goblet cells and eosinophils, and local and circulating antibody responses (Jones *et al*., 2009; Jones *et al*., 2011). However, in this experiment we did not observe an effect of CP supply on mucosal mast cells counts, goblet cell counts or the concentration of any of the immunoglobulins measured. In addition, the observed nutritional sensitivity of eosinophil counts was due to the HP-Met group, and not the LP group. It should be noted that the studies of Jones *et al*. (2009) and Jones *et al*. (2011) were designed to induce lower levels of protein scarcity than the one imposed in the present study, achieving CP supply during lactation as low as 6.6g per day per kg PBW as opposed

to 13.3 g of CP per kg PBW offered in this experiment. Here, we deliberately chose to use a less severe level of CP scarcity in order to minimize the possible effect of AA imbalance *per se* for the essential AA deficient foods. It is well known that parasite expulsion is mediated by a range of effector molecules (Lawrence, 2003; Patel *et al*., 2009) and that no single mechanism is responsible for the expulsion of the parasite population. Therefore, it can not be excluded that our feeding treatments may have affected other than the measured immunological responses.

The restricted consumption of HP-Met food imposed penalties to the dam and litter weight gain and to the expression of the immune response as reflected by the reduced eosinophilia and increased worm burdens and EIC. These effects may be attributed to a large extent to the lower intake achieved in HP-Met rats. In accordance to our data *ad libitum* food intake of SAA deficient foods by adult rats has been shown to be half of a reference food (Nkabyo *et al*., 2006). In our study, 5 out of 11 infected HP-Met rats refused approximately 70% of their already restricted allowances (4 allocated to day 8 and 1 to day 11). Such refusals were noted as soon as the food was offered, i.e. from day 0 onwards and prior to the secondary infection thus their absence in the sham-infected control animals may be considered to be coincidental. The magnitude of these refusals suggests that observed effects on all variables may have arisen from a deficiency in any of the AAs offered and/or any other resource, including ME. In this model system, it has been previously shown that even a moderate ME restriction of 25% imposes penalties on the lactational performance, but not penalties in the parasitological (Sakkas *et al*., 2011 [Chapter 2]) or immunological variables measured (Jones *et al*., 2011). However, data from other experiments in growing parasitized hosts (Koski *et al.,* 1999; Kristan, 2007; Lunn *et*

al., 1988) where the ME restriction was more severe in magnitude and had been applied for a longer period of time, indicate that a ME restriction can penalise host's resistance.

In order to dissociate observed effects arising from the food refusal rather than from a methionine deficiency alone, additional REML statistical analyses were performed following omission of the HP-Met rats that had shown food refusals (HP-Met-Ref). The analyses showed that in the absence of HP-Met-Ref rats, feeding treatments did not affect eosinophil counts (P=0.369) and litter weight gain (P=0.166). Our feeding treatments still affected worm burdens (P=0.002) but this was due to LP and HP-Leu dams harbouring higher worm numbers. HP-met dams had similar worm burdens to HP dams (1.963 vs 2.070; s.e.d 0.178; P=0.554). Nonetheless dam weight gain, litter weight gain and EIC were still affected with those HP-Met dams consuming all their food having lower weight gain (1.41 vs -0.62 g/day; s.e.d. 0.65 g/day; P=0.001) and higher EIC (2.26 vs 3.30 s.e.d 0.46; P<0.001) than HP dams. Further analysis of variance indicates that HP-Met-Ref rats had similar log-transformed EIC (3.30 vs 3.51; s.e.d. 0.271; P=0.461) but higher log transformed worm burdens (2.07 vs 2.575; s.e.d. 0.1675; P=0.015) than their fully fed infected counterparts. This points out that severe undernutrition due to methionine deficiency such as that observed here, further impaired resistance to parasites to some extent. Effects on eosinophil numbers in these animals could be related to the further decrease in methionine intake that occurred in HP-met-Ref dams. This is perhaps not unexpected given the role of n-formyl methionyl leucyl phenylalanine, a methionine containing chemotactic factor, in eosinophil migration (Michail & Abernathy, 2004).

The penalties observed in the animals that consumed all their food are likely not the consequences of a methionine deficiency alone but may have arisen from a general SAA deficiency. Casein is limiting in its sulphur AA content, while having very low levels of cysteine (DeMan, 1999). Cysteine is considered a semi-indispensable AA, the availability of which is dependent on methionine intake and a deficiency in methionine intake can cause a deficiency in cysteine (Stipanuk, 2004). Consequently, it is likely that in addition to methionine scarcity, HP-Met consumption also resulted to a general SAA deficit, as the level of cysteine in casein and in the corresponding AA mixture is very low (Table 4.1). Indeed, it has been shown that methionine restriction in rats caused lower serum cysteine concentrations (Nkabyo *et al*., 2006). In addition, the reduced performance may also have arisen from a reduced efficiency of utilization of protein of the ingested food. It has been shown that adult rats fed methionine restricted foods for 12 weeks significantly reduced their body weight (44%), serum concentrations of sulphur AAs and glutathione (Elshorbagy *et al*., 2010). Restricted feeding at the same level of food intake of a SAA deficient and a SAA supplemented food resulted in a lower weight gain for the former animals comparing to the latter (Nkabyo *et al*., 2006). In the absence of food refusal HP-Met dams showed reduced dam weight gain with no penalties on litter weight gain, which may be indicative of a reduced efficiency of utilization of ingested CP, which in turn may have led to a higher degree of body protein mobilization.

Consumption of our HP-Met food resulted in significantly elevated worm burdens and EIC. Methionine is the only essential SAA and along with cysteine has very important roles for maintenance of intestinal functions as well as for the

immune surveillance of the intestinal epithelial layer and regulation of the mucosal response to foreign antigens (Fang *et al*., 2010). Although cysteine is not an essential AA, it can become conditionally essential during inflammation where it is used for the production of acute-phase proteins and for the synthesis of glutathione, which in turn is required for antioxidant synthesis (Tesseraud *et al*., 2009). Cysteine availability is also important for maintenance of epithelial cell glutathione levels (Bauchart-Thevret *et al*., 2009) and parasite-resistant sheep appear to have a higher demand for glutathione compared to parasite-susceptible sheep (Liu *et al*., 2005). Dietary SAA deficiency in neonatal pigs has been shown to induce small intestinal villus atrophy, lower goblet cell numbers, and lower tissue glutathione, especially in the jejunum (Bauchart-Thevret *et al*., 2009). Furthermore, dietary supplementation with SAA has been shown to enhance many aspects of the immune response. For example, dietary supplementation with N-acetyl-cysteine (a stable precursor of cysteine) is highly effective in enhancing immunity under various disease states in both animal and clinical trials (Grimble, 2006). Experimental studies also support the view that gastrointestinal parasitism may impose additional demands in sulphur containing AAs. It has been shown that 20% of dietary methionine intake is metabolized by the intestine (Riedijk *et al*., 2007). Intestinal methionine oxidation has been linked to the synthesis of mucins by goblet cells, which play an important role in the defence of the host towards GIN infection (Else & Finkelman, 1998; Van Klinken *et al*., 1998). Although in our study we did not observe differences in the goblet cell number between HP-Met and HP rats, differential expression of mucins and/or levels of mucus production can not be excluded. In addition to mucus secretion, methionine has been linked with an indirect role in lymphocyte

proliferation and differentiation via spermidine and spermine metabolism (Liu *et al*., 2007; Flynn *et al*., 2002), and leukocyte metabolism via phosphatidylcholine synthesis (Kim *et al*., 2007). Therefore, a variety of effector molecules in addition to those investigated in the current study may be responsible for the immune-modulatory effects of methionine, or lack thereof, on parasitized rats.

The performance data shows that HP-Leu feeding did not significantly penalize litter and dam weight gain in comparison to the animals on the HP. The uptake of AAs from the mammary gland usually exceeds their quantitative excretion in milk, especially for leucine, in many mammalian species including pigs, goats and rats (Trottier *et al.,* 1997; Bequette *et al.,* 1996; DeSandiago *et al.,* 1998a). A decrease in leucine blood concentration occurs during lactation (Vina & Williamson, 1981) when enzymes which are associated with its catabolism are induced in the mammary gland (DeSandiago *et al*., 1998), directing it for the synthesis of glutamine and glutamate (Li *et al*., 2009), which are abundant AAs in milk proteins (Davis *et al*., 1993). Additionally, the excess net uptake of leucine by the mammary gland is further enhanced by dietary protein supplementation (Bequette *et al*., 1996). As a consequence one would expect that leucine restriction could lead to a penalised pup performance arising from the reduced supply of leucine to the mammary gland and/or an increased mobilization of body protein tissue, which would be reflected in the dam body weight gain. Although, numerically dam and pup weight gain was lower for the HP-Leu dams compared to HP dams this difference failed to reach statistical significance and this may be related to the relatively small sample size. Additionally, weight gain does not give specific information about body composition in general, and lean to fat ratio in particular, of the dams or their litters. For example,

it has been shown that increased supply of leucine in lactating dam's results in a higher lean/fat ratio (López *et al*., 2010) probably due to a reduced degree of tissue mobilization.

Despite the relatively small effect of leucine deficiency on lactational performance as reflected in dam and litter weight gain, HP-Leu dams had significantly higher worm burdens and EIC compared to HP dams, which was associated with some reduction in eosinophil numbers, while none of the other immune measurements was affected. The parasitological data indicate that the penalties induced on the immune response were higher in comparison to the lactational performance under leucine deficiency. This is in agreement with the suggestion that immunity to parasites is given a lower priority than reproductive functions in periparturient hosts when nutrients are scarce (Coop & Kyriazakis 1999). The absence of an effect on the immune parameters measured is contrary to the expectation as cell culture and animal feeding studies, show that there is a dose-response relationship between leucine supply and the immune response to pathogens (Calder *et al*., 2006). Among branch chain AAs, leucine is the sole branched chain AA that can activate the mTOR signaling pathway in intestinal epithelial cells (Ban *et al*., 2004) and it may play an important role in intestinal repair via stimulation of protein growth (Naomoto *et al*., 2005). A damaged mucosa could be associated with the higher number of worm burdens in our HP-Leu dams. Although the number of inflammatory cells in the intestinal mucosa, such as mast cells and eosinophils that are partly responsible for wound repair, did not appear to be affected by the feeding treatment, differential protein or gene expression in these cells cannot be excluded. Finally, compared to a balanced food, a leucine restricted food has been shown to

lead to a 40% decrease in the meal-associated rise in plasma leptin concentrations, while removing either branched chain AA or all AAs from the food did not result in a further decrease (Lynch *et al*., 2006). Leptin is known to be a potent mediator of both the adaptive and the innate immune response (Iikuni *et al.,* 2007) and leptin concentrations have been found to rise in *N. brasiliensis* infected rats (Roberts *et al.,* 1999). Although a recent study has shown that leptin is necessary in the development of immunity to enteric infections with *Entamoeba histolytica* (Guo *et al*., 2010), a role of leptin in ewes showing nutritionally improved resistance to *Teladorsagia circumcincta* could not be demonstrated (Zaralis *et al*., 2009). Whether leptin correlates with AA deficiency induced reduced immunity to GIN parasites in our study cannot be excluded. Therefore, leucine deficiency could have affected repair ability of the intestine or leptin concentrations in the plasma, making hosts more vulnerable to parasitism.

In conclusion, the reduced supplementation of the essential AAs leucine and methionine in a high protein food penalized lactating host's resistance to a secondary infection with gastrointestinal nematodes. In animals offered a methionine deficient diet this concurred with penalties in lactational performance, which was also associated with the occurrence of refusals in some of the infected animals. The response to leucine deficiency indicates that leucine utilization by the mammary gland takes a higher priority than its utilization for immune responses to gastrointestinal parasites. From the immune indicators measured in the study, which have been previously related with the immunomodulatory effects of dietary protein, only eosinophil counts were significantly affected and this was found to be due to the occurrence of food refusals. Consequently, further studies to elucidate the underlying

mechanisms of reduced resistance following leucine and methionine scarcity should concentrate on other indicators of immunity. In light of the role of AAs as immunonutrients and the strong effect that protein supply has on the periparturient host's resistance to GIN, more AAs could be tested using this model. Our results imply that AA balance, and thus protein quality, may be important to consider in immunonutrition strategies for farm animals in which periparturient breakdown of immunity to parasites causes significant economic losses, particularly in the small ruminant sector.

Chapter 5. Effect of plant protein source on periparturient breakdown of immunity to parasites in ewes

5.1. Summary

Effects of increased metabolizable protein (MP) supply on the degree of periparturient relaxation of immunity (PPRI) in sheep may be dependent on the MP quality. Here we hypothesized that additional MP supply from xylose-treated soybean meal would be more effective than from field bean in reducing the degree of PPRI. Multiple bearing ewes were trickle infected with *Teladorsagia circumcincta* and were fed at either 0.8 (LP) or at 1.2 times their respective MP requirements using either xylose-treated soybean meal (HPS) or field beans (HPB) and litter size was standardized to two lambs at parturition. Egg excretion, ewe body weight and condition score were unaffected by the feeding treatments during late pregnancy. During lactation HPS ewes gained more weight than LP ewes with HPB ewes being intermediate; HPS litter weight was higher than HPB the latter being higher than LP and HPS and HPB ewes had higher condition score than LP ewes. Plasma pepsinogen was significantly elevated for LP ewes throughout both pregnancy and lactation being similar for HPS and HPB ewes. Plasma urea concentration was higher for HPS and HPB ewes than LP ewes during both pregnancy and lactation. Plasma albumin concentration was higher for HPS and LP ewes comparing to HPB ewes during late pregnancy only. Egg excretion was higher for LP ewes than HPS ewes, being intermediate for HPB ewes. The results support the view that extra MP supply from field beans is less effective in reducing the degree of PPRI than from xylose-treated soybean meal suggesting that protein source and quality may be important factors to consider for the nutritional control of parasitism.

Keywords: Teladorsagia circumcincta, protein, Faecal egg count, protein quality

5.2. Introduction

The periparturient relaxation of immunity (PPRI) to nematode parasites plays a key role in small ruminant parasite epidemiology. It is characterized by increased faecal egg excretion and/or worm burden in periparturient ewes relative to non-reproducing counterparts (Barger, 1993; Beasley *et al.,* 2010; Houdijk *et al*., 2005b). The degree of PPRI may have a nutritional basis and be sensitive to protein supply (Coop & Kyriazakis 1999).

In ruminant diets, dietary protein is provided in the form of effective rumen degradable protein (ERDP) and undegradable protein. ERDP, which is often the main source of digestible protein for the animals, is converted mainly to ammonia in the rumen where it is the principal starting substrate for microbial protein synthesis (Lobley *et al.,* 1995). The protein that is truly available to the animal comprises the digestible part of microbial and undegradable protein, which flows from the rumen via the abomasum to the small intestine where it is digested and absorbed. These digestible portions are 60–80% of the microbial protein and up to ~85% of the undegradable protein (DUP), which combined is available to the animal as metabolizable protein (MP) (Coleman & Henry, 2002).

It has been shown that levels of metabolizable protein (MP) supply 20-30% greater than the theoretical requirements of uninfected sheep (Sykes & Kyriazakis, 2007) are required to maintain immunity to parasites. This level of supplementation is considerably higher to that required to achieve maximum milk production in infected sheep (Adams & Liu, 2003; Houdijk *et al.,* 2003b). This is attributed to the increased MP requirements during a parasitic infection, as innate and acquired immune responses draw on protein resources for the synthesis of e.g. antigen-

presenting molecules, immunoglobulins, cytokines and acute phase proteins (Calder, 2006; Li *et al.,* 2007). In addition, extra MP is required for the repair and/or replacement of damaged and/or lost host tissue caused by parasitic infestation (Kyriazakis & Houdijk, 2006).

The extent to which increased MP supply reduces PPRI, seems to differ between studies, and may largely depend on the source of supplementation. A qualitative comparison between studies (Sakkas *et al.,* 2010) suggests that the higher the proportion of DUP in the additional MP offered, the more pronounced the reduction in parasitism. Thus, MP supplementation from cottonseed meal (Kahn *et al.,* 2003a), urea (Wallace *et al.,* 1998; Knox & Steel, 1999), conventional soybean meal (Keatinge *et al.,* 2003) and sunflower meal (Van Houtert *et al.*, 1995a), i.e. sources with relatively low DUP levels (Hazzledine, 2008), seem less effective in reducing parasitism than supplementation with xylose-treated soybean meal (Houdijk *et al.,* 2000, 2001b, 2003b, 2005b) or fish meal (Donaldson *et al.,* 1998, 2001), which have high DUP levels (Hazzledine, 2008). In the present study, we tested the hypothesis that additional MP supply from xylose-treated soybean meal (Soypass) based diets would be more effective than from field bean based diets in reducing the degree of PPRI and promoting the host's performance, as the latter contains less DUP than the former.

5.3. Materials and methods

5.3.1. Animals and housing

Twenty four, 4–5-year old Bluefaced Leicester x Scottish Blackface ewes (Mules), scanned for pregnancy and bearing twins or triplets, were used in this experiment. The ewes were recruited from the same flock mated to Suffolk rams, had similar genetic background and common grazing history. The experiment lasted 87 days, i.e. from day -56 to day +31 (day 0 was mean parturition day). Ewes were individually housed during the experiment, in pens sized 1.48 m x 1.88 m. The shed received natural illumination, and was naturally ventilated. Fresh wood shavings was used as bedding and added daily. Fresh water was supplied *ad libitum* and water troughs were checked and cleaned daily. The experimental details described below were approved by the Animal Experiment Committee of Scottish Agricultural College (ED AE 18/2009) and carried out under Home Office regulations (PPL 60/3782).

5.3.2. Feeding treatments

The experiment consisted of three periods: mid pregnancy (day -56 to day -24), late pregnancy (day -24 to day 0) and lactation (day 0 to day +31). The latter two periods were collectively termed the periparturient period (day -24 to day +31). The mid pregnancy period was an adaptation period to reduce body protein reserves prior to the allocation to the periparturient feeding treatments, since high levels of body protein reserves can minimize effects of dietary metabolisable protein scarcity on PPRI (Houdijk *et al.,* 2001b). During mid-pregnancy, all ewes were offered *ad libitum* hay and 250 g of a low protein pregnancy feed. On day -24 ewes were

allocated to one of the three feeding treatment groups (n=8), which were balanced for initial (day-56) faecal egg count (FEC), body weight (BW) and condition score (CS). Feeding treatments were designed to supply 0.8 (LP), 1.2 using Soypass (HPS) and 1.2 using field beans (HPB), times the estimated MP requirement (MPr) and 0.9 times the estimated ME requirement (MEr) of the ewes during the periparturient period through restricted feeding (see below). To estimate the nutrient requirements during pregnancy, we assumed a litter birth weight of 10.3 and 12.4 kg for twin-and triplet bearing ewes, respectively, no maternal bodyweight gain (Houdijk *et al.,* 2005b) and 20.4 g MP/day for wool growth (Agricultural and Food Research Council, 1993). Litter size was standardized to twins at birth. We assumed similar requirements for wool growth during lactation, maternal body weight loss of 100 g/day and milk yields of 3.3 in the 1st, 3.6 in the 2nd and 3.9 kg/day in the 3rd and 4th week of lactation, respectively (Houdijk *et al.,* 2003b). All MP and ME requirement estimates were based on Agricultural and Food Research Council (1993) recommendations. Approximately one third of the daily allowance consisted of medium-quality hay and two-thirds of concentrates (Table 4.1), on as fed basis, during both pregnancy and lactation, which were offered in three approximately equal portions on morning-afternoon-evening basis. The daily lactation allowance offered was gradually increased over a 3 d period after parturition until the calculated lactational allowance was reached.

Feed samples were collected every day during the experiment while weighing daily allowances, and were pooled by feeding treatment before chemical analyses for dry matter (DM), neutral detergent fibre, acid detergent fibre, acid-hydrolysable ether extract, calcium, phosphorus, sulphur and acid-insoluble ash (see below).

Dietary ME and MP contents were calculated using the Agricultural and Food Research Council (AFRC, 1993) recommendations on feeding level effects, and CP, the total diet fermentable ME, ERDP and DUP contents were estimated from feed composition tables (Hazzledine, 2008). Table 5.1 presents the ingredients and the analysed and calculated chemical composition of all feeds.

Table 5.1. Ingredient and chemical composition of the diets used during late pregnancy and lactation.

	Late Pregnancy			Early Lactation		
	LP	HPS	HPB	LP	HPS	HPB
Ingredients[a] (g/kg fresh)						
Barley	253	269	167	181	222	39
Oat feed	100	100	100	100	100	100
Soybean Meal	0	0	0	70	70	70
SoyPass	0.0	70	0	0	120	0
Molasses	32.5	32.5	32.5	32.5	32.5	32.5
Field beans, coloured	0.0	0	240	0	0	400
Straw, partially treated	220	150	100	220	95	20
Urea	12.6	10.5	5.4	10.1	5.8	2.0
DiCal	4.4	1.5	1.0	7.1	1.9	2.0
Limestone	0.0	3.0	4.9	0.0	5.9	8.0
Salt	0.3	2.5	4.2	0.9	4.7	7.2
CalMag	1.20	0.80	1.00	1.10	0.50	0.75
Soya oil	21.0	16.8	12.0	21.0	11.1	4.4
50% Fat Premix	32.5	26.0	18.5	32.5	17.2	6.8
Megalac	20.0	16.0	11.4	20.0	10.6	4.2
Sulphur	0.4	0.0	0.1	1.7	1.0	1.2
MinVit	1.75	1.75	1.75	1.75	1.75	1.75
Hay*	300	300	300	300	300	300
Chemical composition on as fed basis in g/kg unless mentioned						
Dry matter	870	866	853	864	862	844
Crude protein**	101.5	124.4	123.2	121.2	159.4	164.2
NDF	442	410	394	432	393	357
ADF	270.2	236.3	238.1	268.9	232.1	236.9
AHEE (g/kg DM)	64.2	50.3	41.4	68.3	45.6	25.6
Ca	6.82	6.72	6.37	7.06	7.41	6.28
P	2.96	2.89	2.96	3.78	3.48	3.98
S	1.88	1.69	1.65	2.91	2.78	2.67
ME (MJ/kg) ***	8.9	8.8	8.4	8.6	8.9	8.5
Ash	54.3	52.4	48.8	59.0	56.4	50.6
MP ***	55.0	76.3	75.8	70.6	106.7	110.7
ERDP **	70.0	74.0	78.2	78.4	84.6	100.8
DUP **	11.3	30.1	26.9	21.6	53.8	47.7
fME **	8.1	8.5	9.2	8.2	9.0	10.0

LP, low protein; HPS, high protein using SoyPass, HPB high protein using coloured field beans,; Soypass, xylose-treated soyabean meal; DiCal, dicalcium phosphate, 18% P; CalMAg, calcined magnesite (85% MgO); Megalac, calcium soap product; AHEE, acid-hydrolysable ether extract; Crude protein = N x 6.25; NDF, Neutral detergent fibre; ADF, Acid detergent fibre, AHEE, acid-hydrolysable ether extract; ; ME, metabolizable energy; MP, metabolisable protein; fME, fermentable metabolizable energy

* Hay was offered chopped, while other ingredients were mixed in a mash form.

** Estimated from Premier Atlas Ingredients Matrix (Hazzledine, 2008)

*** Predicted from Premier Atlas Ingredients Matrix (Hazzledine, 2008) using Agricultural and Food Research Council assumptions (AFRC,1993)

5.3.3. Experimental infection

Ewes were naturally infected with predominantly *Teladorsagia circumcincta*. All ewes were kept indoors throughout the experimental period when their naturally acquired infection was superimposed with a trickle infection of *T. circumcincta* infective larvae (L3). The *T. circumcincta* strain used was the Moredun Ovine Susceptible Isolate that has been maintained in the laboratory for several years. During late pregnancy and lactation a stepwise increase of the infection dose was applied to mimic increases in infection pressure experienced under grazing conditions arising from pregnancy and lactation associated increases in grass intake (Table 4.2). Each infective dose was suspended in 10 ml water and administered using 10 ml syringes, three days a week (Monday, Wednesday and Friday) from day -56 until day +31 during the morning hours.

Table 5.2. Number of *Teladorsagia circumcincta* larva contained in each infective dose administered on a Monday-Wednesday-Friday basis to multiple bearing and twin rearing ewes throughout the experiment.

Time period (Day relative to parturition date)		
From	To	Number of Larva
-56	-21	1000
-21	-14	2000
-14	-7	2200
-7	-0	2400
0	+7	4000
+7	+14	4400
+14	+31	4800

5.3.4. Animal performance

The ewes were weighed at day -56 and then weekly from day -42 onwards, as well as within 12 h of parturition. The lambs were weighed within 12 h after birth and weekly afterwards. The body condition of the ewes was scored weekly, by lumbar palpation on a 0–5 scale, and to an accuracy of a quarter, as described by Russel (1984). As the ewes were fed restrictedly, refusals were expected to be minimal. However, those that did occur were weighed back to calculate achieved intake of DM, ME, MP, ERDP and DUP.

5.3.5. Faecal egg count and daily nematode egg output

Following the initial FEC on day -56, FEC were performed twice a week from day -49 onwards. Post-lambing FEC was also assessed on samples collected within 12 h after lambing. FEC were determined in the faeces as eggs per gram faeces (epg), by a modified flotation method (Christie & Jackson, 1982) using polyallomer centrifuge tubes to collect the nematode eggs from the meniscus. In addition to FEC, daily nematode egg excretion was also assessed to account for potential variation in faeces production, which may affect FEC *per se* as the FEC is a concentration measure (Vagenas *et al.,* 2007; Houdijk, 2008). To calculate mean daily nematode egg excretion during lactation, total tract DM digestibility was analysed through the use of in-feed acid insoluble ash (AIA) as a marker (Keulen and Young, 1977). To this effect, faeces were collected directly from the rectum of all ewes for four consecutive days during lactation (day +23 to day +26), pooled per individual ewe and kept frozen at -20^{o}C before DM and AIA analysis as described by Keulen and Young (1977). Similarly diet samples from the same days were pooled

per feeding treatment for AIA and DM analyses. Assessed total tract DM digestibility and faecal DM contents were then used to calculate averaged daily fresh faeces production during lactation from achieved mean DM intake. Averaged daily fresh faeces production (g/day) is then multiplied by average FEC (eggs/g) to result in mean daily nematode egg excretion during lactation (eggs/d).

5.3.6. Plasma samples

Blood samples were collected on a weekly basis from the jugular vein of the ewes into heparinised vacutainers from day -56, until the end of the experiment on day + 31. The plasma was separated by centrifuging for 15 min at 2500 rpm and stored at -20 ^{o}C pending analyses. Plasma was analyzed for urea (mmol/l), pepsinogen (expressed in mu/l) and albumin (g/l) concentration. In addition to this, ewes were also monitored for risk of twin lamb disease during late pregnancy with weekly blood sampling to assess beta-hydroxybutyrate (BHOB). Ewes with BHOB level above 2 mmol/l were given propylene glycol as an external energy source until their BHOB level was regarded safe.

5.3.7 Statistical analysis

All data collected over the periparturient period for individual ewes were re-arranged from calendar date to day relative to parturition (day0) prior to analysis. This was done to account for the influence of small differences in parturition dates on the data obtained during the periparturient period. Results obtained during the periparturient period were analyzed separately for late pregnancy and lactation as they were considered to be very distinct in relation to the quantity and composition of feed

offered, as well as to the ewe physiological state. The mean daily DM, ME, ERDP, DUP and MP intake (g/d), DUP/MP ratio, mean daily nematode egg excretion (eggs/d), as well as litter average daily weight gain (g/day) were analysed through analysis of variance (ANOVA) using feeding treatment as a factor. Litter average daily gain (in g/d) was estimated by linear regression. Ewe BW, CS, litter BW, FEC and levels of plasma constituents were analyzed using repeated measures ANOVA to assess possible interactions between feeding treatment and time. Final ewe BW, CS and plasma constituents measurements taken at the end of the adaptation period, and mean FEC during the adaptation period, were used as covariates for the analysis of periparturient ewe BW, CS, plasma constituents and FEC, respectively. When feeding treatment interacted with time, ANOVA was carried out on each time point to locate the interaction. For all the body weight measurements least squares means with standard error of the difference between means (s.e.d.) are reported unless otherwise stated. FEC and total daily nematode egg excretion were transformed via log (x + 1) prior to statistical analysis. These are reported as backtransformed least square means, accompanied by a lower and upper 95% confidence interval. All statistical analyses were performed using Genstat 11 for Windows release 11.1, 2008 (VSN international, Hemel Hempstead, UK).

5.4. Results

5.4.1. Feed intake and digestibility

Table 4.3 presents the achieved average daily intake of DM and CP and the estimated daily intakes ERDP, DUP and MP, as well as the DUP/MP ratio over late pregnancy and early lactation as well as the DM digestibility of the lactation feed

offered. Achieved DM and ME intakes were not affected by the dietary treatments during both late pregnancy (P=0.469 and P=0.427 respectively) and lactation (P=0.741 and P=0.500 respectively). As expected, CP intake was higher during both late pregnancy and lactation for HPS and HPB ewes in LP ewes (P<0.009 and P<0.001 respectively). ERDP (g/d) intake was not affected by the feeding treatments during late pregnancy (P=0.451). However, during lactation, ERDP intake was higher in HPB than HPS ewes, which was, in turn, higher than that in LP ewes (P<0.001). DUP (g/d) intake and DUP/MP ratio were higher during both late pregnancy and lactation for HPS than HPB ewes; they were both higher in the latter than in LP ewes (P<0.001). As expected, achieved calculated MP intake was higher in HPB and HPS ewes than in LP ewes during late pregnancy and lactation (P<0.001 for both periods). Total tract DM digestibility during lactation was higher for HPS than HPB ewes and LP ewes (P=0.014).

Table 5.3. Least squares means for daily intake of crude protein (CP), dry matter (DM), metabolizable energy (ME), effective rumen degradable protein (ERDP), undegredable protein (DUP), ratio of DUP/MP of the food offered during late pregnancy and lactation and DM digestibility during lactation of multiple bearing Mule ewes, trickle infected with *Teladorsagia circumcincta* and fed at either 0.8 (LP) or 1.2 times their respective MP requirements using either xylose-treated soyabean meal (HPS) or field beans (HPB).

		Feeding treatments				
Parameter	Period	HPS	HPB	LP	SED	P
CP (g/day)	Late Pregnancy	180.9	162.7	133.8	14.15	0.009
DM (g/day)		1333	1255	1372	102.7	0.469
ME (MJ/d)		14.3	13.2	14.2	1.1	0.427
ERDP (g/day)		114.4	111.3	103.0	9.4	0.451
DUP (g/day)		45.3	37.3	14.4	2.9	<0.001
DUP/MP ratio		0.38	0.34	0.18	0.004	<0.001
MP (g/day)		116.8	106.8	78.9	8.6	<0.001
CP (g/day)	Lactation	454.5	460.9	339.7	19.1	<0.001
DM (g/day)		2451	2399	2469	100.7	0.741
ME (MJ/d)		26.5	25.3	25.6	1.1	0.500
ERDP (g/day)		236.2	279.3	217.5	11.3	<0.001
DUP (g/day)		153.9	133.4	57.7	5.8	<0.001
DUP/MP ratio		0.50	0.43	0.29	0.006	<0.001
MP (g/day)		305.5	308.1	193.6	10.5	<0.001
DM digestibility (%)		70.8	63.8	60.9	3.2	0.014

5.4.2. Ewe and litter performance

Figure 5.1 shows ewe BW (Figure 5.1a) and CS (Figure 5.1b) during the periparturient period, and litter BW (Figure 5.1c) during lactation. Feeding treatment did not affect gestational ewe BW (P=0.307) or CS (P=0.532) while they were both affected by time (P<0.001) as the ewe BW increased with the progression of gestation while their CS decreased. There were no significant interactions between feeding treatment and time for BW (P=0.428) or CS (P=0.525). On the other hand, lactational CS was affected by feeding treatment (P=0.017) and by the feeding treatment time interaction (P=0.025) as LP ewes showed lower CS than HPB and HPS ewes from day 10 of lactation onwards (Figure 5.1a). Lactational ewe BW was not affected by the feeding treatments (P=0.184) but it was affected by time (P<0.001) and by the feeding treatment and time interaction (P<0.001); feeding treatments affected ewe BW from day 23 onwards (P<0.05), as LP ewes lost significantly more weight than HPB and HPS ewes during this period (Figure 5.1b). Additionally, BW was lower for HPB ewes on day 29 of lactation than that of HPS ewes and similar to that of LP ewes (60.0 vs 63.8 vs 58.3 kg; SED 1.52; P=0.007).

Litter BW was affected by feeding treatment (P=0.041), time (P<0.001) and by their interaction (P=0.018). At day 16 of lactation there was a tendency for HPS litters to be heavier than LP litters, with HPB litters being intermediate (18.89 vs 16.02 vs 17.35 kg; SED 1.136, p=0.063). This trend became significant for the remainder of lactation (Figure 5.1b) with final HPS litters being heavier than HPB litters, the latter being heavier than LP litters. Litter gain was higher for HPS than LP litters, with HPB litters being intermediate, and not different from both LP and HPS (606 vs 550 vs 503 g/d; SED 31; P=0.013).

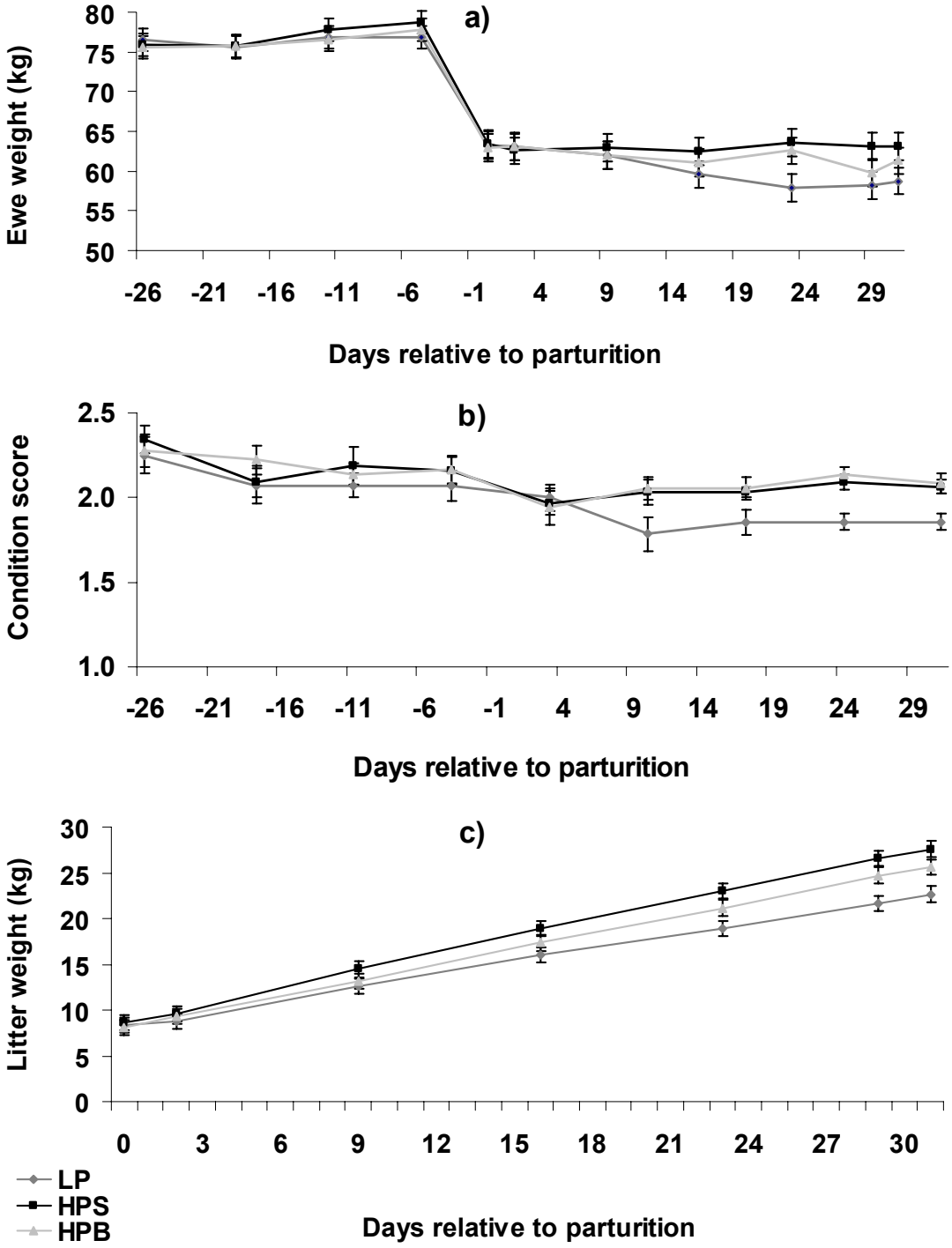


Figure 5.1. Ewe body weight (a) and condition score (b) of multiple bearing and twin rearing ewes trickle infected with *Teladorsagia circumcincta* and body weight of their litters (c), fed at either 0.8 (LP) or 1.2 times their assumed MP requirements using either soypass (HPS) or field beans (HPB) during late pregnancy and early lactation.

5.4.3. Faecal egg count and daily nematode egg output

Figure 2 shows backtransformed FEC during late pregnancy were not affected by our feeding treatments (P=0.659), by time (P=0.144) or by the interaction between feeding treatment and time (P=0.709). However, during lactation, FEC were affected by our feeding treatments (P=0.017), but not by time (P=0.381) or their interaction (P=0.543). HPS ewes had lower FEC over the lactation period than LP ewes while HPB ewes were intermediate, and did not differ from LP and HPS ewes. Likewise, daily nematode egg output (eggs x 1000/d) was lower for HPS ewes (742 [167-136]) than LP ewes (1702 [447-354]) with HPB ewes being intermediate (1233 [190-165]), and not different from LP and HPS ewes (P=0.029).

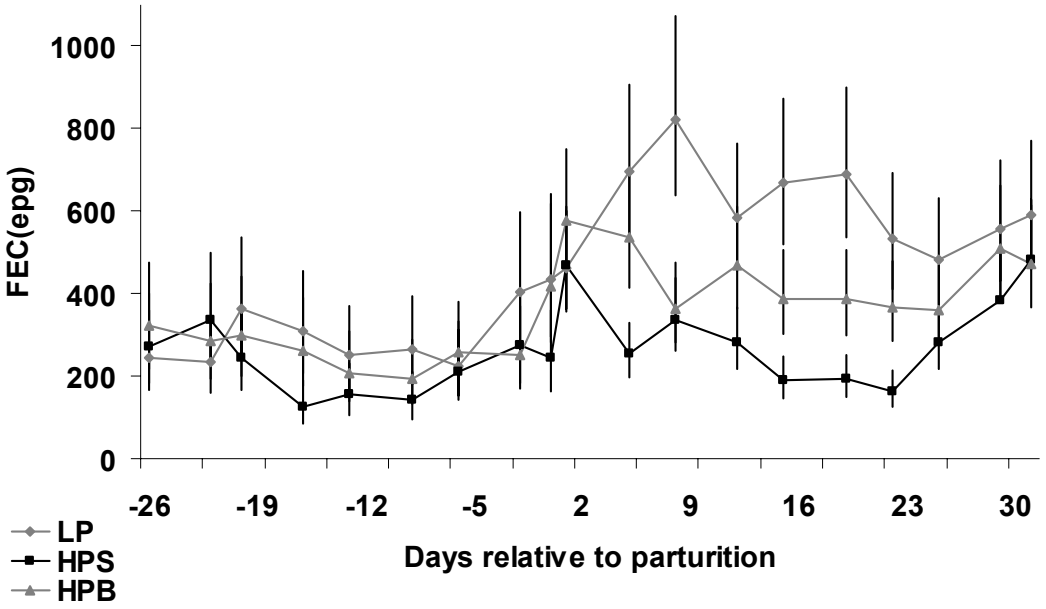


Figure 5.2. Backtransformed faecal egg count (FEC, in eggs/g (epg) faeces) with 95% CI of ewes, trickle infected with *Teladorsagia circumcincta* and fed at either 0.8 (LP) or 1.2 times their assumed metabolisable protein requirements using either soypass (HPS) or field beans (HPB) during late pregnancy and early lactation.

5.4.4. Plasma constituents

Figure 5.3 presents ewe plasma albumin (Figure 5.2a), urea (Figure 5.2b) and pepsinogen (Figure 5.2c) concentrations. During late pregnancy, albumin concentration was affected by feeding treatment ($P=0.019$), time ($P<0.001$) and there was a tendency to be affected by their interaction ($P=0.065$). It was lower for HPB ewes than HPS and LP ewes and its concentration decreased. Urea concentration was affected by feeding treatment ($P<0.001$) and time ($P<0.001$) while it was not affected by their interaction ($P=0.101$); it was higher for HPS and HPB ewes than for LP ewes and it declined as gestation progressed. Plasma pepsinogen concentration was affected by the feeding treatment ($P=0.003$) while it was not affected by time ($P=0.358$) or by the feeding treatment and time interaction ($P=0.737$). HPS ewes and HPB ewes had significant lower levels of pepsinogen throughout late pregnancy.

During lactation, albumin concentration was not affected by feeding treatment ($P=0.149$) or by the feeding treatment and time interaction ($P=0.266$), while it was affected by time ($P=0.001$) as there was an increase in the concentration of albumin from day 8 of lactation onwards. Urea concentration was affected by feeding treatment ($P<0.001$), time ($P<0.001$) and their interaction ($P=0.015$). Urea was significantly elevated for HPS and HPB ewes compared to LP ewes throughout lactation and its concentration increased with time. Pepsinogen was affected by the feeding treatments ($P=0.012$) and time ($P=0.008$) while it was not affected by the feeding treatment and time interaction ($P=0.524$). HPS and HPB ewes had consistently higher pepsinogen concentrations than LP ewes whilst overall pepsinogen levels tended to decrease.

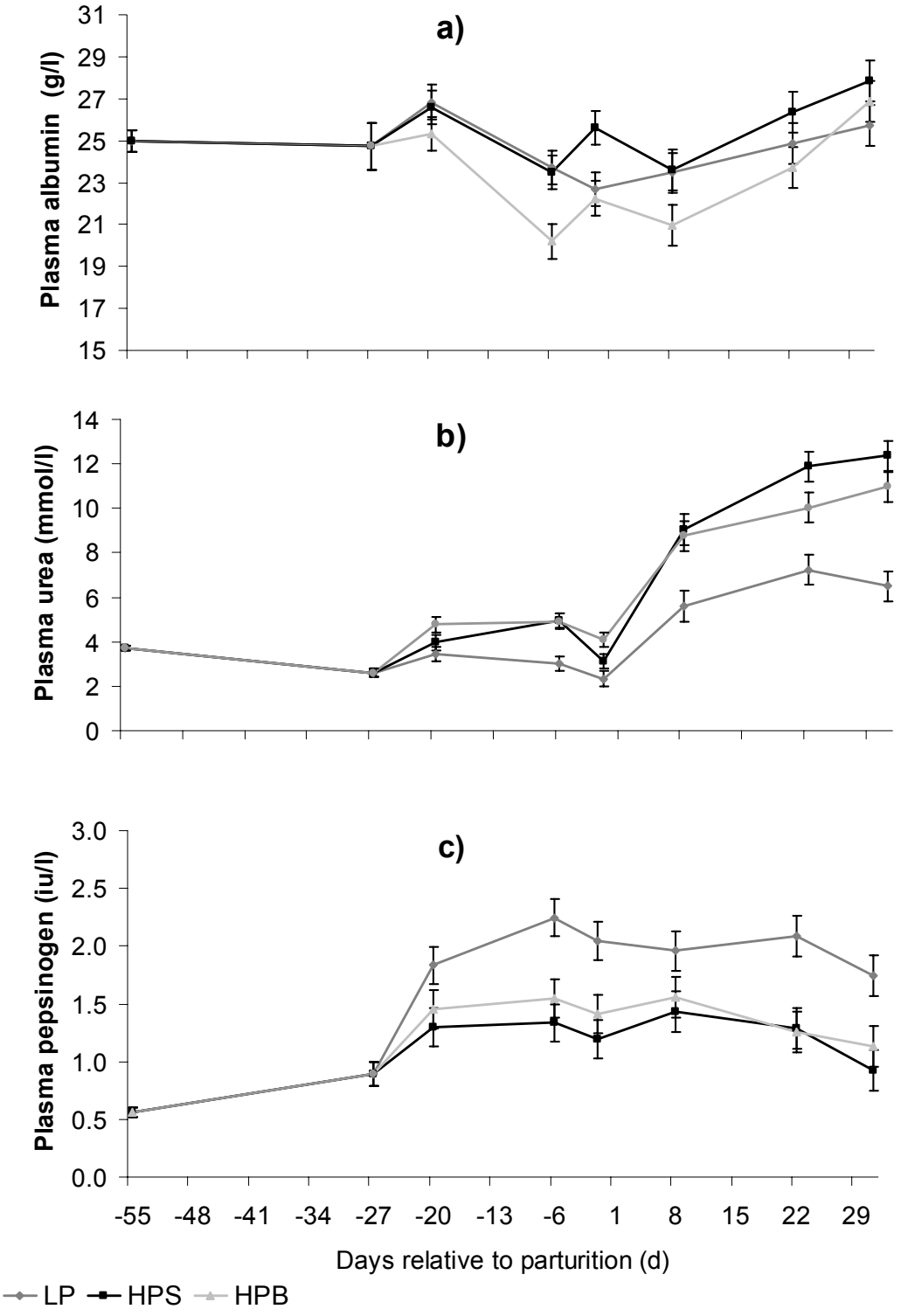


Figure 5.3. Plasma albumin (a), urea (b) and pepsinogen (c) concentrations of ewes, trickle infected with *Teladorsagia circumcincta* and fed at either 0.8 (LP) or 1.2 times their assumed metabolisable protein requirements using either soypass (HPS) or field beans (HPB) during late pregnancy and early lactation.

5.5. Discussion

The objective of the present experiment was to test the hypothesis that a high MP ratio based on Soypass (xylose-treated soybean meal) would be more effective to reduce the degree of PPRI in sheep than a high MP ration based on field beans. This would arise from the higher DUP/MP ratio in Soypass compared to field beans. The results support this hypothesis; HPS ewes were more effective than the LP ewes in both reducing the degree of PPRI and improving lactational performance, while HPB outcomes were intermediate to them. Whilst in our experiment HPB ewes did not statistically differ from HPS and LP ewes, the differences observed are biologically relevant, and suggest that HPB ewes would have differed from both LP and HPS ewes at a higher degree of replication.

The experimental design and conditions allowed us to test our hypothesis as the feeding treatments resulted in similar DM and ME intakes during both pregnancy and lactation (Table 5.3). As a result, observed differences in the degree of PPRI and in the periparturient performance of the ewes are attributed to the differential level of MP supply and DUP/MP ratio of our feeding treatments. As expected, both HPS and HPB feeding treatments offered more MP than our LP food during both late pregnancy and lactation. This led to increased lactational performance of the HPS ewes, and to a lesser extent of the HPB ewes, suggesting that MP was scarcer in the LP feeding treatment than in the high MP treatments. During both late pregnancy and lactation estimated achieved DUP intake and the ratio of DUP/MP intake were higher for HPS than HPB ewes, and these were both higher in the latter than in the LP ewes ($P<0.001$).

In agreement with the current study, most studies involving the effect of nutrition on PPRI to gastrointestinal nematode parasites show an absence of effect of MP nutrition on FEC during pregnancy (Houdijk *et al.,* 2000; Houdijk *et al.,* 2003b; Houdijk *et al.,* 2005b). The absence of an effect of MP nutrition and of the DUP/MP ratio on parasitism during pregnancy may be explained by the fact that when pregnant ewes are fed inadequate levels of MP they may mobilise amino acids from the skin and the muscle, sparing protein from visceral tissues to support fetal growth (McNeill *et al.,* 1997) and it has been proposed that this may facilitate the gut immune response to gastrointestinal nematodes (Adams & Liu, 2003).

However, again in agreement with many other studies (Houdijk *et al.,* 2000; Houdijk *et al.,* 2003b; Houdijk *et al.,* 2005b; Donaldson *et al.,* 1998; 2001), HPS ewes had consistently lower egg counts than LP ewes during lactation, both in terms of the average daily nematode egg excretion and FEC in absolute numbers. This further confirms that MP was scarce for LP ewes both in terms of their lactational performance and the expression of acquired immunity. In agreement to our hypothesis, HPB ewes showed an intermediate egg excretion to that of HPS and LP ewes although it was not significantly different than both. On the other hand, we observed that pepsinogen concentration of HPB and HPS ewes did not differ and were both consistently lower than that of LP ewes. It will be discussed below that this observation may suggest that variation in protein quality impacted on fecundity rather than worm burden.

Pepsinogen is secreted by chief cells and leaks into the circulatory system through epithelial cell tight junctions that are disrupted by mast cell proteases, which are released through Th2-cytokine action during parasitism (Patel *et al.,* 2009; Stear

et al., 1999). Our results are in agreement with previous studies where reduced pepsinogen concentration has been observed in high protein fed parasitized ewes as compared with their low protein fed counterparts (Houdijk *et al.,* 2005b). The lower pepsinogen concentrations of HPB and HPS ewes, relative to LP ewes, suggests that excess MP can to some extent restore abomasal integrity (Houdijk *et al.,* 2003b). It has been proposed that the rise in pepsinogen concentration observed in abomasal parasitism is caused by the presence of adult worms in the abomasal lumen (Scott *et al.,* 2000; Simpson, 2000) and may correlate with mean length of the adult female worms as well as with the number of nematodes present (Stear *et al.,* 1999). Thus, it is reasonable to speculate that both HP ewes had either a lower number of adult worms compared to LP ewes burrowed in their abomasums, or that they were less damaging to the host due to an enhanced expression of acquired immunity, or that additional MP was preferentially directed towards maintaining abomasal integrity.

The difference in worm egg output at apparent similar levels of worm burdens could be attributed to a lower expression of immunity in the HPB ewes comparing to the HPS ewes towards the fecundity of the established worm population. It is likely that this variability in response is due to variation in MP quality, defined as its amino acid (AA) composition. The relatively unbalanced AA profile of microbial protein, compared to that of animal protein in general and immune-proteins in particular, may account for their limited effect on reducing PPRI, as parasitism increases specific AA requirements to synthesize proteins to maintain homeostasis (e.g. albumin) and to mount immune responses (e.g. inflammatory agents, mucins and antibodies) (Houdijk & Athanasiadou, 2003). It has been suggested that regulating nematode expulsion may be of less importance than

regulating fecundity to the host, and that consequently fecundity, observed as FEC, is expected to be more severely affected by nutrient scarcity (Houdijk *et al.,* 2003b). Although worm burdens were not assessed the pepsinogen level of HPB and HPS ewes indicate a similar level of induced damage by the parasite population, which may indicate similar numbers of established worms. Thus, our data agree with a prioritisation of expression of immunity towards fecundity over worm expulsion.

It should be noted that field beans usually contain significant levels of condensed tannins (CT), substances with both antiparasitic and antinutritional properties (Athanasiadou *et al.,* 2001). However, high levels of field beans in ruminant diets have been used without detrimental effects on productivity (Crépon *et al.,* 2010; Brunschwig *et al.,* 2004). Min and Hart (2003) summarized effects of CT content on gastrointestinal parasitism from various studies and concluded that at FEC reduce by 50% at levels of 45 to 55 g of CT/kg DM. These levels are well above the estimated 7.5 g of CT/kg DM in our experiment (Smith, personal communications), and suggest that although CT were likely present, their levels were too low to impact on FEC. This is further supported by the absence of any FEC reduction upon introducing the HPB treatment during late pregnancy.

Our feeding treatments did not affect ewe BW and CS during late pregnancy, meaning that neither the ratio of DUP/MP nor the level of MP supply influenced any of these parameters. It has been previously shown that offering diets to ewes during late pregnancy with increasing ratio of DUP/kg of DM supply does not influence lamb birth weight (Annett *et al.,* 2005; Annett *et al.,* 2008; Dawson *et al.,* 1999). Similarly, studies on periparturient ewes infected with *T. circumcincta* (Houdijk *et*

al., 2005b; Kidane *et al.,* 2009) receiving different levels of MP during late pregnancy, did not result in differences in any of the aforementioned parameters.

On the other hand, the feeding treatment resulted in pronounced differences in ewe performance during the lactation period. As lactation progressed, HPS ewes gained more weight than LP ewes with HPB ewes being intermediate, while CS was higher for HPS and HPB ewes compared to LP ewes. The weight loss of LP ewes reflects a higher degree of MP scarcity compared to HPS ewes, which led the former to mobilize more body reserves compared to the latter in order to satisfy the energetic and protein demands of the progressing lactation. This was also associated with a reduction of the CS of LP ewes from day 10 of lactation onwards. Although this is attributed mainly to MP scarcity, the higher rate of BW loss in LP ewes could lead to more amino acids being oxidised in order to satisfy the energetic demands of lactation and to an augmented degree of body protein and fat mobilization (Liu *et al.,* 2003). Nonetheless fat mobilization has not been shown to influence the degree of PPRI in periparturient ewes (Houdijk *et al.,* 2001b). Therefore, observed effects of our feeding treatment on egg excretion during the lactation period (see below) are attributed solely to the higher degree of MP scarcity.

HPS litter weight became significantly higher than that of HPB litters, the latter being higher than the weight of LP litters with the progression of lactation. This shows that increased MP supply promoted milk production of HPS ewes and HPB ewes to a higher degree than LP ewes due to the increased availability of AA for production purposes. However, HPB ewes failed to perform as well as their HPS counterparts, likely due to the lower DUP/MP ratio of their diet. The observed improved lactational performance indicates that the HPS feeding treatment offered

MP with an amino acid profile closer to the desired for milk production. Our results agree with those of Mikolayunas-Sandrock *et al*. (2009) where increasing the DUP/MP ratio of the food offered to lactating sheep has been shown to increase milk production. It is also known that less degradable protein sources such as Soypass may give rise to more efficient protein utilization for the production of milk protein (Ramos Morales *et al.,* 2010). On the other hand, a decrease in the protein content of the milk has been previously observed when feeds with high content in faba beans were offered to dairy cows (Trommenschlager *et al.,* 2003).

Levels of plasma albumin decreased for all dietary treatments as a result of increasing infection pressure and were significantly lower for HPB than HPS and LP ewes during the late pregnancy period. A decrease of the concentration of albumin is a common trait of gastrointestinal nematode infections (Farid *et al.,* 1969) as a result of increased loss of endogenous protein into the GI tract (Parkins & Holmes, 1989; Holmes, 1993). Albumin concentrations have been shown to increase as a result of an increase in the level of MP offered in parasitized animals (Houdijk *et al.,* 2000; Houdijk *et al.,* 2005b; Zaralis *et al.,* 2009) or to remain unaltered (Kidane *et al.,* 2009; Kyriazakis *et al.,* 1996; Houdijk *et al.,* 2009; Houdijk *et al.,* 2003b). The expectation was that ewes in both HP diets would be able to use the additional MP offered in order to partly synthesise albumin and to reduce the damage or loss of endogenous protein that is induced by abomasal parasitism (Holmes, 1993) through an improved expression of acquired immunity (Kyriazakis & Houdijk, 2006) and therefore to have higher albumin concentration than LP ewes. Although the acquired difference in albumin concentration during late pregnancy was significant, during lactation it was similar between the feeding treatments. The acquired differences in

the concentration of urea during both late pregnancy and lactation are further indicative of the higher level of protein nutrition of HPB and HPS ewes, as compared to LP ewes.

In conclusion, this study supports the view that the degree of PPRI is sensitive to both MP supply and MP quality, suggesting that protein source is important issue to consider in non-chemical parasite control strategies in small ruminant production systems. Further research to assess variation in the ability of protein sources to improve host resistance to gastrointestinal nematodes is required, especially if reliance on imported soybean meal is to be reduced. Finally the effects of processing, such as pressure toasting, toasting, and pelleting, which have been shown to reduce the degradability of the protein fraction of legumes (Goelema *et al.,* 1998), on the degree of PPRI could be assessed in a future experiment.

Chapter 6. General Discussion

6.1. Introduction

This thesis has provided novel information on the nutritional sensitivity of PPRI to gastrointestinal nematode infections in monogastric and ruminant hosts. The hypothesis that has been developed to account for the effects of increasing scarce nutrient supply on PPRI is based on a nutrient-partitioning framework. This framework proposes that PPRI occurs due to an increased nutrient requirement of the prioritised reproductive effort over immunity to parasites when nutrient supply is scarce, thus ensuring the preservation of the host's genetic material (Coop & Kyriazakis, 1999; Kyriazakis & Houdijk, 2006). As a result, during late pregnancy and lactation, if the periparturient mammal receives a secondary nematode infection, it will be unable to mount an effective immune response due to the preferential allocation of scarce nutrients to the reproductive effort, (Coop & Kyriazakis, 1999).

In order to test the sensitivity of resistance and resilience of periparturient immune animals to increased nutrient supplementation or decreased nutrient demand a *Nippostrongylus brasiliensis* re-infected lactating rat model was employed in Chapters Two, Three and Four. The rat was chosen as the experimental model as it is easy to manipulate the host's nutritional status due to the substantially elevated nutrient requirement during lactation (up to tenfold relative to maintenance (Pine *et al*, 1994). Moreover, its monogastric nature avoids the effect rumen fermentation has on ingested protein, and its lactational response to changes in endogenous and dietary protein supply are well established (Jessop, 1997). In addition, rats acquire a strong immunity to the small intestinal nematode *N. brasiliensis*, and immune rats rapidly expel adult *N. brasiliensis* during a secondary infection (Jarrett *et al.,* 1968). However, during lactation dams with previous exposure to this parasite experience

PPRI, expressed through delayed expulsion of the parasite population and increased nematode egg excretion comparing with their non lactating counterparts (Houdijk *et al.,* 2003a). Additionally, an increased nutrient supply (Houdijk *et al.,* 2005a) or a reduced nutrient demand (Normanton *et al.,* 2007) have been shown to reduce the degree of PPRI as a result of an enhanced expression of immunity (Jones *et al.,* 2009) in *N. brasiliensis* re-infected lactating rats. This model would also enable to identify which effector mechanisms are affected by reducing nutrient scarcity, through the assessment of the numbers of mucosal inflammatory cells (**Chapter Three**, **Four**) and the concentration of anti-*N. brasiliensis* antibodies at different time points during the course of infection (**Chapter Four**).

In **Chapter Five** a periparturient sheep-*Teladorsagia circumcincta* model was employed. *T. circumcincta* is a parasitic nematode that inhabits the abomasum of sheep, recognized as an important parasite in temperate regions in terms of animal welfare and productivity (Halliday *et al.,* 2007). It is the predominant nematode in cool, temperate climates, such as Scotland (Stear *et al.,* 2009). PPRI to this nematode is well established and effects of increased MP supply and reduced nutrient demand have been repeatedly observed (Houdijk *et al.,* 2000; 2001b; 2001c; 2003b; 2005b; 2006; Kidane *et al.,* 2009; Zaralis *et al.,* 2009).

The first hypothesis of this thesis was that protein scarcity would have a stronger effect on the resistance of lactating parasitized hosts than energy has (**Chapter Two**). A subsequent experiment explored the rate of improvement of immunity to GIN as a result of a reduction of the degree of nutrient scarcity by reducing nutrient demand (**Chapter Three**). Since protein is comprised of AAs, we hypothesized that the relative deficiency of two selected essential AAs, leucine and

methionine, would impose penalties to the degree of PPRI due to their reduced availability for the developing immune response and the progressing lactation (**Chapter Four**). Finally, the implications of AA supply on the resistance to parasites in our rodent-nematode model were investigated on ruminant hosts by offering diets differing in their DUP/MP proportion and as a result in the protein quality of the MP offered (**Chapter Five**).

As each experimental **Chapter** (**Two, Three, Four and Five**) contains a comprehensive discussion of the results, the purpose of this general discussion will be to draw together the experimental results and discussions and suggest possible future work in the field of nutritional modulation of immunity in parasitized hosts.

6.2. Outcomes of the PhD

6.2.1 Nutrient partitioning between reproduction and immunity

Lactating rat- N. brasiliensis re-infection model

In **Chapter Two, Three and Four** dam and litter weight was measured in order to assess the reproductive effort of the dam, as well as worm burdens and the number of nematode eggs present in the colon in order to assess the expression of resistance to the worm population. Assessing the relative responses of overcoming nutrient scarcity on lactational performance and resistance to parasitism enables the investigation of the rules of nutrient partitioning.

In **Chapter Two** increased protein supply at two levels of energy resulted in a decreased degree of PPRI, illustrated through a reduction of worm burdens and a lower female/male ratio. Increased protein supply lead to an improvement of the lactational performance of the dams expressed in higher dam and litter weight gain.

This supports the view of a partial allocation of scarce protein supply on both reproductive and immune functions in periparturient hosts with prior exposure to parasites (Kyriazakis & Houdijk, 2006). Our results come in agreement with that of many studies on periparturient ruminant host's that illustrate a positive effect of increased protein supply on the degree of PPRI (Donaldson *et al.,* 2001; Houdijk *et al.,* 2003b; 2005b; Kahn *et al.,* 2003b; Chartier *et al.,* 2000; Etter *et al.,* 1999). This is expected since lactation imposes significant protein demands on mammals (Pine *et al.,* 1994; Jessop, 1997). At the same time, parasitised hosts have additional protein requirements to activate and maintain an effective protective response to parasites (Kyriazakis & Houdijk, 2006). Although additional protein may be derived from labile body protein its amount and the extent to which it can be mobilised is limited (Pine *et al.,* 1994).

On the other hand, the application of an energy restriction penalised performance in the form of reduced dam and litter weight gain, but did not induce penalties on the degree of PPRI. It is well established that activation and maintenance of an immune response are energetically demanding processes (Colditz, 2008) and parasitism imposes energetic demands on the host, evidenced by the lower amount of fat deposits in parasitized mice compared with their uninfected counterparts (Kristan & Hammond, 2000; Coltherd *et al.,* 2009). However, the amount of fat deposits acquired during pregnancy can provide energy if an energy deficit exists during lactation (Oftedal, 2000). Our results show that energy is not likely to be scarce for the development of immunity to parasites relative to protein in lactating parasitized hosts, although it highly affects the lactational performance of the host.

In **Chapter Three** a reduced nutrient demand arising from rearing a smaller number of offspring resulted in a reduced degree of PPRI. This was expressed through both reduced nematode egg excretion and worm burdens in dams which were nursing three instead of twelve pups. Effects on parasitological parameters were similar in animals that had their litter size reduced either at the onset, or at a later time point in lactation. This reduced degree of PPRI was accompanied by an increased dam weight gain and a reduced litter weight gain following litter size reduction. Our results suggest that immune functions take a lower priority than reproductive functions and come in agreement with studies with lactating ruminant hosts infected with various species of gastrointestinal nematode parasites which illustrate that a lower nutritional demand arising from rearing fewer offspring (Romjali *et al.,* 1997; Donaldson *et al.,* 1998; Houdijk *et al.,* 2001c; Kahn *et al.,* 2003b; Baker *et al.,* 1998) significantly reduces the degree of PPRI. This is related to a lower lactational demand as rearing a smaller litter decreases milk production, and as a result the amount of nutrients directed to reproductive effort in both sheep (Alexander & Davies, 1959; Gardner & Hogue, 1964) and rats (Morag *et al.,* 1975). Similarly, cessation of lactation results in an even more pronounced enhancement of resistance (Houdijk *et al.,* 2005b, O'Sullivan & Donald 1973; Lloyd, 1983) and low producing goats exhibit lower FEC than their high producing counterparts (Hoste & Chartier 1993; Chartier *et al.,* 2000). Additionally, results on parasitological figures show that a reduction of nutrient scarcity at a later time point results in a rapid reduction in the degree of PPRI associated with an increased dam weight gain and lower litter growth. Our results are in agreement with that of Houdijk *et al*. (2006)

where removing one lamb from parasitized twin rearing ewes during lactation rapidly reduced the degree of PPRI.

In **Chapter Four** offering high protein diets restricted either in their leucine or their methionine content penalised resistance to *N. brasiliensis*. Dams on these diets had higher worm burdens and eggs in colon comparing to dams which were fed a diet containing the full AA profile of casein protein and similar to that of dams allocated to a low protein diet. Methionine deficient diets resulted in extensive food refusals in a number of dams. Excluding these dams from the statistical analysis indicated that dams fed methionine deficient diets had reduced dam weight gain but not significantly lower litter weight gain in relation to dams consuming diets with adequate amounts of methionine. These results show that methionine was scarcer for immune rather than reproductive functions i.e if reproductive functions had a lower priority for the allocation of scarce methionine supply one would expect them to be penalised to a greater extent than resistance to parasites, which was not the case in the present experiment. Our findings support the view that there is an increased demand for SAA during GIN infections (Hoskin *et al.,* 2002; Liu *et al.,* 2007) possibly due to loss of SAA-rich endogenous protein via increased sloughing of epithelial cells and especially mucin secretion (Poppi *et al.,* 1986). Furthermore, they come in agreement with the results of Cummins *et al*. (1986) where methionine supplementation of growing rats infected with *N. brasiliensis* resulted in a reduction of worm burdens.

Leucine deficiency exerted smaller effects on the performance of the dams and their litters, which is also indicative of its preferential allocation on reproductive rather than immune functions. Lactation increases leucine requirements, as an

increased channelling of leucine from the blood flow to the mammary gland has been observed (Trottier *et al.,* 1997, DeSandiago *et al.,* 1998a). At the same time an increased intestinal metabolism of leucine takes place during parasitism (Yu *et al.,* 2000; Liu *et al.,* 2007) and its dietary intake has been directly related to the immune response in both *in vitro* and *in vivo* studies (Calder *et al.,* 2006).

Presence vs absence of secondary infection

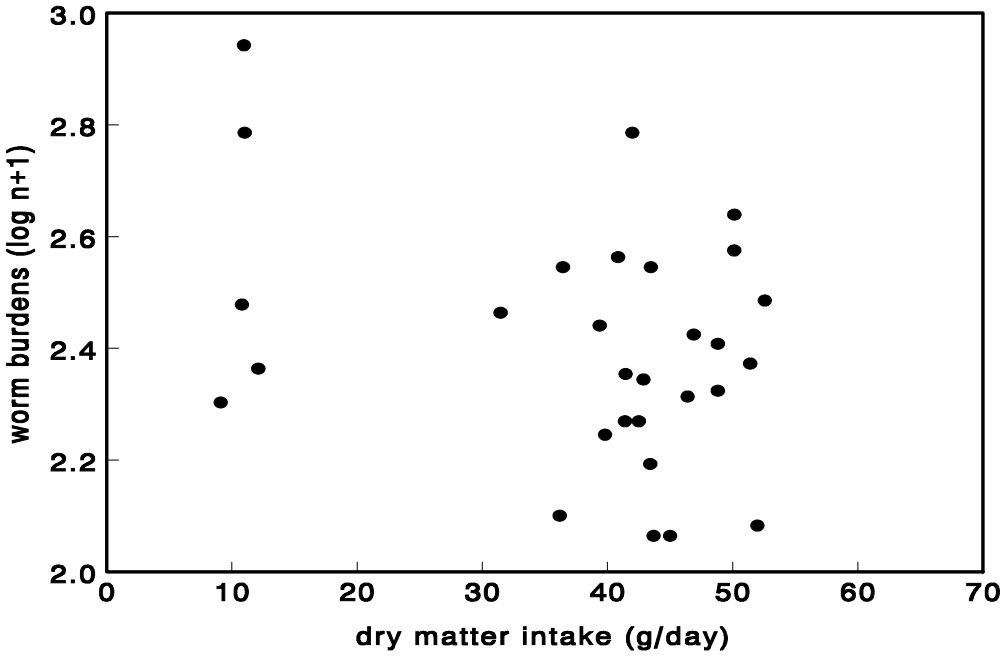
In **Chapter Two** and **Four** non infected animals were included so as to investigate whether the expression of acquired immunity to parasites imposes penalties in lactating mammals. The expectation, on the basis of the nutrient partitioning framework, would be that infection wouldn't exert any effects on dam and litter weight gain since the expression of acquired immunity takes lower priority than the reproductive effort (Coop & Kyriazakis, 1999). Results of **Chapter Two** are in accordance with this hypothesis as infection did not induce penalties on the performance of the dams or their litters when dams were fed adequate amounts of protein and energy. However, when sufficiently low level of energy and protein were offered an inferior performance was recorded for infected animals compared to their non infected counterparts. Under such conditions of malnutrition protein, of dietary and endogenous origin, would be expected to become even scarcer as it is expected to be increasingly catabolised for yielding energy. This would result to a further drawdown on protein reserves. It is known that protein deficiency causes depletion of the gut protein mass more rapidly than the muscle (Murray & Slezacek, 1994) and that it causes villus and mesenteric lymph node (MLN) atrophy and crypt hypoplasia (Woodward & Miller, 1991; Deitch *et al.,* 1992). At the same time GIN parasites

cause mucosal damage, leading to loss of plasma proteins and epithelial cells, which are common features of intestinal parasitism (Coop & Holmes, 1996; Van Houtert & Sykes, 1996). It is possible that under such conditions of protein and energy malnutrition the infected dams diverted a significantly higher proportion of scarce CP for the restoration of intestinal integrity comparing to their non infected counterparts in order to ensure the hosts survival, which in turn explains why the former animals had inferior productivity than the latter. In **Chapter Four,** excluding infected methionine deficient dams that showed extensive, though coincidental food refusals, similarly showed that infection did no affect the dams lactational performance.

Effects of malnutrition

The significantly reduced food intake of methionine deficient diets in some of the infected dams offered additional information on the effects malnutrition may exert on the lactational breakdown of immunity. One would expect that those dams would have significant penalties on both resistance to parasitism and lactational performance due to the magnitude of reduced availability of nutrients. Indeed these dams showed decreased litter and dam weight gain, increased worm burdens. Moreover, significantly reduced numbers of eosinophils were found in the mucosa of those dams. This could be related to the reduced production of n-formyl methionyl leucyl phenylalanine, a methionine containing chemotactic factor, in eosinophil migration (Michail & Abernathy, 2004). Nonetheless, worm burdens were not as high as one would expect considering the magnitude of food reduction (Figure 6.1) and egg excretion remained unaffected.

Figure 6.1. Worm burdens (*Nippostrongylus brasiliensis*) in lactating rats achieving different levels of dry matter intake.



This is in accordance with the results of Coltherd *et al*. (2009) where *H. polygyrus* infected mice allocated on very low protein diets did not show significantly higher worm burdens and egg excretion. These observations may suggest that very low levels of nutrient supply can limit the parasite, as well as the immunological response of the host towards it (Houdijk & Athanasiadou, 2003). *N. brasiliensis* adults attach to the intestinal mucosa and feed on host villus tissue (Bansemir & Sukhdeo, 2001). However, villous atrophy that occurs for example under protein scarcity (Tu *et al.,* 2007), may have limited their ability to attach and to feed and as a result their survival, regardless of the significantly reduced eosinophilia observed in these dams.

Periparturient ewe-T. circumcincta model

In **Chapter Five** ewe and litter weight were measured in order to assess the reproductive effort of the ewe, level of plasma urea as it is reflective of the protein status of the ewe, pepsinogen in order to assess the degree of abomasal damage due to parasitism and faecal egg excretion in order to measure the expression of resistance to the worm population. By assessing the relative responses of overcoming nutrient scarcity and of differential postruminal AA supply on lactational performance and resistance to parasitism we were enabled to investigate the rules of nutrient partitioning.

In **Chapter Five** the effect of two feeding treatments differing in their AA profile due to their different DUP/MP ratio on the degree of PPRI was assessed. Dams were fed either a high protein diet with soypass as the protein source (HPS) that offered a higher DUP/MP ratio relative to a high protein diet with field beans (HPB), or a low protein diet. During gestation levels of plasma urea of dams fed the high protein diets were higher than that of ewes fed a low protein diet. During gestation the same was the case for the level of plasma pepsinogen, indicative of lower abomasal damage inflicted by the established parasite population (Houdijk *et al.,* 2003b) in those dams fed the high protein diets. However, the feeding treatments did no result in significant differences in dam weight gain, condition score and egg excretion during late gestation supporting the view that nutritional effects on PPRI are more pronounced during the lactation period (Adams & Liu, 2003). This could be associated with the fact that under dietary protein scarcity ewes preferentially mobilise amino acids from the skin and the muscle, sparing protein from visceral tissues to support fetal growth (McNeill *et al.,* 1997).

During lactation levels of plasma urea and pepsinogen remained consistently higher for high protein fed ewes comparing to their low protein fed counterparts. On the other hand, litter weight gain was higher for ewes fed the diet with the higher DUP/MP ratio and this was associated with a numerically lower egg excretion, which nonetheless failed to reach statistical significance. These results indicate that the increased postruminal supplementation of AA through an increase of the undegradable protein fraction of the diet improved the resilience and to some extent the resistance of the ewes to the parasite population. Increased DUP/MP ratio has been recently shown to improve the lactational performance of lactating ewes (Mikolayunas-Sandrock *et al.,* 2009). Additionally, the egg excretion data partially support the higher effectiveness on host resistance to GIN observed in studies where additional MP supply derives from diets high DUP to ERDP ratio (e.g. Donaldson *et al.,* 1998; Houdijk *et al.,* 2003b; Van Houtert *et al.,* 1995b) in relation to studies where it derives from diets with a low DUP to ERDP ratio (Kahn *et al.,* 2003a, 2003b, Knox & Steel, 1996; Shaw *et al.,* 1995; Van Houtert *et al.,* 1995a). Results of this study suggest a partial allocation of immunity for both immune and reproductive functions during the lactation period. It is my belief that given a higher number of experimental animals per treatment group, effects of feeding treatments with differing DUP/MP ratio on nematode egg excretion would have been more significant.

6.2.2 Timing and effects on associated immune responses

In **Chapter Two, Three and Four** two serial slaughter points, on day six and nine post secondary infection, were applied so as to reveal the timing of effects of

decreased nutrient scarcity on resistance to parasites. Additionally, mucosal eosinophil, mast cells and goblet cell numbers (**Chapter Three and Four**) and systemic levels of parasite-specific serum levels of *N. brasiliensis* specific IgA, IgE, IgG1, IgG2a, IgG2b and total IgG were assessed (**Chapter Five**) so as to identify the effect of our treatments on Th2 type immune responses, typically involved in resistance to gastrointestinal nematode parasitism (Shea Donohue & Urban, 2004).

Timing of nutritional effects on parasitism

In **Chapters Two** and **Four** increased nutrient supply enhanced resistance to *N. brasiliensis* within six days post secondary infection and persisted throughout the lactation period as indicated by the absence of treatment and time interactions. This enhancement of resistance was illustrated through reduced worm burdens (**Chapters Two** and **Four),** reduced nematode egg excretion (**Chapter Four**) and a lower female/male ratio (**Chapter Two**). Effects of increased nutrient supply on resistance to *N. brasiliensis* have been previously shown to take place within ten days post secondary infection and have been associated with reduced worm burden and nematode egg excretion (Houdijk *et al.,* 2005a) or a lower female/male ratio (Houdijk *et al.,* 2003a). However, in the latter studies only one serial slaughter point was applied. Our results are in accordance with that of a subsequent experiment (Jones *et al.,* 2009) where the same serial slaughter protocol was applied and effects of increased nutrient supply were also mediated within six days post secondary infection. A reduction of nutrient demand has been similarly shown to increase resistance within ten days after a reduction of litter size took place (Normanton *et al.,* 2007). However, in the latter study the application of one endpoint did not allow

assessing the timeframe within which effects took place. In **Chapter Three** a reduction in nutrient demand affected PPRI within three days post secondary infection through a reduction of both worm burdens and nematode egg excretion. These findings illustrate clearly that the beneficial impact of improving host's nutritional status on resistance to parasitism can be mediated within a short period of time, influencing the parasites fecundity, population size and the rate of its expulsion.

Timing of nutritional effects on immune responses

During protein deficiency, gut and intestinal morphology is altered, crypt hypoplasia and atrophy of the villi, decreased mast and goblet cell response, and a more permeable gut mucosal barrier is observed (Tu *et al.,* 2007). Alongside the morphological changes, atrophy of the mesenteric lymph nodes along with decreased B lymphocytes and CD4+ cells are observed (Tu *et al.,* 2007). These morphological and intestinal changes impair the hosts' ability to limit, damage and expel the resident worm population.

In **Chapter Three** reducing nutrient scarcity (primarily of protein as the diet offered was sufficiently providing all other nutrients) resulted in increased number of mucosal mast cells and these effects were observed within three days following a litter size reduction. Mast cell hyperplasia is a feature of almost all helminth infections (Miller, 1996). Mast cells degranulate during GIN infections and release a number of mediators including mast cell proteases, histamine and prostaglandins (Kalesnukoff and Galli, 2008) and they are a source of cytokines such as IL-4 and IL-13 (Gessner *et al.,* 2005). Our results are in agreement with previous studies using this host parasite model system; increased nutrient supply (Jones *et al.,* 2009) or CP

supply (Jones *et al.,* 2011) resulted in increased mast cell numbers on both day six and nine post secondary infection. Protein malnutrition has been previously linked to a reduction in mucosal mast cell numbers in growing mice infected with *H. polygyrus* (Ing *et al.,* 2000). Similarly increasing the level of protein supply has resulted in mast cell hyperplasia in experiments involving parasitized lambs and ewes (Van Houtert *et al.,* 1995a; Coop & Holmes, 1996; Houdijk *et al.,* 2003b). It is likely that increased mast cell numbers and subsequent degranulation due to a higher availability of scarce resources could contribute to a higher rate of expulsion and a limitation in the fecundity of the parasite population.

Moreover, in **Chapter Three** effects of smaller magnitude were observed on the number of mucosal eosinophils, which tended to increase as a result of reduced nutrient demand also within three days following litter size reduction. Both peripheral and tissue eosinophilia are characteristic features of GIN infections (Lawrence, 2003, Behm & Ovington 2000). Upon stimulation they degranulate and release highly toxic proteins, oxidizing agents and neurotoxins in proximity to the nematodes in tissue. Increasing protein supply and rearing smaller litters have, to date, not affected eosinophil numbers in this rodent parasite system (Normanton *et al.,* 2007; Jones *et al.,* 2009; Jones *et al.,* 2011) although effects have been observed in sheep-parasite models of infection (Kambara *et al.,* 1993; Coop *et al.,* 1995; Van Houtert *et al.,* 1995b; Datta *et al.,* 1998; Houdijk *et al.,* 2000).

The effect on mucosal eosinophil numbers observed in **Chapter Three** was also related with a reduction in egg excretion of higher magnitude as compared to the aforementioned studies carried out with this model system. This may be related to the higher degree of nutrient scarcity imposed in this experiment as compared to

Normanton *et al*. (2007); dams under nutrient scarcity were rearing a litter of twelve instead of nine pups. In addition although all nutrients other than protein were offered in abundance to all dams such as minerals and vitamins, dams rearing twelve pups would have diverted more minerals towards milk production than dams rearing three pups. That is because lactation increases the fluxes of the primary bone-forming minerals, calcium, phosphorus, magnesium and zinc, across the placenta and through breast milk, placing considerable demands on maternal mineral economy (Prentice, 2003). Parasitism has been also found to alter concentrations of a wide range of liver minerals, including iron, zinc, calcium, phosphorus, potassium, sodium, and sulfur, despite adequate dietary intakes of these minerals (Bourgeois *et al.,* 2007) and these effects are more pronounced when a protein deficiency takes place (Tu *et al.,* 2009). This regarded in relation to the existing evidence for the influence of mineral supplementation in GIN infected host's (McClure, 2008; Koski & Scott, 2003) may imply a possible effect of increased mineral supplementation on host's immune responses.

In **Chapter Three** no effects were observed on goblet cell hyperplasia. Our results are in agreement with Jones *et al*. (2011) and Normanton *et al*. (2007) where there was an absence of effect on their numbers following increased CP supply and rearing smaller litters, respectively. However, Jones *et al*. (2009) observed higher number of goblet cells due to increased dietary CP contents. An increase in the numbers of goblet cells (GC) (De Veer *et al.,* 2007; Mahida, 2003) is a common feature of GIN infections. Mucins from goblet cells play an important role in the trapping of worms in the mucus layer and inhibiting worm motility and feeding (Yamauchi *et al.,* 2006, Tsubokawa *et al.,* 2009). One cannot exclude that the level

of mucin secretion or their profile might have been affected as a result of our dietary treatments.

In **Chapter Four** none of the mucosal inflammatory population numbers was affected as a result of reduced supply either of protein, leucine or methionine, regardless of the penalties imposed on both the development of resistance and host's performance. In terms of the absence of an effect on goblet and eosinophils mucosal numbers this comes as no surprise; nutritional manipulations do not exert consistent effects on their accumulation in the intestinal mucosa in this model system (Jones et a., 2009; Jones *et al.,* 2011; Normanton *et al.,* 2007; Chapter Three). Additionally, diets offered in previous experiments in this model system (Jones *et al*. 2009; 2011), where effects on mucosal inflammatory cell numbers have been observed, were designed to induce lower levels of protein scarcity than the ones imposed in the current study. However, in **Chapter Four** this was avoided as individual indispensable amino acid deficiency of higher magnitude could potentially result in unacceptable food refusals (Gietzen, 1993; Gietzen *et al.,* 2007).

In a similar fashion, in **Chapter Four** levels of serum anti- *N.brasiliensis* antibodies were not affected by our dietary treatments. Immunoglobulins are considered as mediators of immunity in nematode infections (Harris & Gause, 2011) and antibody synthesis is likely to draw on protein resources (Houdijk *et al.,* 2001a). Results of studies in parasitized periparturient hosts show that increased protein supplementation can lead to an increased antibody production in parasitized hosts (Athanasiadou & Houdijk, 2010). However, in our model system, effects on serum levels of antibodies as a result of increased CP supply have been minor (Jones *et al.,* 2011). On the other hand, an early upregulation of the mucosal levels of IG2b has

been observed at day three post secondary infection in dams fed HP diets and this implies that nutritional effects may had taken place earlier in the infection in this model system (Jones *et al.,* 2009). Nevertheless, antibody production in *N. brasiliensis* primary and secondary infected rats has been recently discarded as being important for the expulsion of this nematode species (Liu *et al.,* 2010). Therefore, even if an earlier upregulation of their production at the mucosal level had occurred, it is not likely to have affected the resistance of the host towards this parasite species.

Collectively, results of **Chapter Three** indicate that effects of decreased nutrient scarcity on the resistance of the host are mediated through an increased expression of immunity. Nevertheless, the absence of observed effects on the effector mechanisms measured in **Chapter Four** implies that other effector cells could have been affected by reduced specific AA deficiency.

Other effector mechanisms

The absence of an observed effect on the expression of immunity in **Chapter Four** points out that other effector mechanisms, not measured in this experiment, could have been affected by our dietary treatments. Although the *N. brasiliensis* nematode model has been widely used to unravel the key immunological factors that underlie Th2 immune responses (Marsland *et al.,* 2008), ultimately mechanisms contributing to helminth parasite clearance remain elusive (Anthony *et al.,* 2007). Recent advances in the field of immunoparasitology identify effector mechanisms that were not thought to be important in the immune response to nematodes, while new ones are constantly being discovered. Their activation, proliferation and the

synthesis of their products could be sensitive to nutrient scarcity and could have therefore been affected in our model.

Recently, a new innate effector cell line critically important in Th2 immune responses to helminthes has been discovered in *N. brasiliensis* infected mice, called nuocytes. They have been shown to augment T-cell cytokine production, and augment T-cell responses in *N. brasiliensis* infected mice (Neill *et al.,* 2010).

Basophils have long been considered as minor effector cells in helminth infections (Karasuyama *et al.,* 2011). Nevertheless, their numbers have been shown to increase in several animal models of helminth infection (Mitre & Nutman, 2006). Recent studies show that although they have minor contribution to worm expulsion in primary infection with *N. brasiliensis*, they play a host-protective role in secondary infections of the same parasite (Ohnmacht *et al.,* 2010; Ohnmacht & Voehringer 2010). Nonetheless, it remains elusive as to how they contribute to worm expulsion in secondary infections (Karasuyama *et al.,* 2011).

Until recently, macrophages were thought to be relatively unimportant during Th2-type responses (Maizels *et al.,* 2009). However, they have been recently recognised as effector mechanisms in GIN infections (Anthony *et al.,* 2007) being activated in the presence of IL-4 and IL-13, cytokines produced in Th2 type immune responses (Zhao *et al.,* 2008). At least three principal functions in the protective immune response towards nematode infections have been attributed to them: regulation of the immune response, wound healing and resistance to parasite invasion (Anthony *et al.,* 2007).

Methionine deficiency in particular could have affected the production of mucins from goblet cells. Mucins are cysteine rich proteins that are increasingly

produced during parasitic infections. They contribute in worm expulsion of damaged worms, and inhibit their migration and feeding at the mucosal surfaces (Kim & Ho, 2010) and they are considered essential for *N. brasiliensis* expulsion (Herbert *et al.,* 2009). In addition methionine deficiency could have affected lymphocyte proliferation and differentiation via spermidine and spermine metabolism (Liu *et al.,* 2007; Flynn *et al.,* 2002), and leukocyte metabolism via phosphatidylcholine synthesis (Kim *et al.,* 2007).

Leucine on the other hand, is the only BCAA that can activate the mTOR signaling pathway in intestinal epithelial cells (Ban *et al.,* 2004), its intake affects the morphology of the intestinal epithelium (Sheibak *et al.,* 2007) and may influence the gut's repair ability. A damaged mucosa could lead to an impaired expression of immunity and as a consequence result in higher worm burdens in the leucine deficient dams. Furthermore, leucine deficiency causes a decrease on the level of plasma leptin concentration (Lynch *et al.,* 2006). Leptin is a potent modulator of the immune response (Iikuni *et al.,* 2007) and its concentration in the rat has been found to rise as a result of infection with *N. brasiliensis* (Roberts *et al.,* 1999), while lactation suppresses its levels (Brogan *et al.,* 1999). Interest in the role leptin might play in GIN infections has been recently raised (Sykes, 2008) and although it has been found to be crucial for gut immunity (Guo *et al.,* 2010) a role has not yet to be revealed for GIN infections. These information indicate that leucine deficiency may have affected immunity via different mechanisms which demand further investigation.

6.2.3. Implications of the thesis for small ruminants

Chapters Two, Three and Four have provided novel insights in the nutritional manipulation of parasitism and important implications for its application in sustainable control strategies in ruminant production systems.

Chapter Two provided novel information on the relevant importance of protein and energy nutrition in the regulation of parasitism. It enhanced the view that protein is more important than energy supply in parasitized hosts. This does not mean that energy should be disregarded in small ruminant production systems; sufficient levels of fermentable metabolizable energy are needed for the production of microbial protein from the bacteria that reside in the rumen. Nevertheless, temporal increase in the level of MP supply offered to periparturient parasitized hosts is deemed more important than a temporal increase in the level of ME supply.

Chapter Three confirms the observation that nutrient demand affects the degree of PPRI. It illustrated that increased parasitism as a result of increased nutrient scarcity induces penalties on the immune response and as a consequence on the resistance of the host. Moreover, it showed that a reduction of nutrient scarcity can rapidly reduce the degree of PPRI by directly enhancing the expression of immunity in the level of the gut. These findings support the implementation of strategic protein supplementation of multiple rearing, or high producing, ruminant hosts in sustainable worm control programmes with the aim to lower the dependency on chemoprophylaxis, and those benefits can be observed rapidly.

Chapter Four implies that AA balance, and thus protein quality, may be important to consider in immunonutrition strategies for small ruminant species. It shows for the first time that reduced supply of AA, for which there is an increased

demand for the expression of acquired immunity and the reproductive effort, may have deleterious effects on immunity to parasites.

Chapter Five verifies the results of **Chapter Four** and further shows that the degree of PPRI is sensitive to both MP supply and MP quality, suggesting that protein source is an important issue to consider in non-chemical parasite control strategies in small ruminant production systems.

6.3. Future directions

The thesis presented provides novel findings in the field of the nutritional manipulation of parasitism. Nonetheless, there are points to be considered and future directions to be taken that are briefly presented below.

Chapter Two showed that moderate ME scarcity did not affect the degree of breakdown of immunity. The level of accumulation of fat reserves during pregnancy has not been shown to be important for resistance to GIN (Houdijk *et al.,* 2001b). However, the results of studies on growing hosts, which show that energy restriction affects immunity to parasites when it is more prolonged and of higher magnitude (Koski *et al.,* 1999; Kristan, 2007; Lunn *et al.,* 1988), poses the need for further investigation. I believe that an experiment involving dietary treatments that manifest the accumulation of fat reserves during pregnancy and offer different levels of energy supply during the lactation period might reveal an effect of energy on the lactational breakdown of immunity. In such an experiment monitoring the effects of our dietary treatments on leptin concentrations is necessary as it has an emerging role as a modulator of gut associated immune responses (Yarandi *et al.,* 2011) and its role in

immunity to GIN infections has not yet been explored in a periparturient monogastric model of disease.

Chapter Four showed that reduced AA supplementation of leucine or methionine penalises immunity to parasites. It would be interesting to assess the effects of the differential supply of other amino acids such as threonine or arginine on the degree of PPRI. Threonine is a major component of intestinal mucin and plasma γ-globulin in animals (Li *et al.,* 2007) and is regarded as crucial for mucin production and maintenance of gut function (Le Floc'h *et al.,* 2004). Dietary threonine restriction has been shown to dramatically impair the synthesis of mucins in the small intestine (Faure *et al.,* 2005). Such an effect of reduced supplementation of threonine in this model system is expected to exert significant penalties on immunity to parasites and has to be investigated. Arginine on the other hand, has been identified by a large number of animal and human studies as important in intestinal immune response (Li *et al.,* 2007; Wang *et al.,* 2009). Studies indicate that arginase activity is a limiting factor for polyamine synthesis and proliferation in macrophage cell lines (Kepka-Lenhart *et al.,* 2000), and smooth muscle cells (Wei *et al.,* 2001) (see Wu *et al.,* 2009 for a recent review) and both smooth muscle contractility and macrophages are now considered important features of the immune response to GIN. Moreover, utilization of arginine is high in mammary gland of rats (Mezl & Knox, 1977), sows (O'Quinn *et al.,* 2002) and ruminants (Mepham, 1982). A competition between immune and reproduction functions therefore exists and reduced arginine supply could potentially affect resistance to GIN nematodes in periparturient hosts.

Mineral nutrition could also be investigated in this model system. An experiment involving different levels of supplementation of minerals for which there

is evidence that their supply can affect the host's resistance to GIN and which are increasingly utilised during lactation could be performed. Such an experiment would provide novel information on the nutritional modulation of parasitism and could lead to the application of an experiment on ruminant hosts.

I believe that future experiments using this model should exploit the recent findings on the field of immunology. Assessing the activation and proliferation of macrophages, basophils or nuocytes and the level of mucus secretion and its products could reveal effects of nutritional supplementations on their numbers. On the other hand, amino acids have distinct roles in the regulation of gene expression (Wu, 2009) and gene expression has been shown to be influenced by increased levels of protein in this model system (Athanasiadou *et al*. 2011). Future studies could implement gene expression analysis, which could provide insights as to how AA supplementation affects the expression of immunity to parasites.

This thesis raises questions concerning the mode of effect of protein supplementation. It is possible that the observed effects could have been exerted through other mechanisms, in addition to a direct enhancement of immunity. Assessment of the changes that are induced in the intestinal function, size and structure could lead to a better understanding as to how the interaction between lactation-nematode infection-and host nutrition affects the level of parasitism. It is possible that increased nutrient supplementation manipulates the intestinal morphology in such a way that it could mediate cytokine induced muscle contractility more efficiently and therefore contribute to the expulsion of the worm population.

Although dam and litter weight gain are useful in estimating host's performance, carcass analysis will provide more specific answers regarding the nutrient partitioning during secondary infections. Serial blood sampling throughout the lactation period for assessing plasma amino acid concentrations could potentially reveal altered patterns of inter organ amino acid fluxes as a result of infection, or could indicate increased amino acid utilization. This technique has already been performed in our lab, but it was applied only at terminal slaughter point which does not allow for a proper interpretation of the results. Analysis of the blood plasma concentration of amino acids collected from **Chapter Two** and **Chapter Four** did not reveal an effect of infection on their concentrations, although they were significantly affected by our dietary treatments.

In a future experiment carried on our periparturient ruminant host model, other legumes such as peas and lupins, which can be homegrown, can be tested for their efficacy in reducing the degree of PPRI. Finally, the effect of heat treatment such as pressure toasting, expander treatment and pelleting on the effectivity of legumous seeds in improving the resistance and resilience of parasitized ewes should be investigated. These treatments can potentially increase the DUP proportion of the protein fraction of the diet (Goelema *et al.,* 1998). It is my belief that these sources can be exploited in such a way that dependency on soya, a source of imported protein, could be diminished.

6.4 General conclusions

- This thesis provides evidence that improved protein but not energy nutrition can be used to improve/restore the resistance of periparturient hosts to GIN parasites.
- It also provides evidence that benefits on resistance, arising from improving the host's nutritional status, are associated with an improvement of gut immunity. It further shows that such improvement occurs rapidly, i.e. within a few days after overcoming nutrient scarcity.
- In addition, it provides evidence that periparturient host's resistance is sensitive to the protein quality of the ingested protein.
- Overall, this thesis contributes to the experimental evidence that improved protein nutrition can be useful as part of a multi-faceted non-chemical means of parasite control. It further implies that future studies should concentrate on the nature of the protein offered to grazing ruminants. This multi-faceted approach to parasite control could reduce reliance on anthelmintics to improve production and reduce parasite egg contamination of the environment (pasture or paddock) in livestock species.

Future studies should provide more information concerning the mode of effect of AAs, which are shown to be important immunonutrients in cases of parasitism, and further explore the effects of nutrition on the pathophysiology of GIN infections in immune animals. Additionally, they should test more alternative sources of protein for their effectivity in improving resistance and resilience to parasitism.

References

Adams NR & Liu SM (2003) Principles of nutrient partitioning for wool, growth and reproduction: implications for nematode parasitism. *Aust J Exp Agr* **43,** 1399-1407.

AFRC (1993) *Energy and Protein Requirements of Ruminants. An Advisory Manual Prepared by the AFRC Technical Committee on Responses to Nutrients.* Wallingford: CAB International.

Africa CM (1931) Studies on the host relations of *Nippostrongylus muris*. *J Parasitol* **18**, 1-13.

Alexander G & Davies HL (1959) Relationship of milk production to number of lambs born or suckled. *Australian J Agr Res* **10,** 720-724.

Albers GAA, Gray GD, Piper LR, Barber JSF, LeJambre LF & Barger IA (1987) The genetics of resistance and resilience to *Haemonchus contortus* infection in young Merino sheep. *Int J Parasitol* **17**, 1355-1367.

Annett RW, Carson AF & Dawson LER (2005) The effect of digestible undegradable protein (DUP) content of concentrates on colostrums production and lamb performance of triplet bearing ewes on grass-based diets during late pregnancy. *Anim Sci* **80**, 101-110.

Annett RW, Carson AF & Dawson LER (2008) Effects of digestible undegradable protein (DUP) supply and fish oil supplementation of ewes during late pregnancy on colostrums production and lamb output. *Anim feed Sci Tech* **146**, 270-288.

Anthony RM, Rutitzky LI, Urban JF Jr, Stadecker MJ & Gause WC (2007) Protective immune mechanisms in helminth infection. *Nat Rev Immunol* **7**, 975-987.

Artis D (2006) New weapons in the war on worms: Identification of putative mechanisms of immune-mediated expulsion of gastrointestinal nematodes. *Int J Parasitol* **36**, 723-733.

Astrup A & Tremblay A (2009) Energy metabolism. In Introduction to Human Nutrition, 2nd ed., pp. 31–48 [MJ Gibney, SA Lanham-New and HH Cassidy Vorster, editors]. Oxford: Wiley-Blackwell.

Athanasiadou S & Houdijk JGM (2010) Nutrition and immunity in animal disease: lessons from parasitic gastroenteritis. In: Watson, R.R., Preedy, V., (Eds.), Dietary components and immune function-prevention and treatment of disease and cancer. Springer Humana Press, New York. pp. 63-74.

Athanasiadou S, Houdijk J & Kyriazakis I (2008) Exploiting synergisms and interactions in the nutritional approaches to parasite control in sheep production systems. *Small Rumin Res* **76**, 2-11.

Athanasiadou S, Jones LA, Burgess ST, Kyriazakis I, Pemberton AD, Houdijk JG & Huntley JF (2011) Genome-wide transcriptomic analysis of intestinal tissue to assess the impact of nutrition and a secondary nematode challenge in lactating rats. *PLoS One* **6,** e20771.

Athanasiadou S, Kyriazakis I, Jackson F & Coop RL (2001) Direct anthelmintic effects of condensed tannins towards different gastrointestinal nematodes of sheep: in vitro and in vivo studies. *Vet Parasitol* **99**, 205-219.

Athanasiadou S, Tzamaloukas O, Kyriazakis I, Jackson F & Coop RL (2005) Testing for direct anthelmintic effects of bioactive forages against *Trichostrongylus colubriformis* in grazing sheep. *Vet Parasitol* **127**, 233-243.

del Rosario Ayala M, Racotta R, Hernández-Montes H & Quevedo L (2006) Some metabolic effects on lactating rats of a low-energy diet restricted in good-quality protein. *Br J Nutr* **96**, 667-673.

Baker RL, Mwamachi DM, Audho JO, Aduda EO & Thorpe W (1998) Resistance of Galla and Small East African goats in the subhumid tropics to gastrointestinal nematode infections and the periparturient rise in faecal egg counts. *Vet Parasitol* **79**, 53-64.

Ball G, Selkirk ME & Knox DP (2007) The effect of vaccination with a recombinant *Nippostrongylus brasiliensis* acetylcholinesterase on infection outcome in the rat. *Vaccine* **25**, 3365-3372.

Ban H, Shigemitsu K, Yamatsuji T, Haisa M, Nakajo T, Takaoka M, Nobuhisa T, Gunduz M, Tanaka N & Naomoto Y (2004) Arginine and leucine regulate p70 S6 kinase and 4E-BP1 in intestinal epithelial cells. *Int J Mol Med* **13**, 537-543.

Bansemir AD & Sukhdeo MV (2001) Intestinal distribution of worms and host ingesta in *Nippostrongylus brasiliensis. J Parasitol* **87**, 1470-1472.

Barger IA (1993) Influence of sex and reproductive status on susceptibility of ruminants to nematode parasitism. *Int J Parasitol* **23**, 463-469.

Barry TN & McNabb WC (1999) The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. *Br J Nutr* **81**, 263-272.

Bauchart-Thevret C, Stoll B, Chacko S & Burrin DG (2009) Sulfur amino acid deficiency upregulates intestinal methionine cycle activity and suppresses epithelial growth in neonatal pigs. *Am J Physiol Endocrinol Metab* **296**, E1239-E1250.

Beasley AM, Kahn LP & Windon RG (2010) The periparturient relaxation of immunity in Merino ewes infected with *Trichostrongylus colubriformis*: Parasitological and immunological responses. *Vet Parasitol* **168**, 60-70.

Behm CA & Ovington KS (2000) The role of eosinophils in parasitic helminth infections: insights from genetically modified mice. *Parasitol Today* **16**, 202-209.

Bell AW & Ehrhartd RA (2000) Regulation of macronutrient partitioning between maternal and conceptus tissues in the pregnant ruminant. In Ruminant Physiology. Digestion, metabolism, growth and reproduction pp.275-293 [PB Cronje, editor]. CABI Publishing: Wallingford.

Bequette BJ, Metcalf JA, Wray-Cahen D, Backwell FR, GFtton JD, Lomax MA, Macrae JC & Lobley GE (1996) Leucine and protein metabolism in the lactating dairy cow mammary gland: Responses to supplemental dietary crude protein intake. *J Dairy Res* **63**, 209-222.

Bocquier F, Atti N, Purroy A & Chilliard Y (2000) The role of body reserves in the metabolic adaptation of different breeds of sheep to food shortage. In Livestock Production and Climatic Uncertainty in the Mediterranean. European Association of Animal Production Publication no. 94, pp. 75-93 [Guessous F, Rihani N, and Ilham A, editors]: Wageningen Pers: Wageningen.

Boes J & Helwigh AB (2000) Animal models of intestinal nematode infections of humans. *Parasitology* **121**, S97-S111.

Bourgeois AC, Scott ME, Sabally K & Koski KG (2007) Low dietary boron reduces parasite (Nematoda) survival and alters cytokine profiles but the infection modifies liver minerals in mice. *J Nutr* **137**, 2080-2086.

Bown MD, Poppi DP & Sykes AR (1991) The effect of post ruminal infusion of protein or energy on the pathophysiology of *Trichostrongylus Colubriformis* infection and body composition in lambs. *Aust J Agric Res* **42**, 253-267.

Brendemuhl JH, Lewis AJ & Peo ER (1987) Effect of protein and energy intake by primiparous sows during lactation on sow and litter performance and sow serum thyroxine and urea concentrations. *J Anim Sci* **64**, 1060-1069.

Brendemuhl JH, Lewis AJ & Peo ER (1989) Influence of energy and protein intake during lactation on body composition of primiparous sows. *J Anim Sci* **67**, 1478-1488.

Brogan RS, Mitchell SE, Trayhurn P & Smith MS (1999) Suppression of leptin during lactation: contribution of the suckling stimulus versus milk production. *Endocrinology* **140**, 2621-2627.

Brunschwig P, Lamy JM, Peyronnet C & Crepon K (2004) Faba bean valorisation in complete diet for dairy cows. *Renc Rech Ruminants* **7**, 275.

Calder PC (2006) Branched-chain amino acid and immunity. *J Nutr* **136**, 288S–293S.

Chartier C, Etter E, Hoste H, Pors I, Mallereau MP, Broqua C, Mallet S, Koch C & Masse A (2000) Effects of the initial level of milk production and of the dietary protein intake on the course of natural nematode infection in dairy goats. *Vet Parasitol* **92**, 1-13.

Chemical Rubber Company. Handbook of Chemistry and Physics. London, CRC Press, 2001.

Christie M & Jackson F (1982) Specific identification of strongyle eggs in small samples of sheep faeces. *Res Vet Sci* **32**, 113-117.

Clowes EJ, Aherne FX & Baracos VE (2005) Skeletal muscle protein mobilization during the progression of lactation. *Am J Physiol Endocrinol Metab* **288**, 564-572.

Colditz IG (2008) Six costs of immunity to gastrointestinal nematode infections. *Parasite Immunol* **30**, 63-70.

Coleman SW & Henry DA (2002) Nutritive Value of Herbage. In Sheep Nutrition, pp1-27 [M Freer and H Dove, editors]. CABI International.

Coltherd JC, Bünger L, Kyriazakis I & Houdijk JG (2009) Genetic growth potential interacts with nutrition on the ability of mice to cope with *Heligmosomoides bakeri* infection. *Parasitology* **136**, 1043-1055.

Coop RL & Holmes PH (1996) Nutrition and parasite interaction. *Int J Parasitol* **26**, 951-962.

Coop RL & Kyriazakis I (1999) Nutrition-parasite interaction. *Vet Parasitol* **84**, 187-204.

Coop RL & Kyriazakis I (2001) Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. *Trends Parasitol* **17**, 325-330.

Cowan RT, Robinson JJ, McHattie I & Fraser C (1980) The prediction of body composition in live ewes in early lactation from live weight and estimates of gut contents and total body water. *J Agric Sci* **95**, 515-522.

Crépon K, Marget P, Peyronnet C, Carrouée B, Arese P, Duc G (2010) Nutritional value of faba bean (Vicia faba L.) seeds for feed and food. *Field Crop Res* **115**, 329-339.

Crowle PK & Reed ND (1981) Rejection of the intestinal parasite *Nippostrongylus brasiliensis* by mast cell-deficient w-w-upsilon anemic mice. *Infect Immun* **33**, 54-58.

Cuaron JA, Chapple RP & Easter RA (1984) Effect of lysine and threonine supplementation of sorghum gestation diets on nitrogen balance and plasma constituents in 1st litter gilts. *J Anim Sci* **58**, 631-637.

Cummins AG, Bolin TD, Duncombe VM & Davis AE (1986) The effect of methionine and protein deficiency in delaying expulsion of *Nippostrongylus brasiliensis* in the rat. *Am J Clin Nutr* **44,** 857-862.

D'Mello JFP, (2003) Amino acids as multifunctional molecules, In Amino Acids in Animal Nutrition, 2nd ed, pp 1-14. [D'Mello JPF, editor] CABI Publishing, Cambridge, MA.

Datta, FU, Nolan JV, Rowe JB & Gray GD (1998) Protein supplementation improves the performance of parasitised sheep fed a straw-based diet. *Int J Parasitol* **28**, 1269-1278.

Davis TA, Fiorotto ML & Reeds PJ (1993) Amino acid compositions of body and milk protein change during the suckling period in rats. *J Nutr* **123**, 947-956.

Dawson LER, Carson AF & Kilpatrick DJ (1999) The effect of the digestible undegradable protein concentration of concentrates and protein source offered to ewes in late pregnancy on colostrum production and lamb performance. *Anim feed Sci Tech* **82,** 21-36.

De Veer MJ, Kemp JM & Meeusen ENT (2007) The innate host defence against nematode parasites. *Parasite Immunol* **29**, 1-9.

Dehlawi MS & Goyal PK (2003) Responses of inbred mouse strains to infection with intestinal nematodes. *J Helminthol* **77**, 119-124.

Deitch EA, Xu DZ, Qi L, Specian RD & Berg RD (1992) Protein malnutrition alone and in combination with endotoxin impairs systemic and gut-associated immunity. *J Parenter Enteral Nutr* **16**, 25-31.

DeSantiago S, Torres N & Tovar AR (1998a) Leucine catabolism in mammary tissue, liver and skeletal muscle of dam rat during lactation and weaning. *Arch Med Res* **29**, 25-32.

DeSantiago S, Torres N, Suryawan A, Tovar AR & Hutson SM (1998b) Regulation of branched-chain amino acid metabolism in the lactating rat. *J Nutr* **128**, 1165-1171.

Domeneghini C, Giancamillo AD, Bosi G & Arrighi S (2006) Can nutraceuticals affect the structure of intestinal mucosa? Qualitative and quantitative microanatomy in L-glutamine diet supplemented weaning piglets. *Vet Res Comm* **30**, 331-342.

Donaldson J, Van Houtert MFJ & Sykes AR (1998) The effect of nutrition on the periparturient parasite status of mature ewes. *Anim Sci* **67**, 523-533.

Donaldson J, Van Houtert MFJ & Sykes AR (2001) The effect of dietary fish-meal supplementation on parasite burdens of periparturient sheep. *Anim Sci* **72**, 149-158.

Else KJ & Finkelman FD (1998) Intestinal nematode parasites, cytokines and effector mechanisms. *Int J Parasitol* **8**, 1145-58.

Elshorbagy AK, Valdivia-Garcia M, Refsum H, Smith AD, Mattocks DA & Perrone CE (2010) Sulfur amino acids in methionine restricted rats: Hyperhomocysteinemia. *Nutrition* **26**, 1201-1204.

Etter E, Chartier C, Hoste H, Pors I, Bouquet W, Lefrileux Y & Borgida LP (1999) The influence of nutrition on the periparturient rise in fecal egg counts in dairy goats: results from a two-year study. *Rev Med Vet* **150**, 975-980.

Fang Z, Yao K, Zhang X, Zhao S, Sun Z, Tian G, Yu B, Lin Y, Zhu B, Jia G, Zhang K, Chen D & Wu D (2010) Nutrition and health relevant regulation of intestinal sulfur amino acid metabolism. *Amino Acids* 3**9**, 633-640.

Farid Z, Patwardhan VN & Darby WJ (1969) Parasitism and anemia. *Am J Clin Nutr* **22**, 498-503.

Faure M, Moennoz D, Montigon F, Mettraux C, Breuille´ D & Balle`vre O (2005) Dietary threonine restriction specifically reduces intestinal mucin synthesis in rats. *J Nutr* **135,** 486-491.

Ferguson EGW, Mitchell GBB & MacPherson A (1989) Cobalt deficiency and *Ostertagia circumcincta* infection in lambs. *Vet Rec* **124**, 20-24.

Field CJ, Johnson IR & Schley PD (2002) Nutrients and their role in host resistance to infection. *J Leukoc Biol* **71**, 16-32.

Finkelman FD, Shea-Donohue T, Morris SC, Gildea L, Strait R, Madden KB, Schopf L & Urban JF (2004) Interleukin-4-and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunol Rev* **201**, 139-155.

Flynn NE, Meininger CJ, Haynes TE & Wu G (2002) The metabolic basis of arginine nutrition and pharmacotherapy. *Biomed Pharmacother* **56**, 427-438.

Fontenot ME, Miller JE, Peña MT, Larsen M & Gillespie A (2003) Efficiency of feeding *Duddingtonia flagrans chlamydospores* to grazing ewes on reducing availability of parasitic nematode larvae on pasture. *Vet Parasitol* **118**, 203-213.

Fox CJ, Hammerman PS & Thompson CB (2005) Fuel feeds function: Energy metabolism and the T-cell response. *Nat Rev Immunol* **5**, 844-852.

Friggens NC, Hay DEF & Oldham JD (1993) Interactions between major nutrients in the diet and the lactational performance of rats. *Br J Nutr* **69**, 59-71.

Gardner RW & Hogue DE (1964) Effects of energy intake and number of lambs suckled on milk yield, milk composition and energetic efficiency of lactating ewes. *J Anim Sci* **23,** 935-942.

Gessner A, Mohrs K & Mohrs M (2005) Mast cells, basophils, and eosinophils acquire constitutive IL-4 and IL-13 transcripts during lineage differentiation that are sufficient for rapid cytokine production. *J Immunol* **174**, 1063-1072.

Gietzen DW (1993) Neural mechanisms in the responses to amino acid deficiency. *J Nutr* **123,** 610-625.

Gietzen DW, Hao S & Anthony TG (2007) Mechanisms of food intake repression in indispensable amino acid deficiency. *Annu Rev Nutr* **27,** 63-78.

Gilleard JS (2006) Understanding anthelmintic resistance: The need for genomics and genetics. *Int J Parasitol* **36**, 1227-1239.

Goelema JO, Spreeuwenberg MAM, Hof G, van der Poel AFB & Tamminga S (1998) Effect of pressure toasting on the rumen degradability and intestinal digestibility of whole and broken peas. *Anim Feed Sci Technol* **76**, 35-50.

Good B, Hanrahan JP, Crowley BA & Mulcahy G (2006) Texel sheep are more resistant to natural nematode challenge than Suffolk sheep based on faecal egg count and nematode burden. *Vet Parasitol* **136**, 317-327.

Greiner EF, Guppy M & Brand K (1994) Glucose is essential for proliferation and the glycolytic enzyme induction that provokes a transition to glycolytic energy production. *J Biol Chem* **269**, 31484-31490.

Grimble RF & Grimble GK (1998) Immunonutrition: Role of sulfur amino acids, related amino acids, and polyamines. *Nutrition* **14**, 605-610.

Grimble RF (1998) Nutritional modulation of cytokine biology. *Nutrition* **14**, 634-640.

Grimble RF (2001) Symposium on 'evidence-based nutrition' - Nutritional modulation of immune function. *Proc Nutr Soc* **60**, 389-397.

Grimble RF (2006) The effects of sulfur amino acid intake on immune function in humans. *J Nutr* **136**, 1660S–1665S.

Guo X, Roberts MR, Becker SM, Podd B, Zhang Y, Chua SC Jr, Myers MG Jr, Duggal P, Houpt ER & Petri WA Jr (2011) Leptin signaling in intestinal epithelium mediates resistance to enteric infection by *Entamoeba histolytica*. *Mucosal Immunol* **4**, 294-303.

Halliday AM, Routledge CM, Smith SK, Matthews JB & Smith WD (2007) Parasite loss and inhibited development of *Teladorsagia circumcincta* in relation to the kinetics of the local IgA response in sheep. *Parasite Immunol* **29**, 425-434.

Harris N & Gause WC (2011) To B or not to B: B cells and the Th2-type immune response to helminths. *Trends Immunol* **32**, 80-88.

Hazzledine M (2008) Premier Atlas Ingredients Matrix. Rugeley: Premier Nutrition Products Ltd.

Heath GBS & Michel JF (1969) A contribution to the epidemiology of parasitic gastroenteritis in lambs. *Vet Rec* **85**, 305-308.

Herbert DR, Yang JQ, Hogan SP, Groschwitz K, Khodoun M, Munitz A, Orekov T, Perkins C, Wang Q, Brombacher F, Urban JF Jr, Rothenberg ME & Finkelman FD (2009) Intestinal epithelial cell secretion of RELM-beta protects against gastrointestinal worm infection. *J Exp Med* **206,** 2947-2957.

Holmes PH (1993) Interactions between parasites and animal nutrition: the veterinary consequences. *Proc Nutr Soc* **52**, 113-120.

Hornick JL, Van Eenaeme C, Gérard O, Dufrasne I & Istasse L (2000) Mechanisms of reduced and compensatory growth. *Domest Anim Endocrinol* **19**, 121-132.

Hoskin SO, Lobley GE, Coop RL & Jackson F (2002) The effect of cysteine and glutamine supplementation on sheep infected with *Trichostrongylus colubriformis*. *Proc the New Zealand Society Anim Pr* **62**, 72–76.

Hoste H & Chartier C (1993) Comparison of the effects on milk production of concurrent infection with *Haemonchus contortus* and *Trichostrongylus colubriformis* in high- and low-producing dairy goats. *Am J Vet Res* **54,** 1886-1893.

Houdijk JGM & Athanasiadou S (2003) Direct and indirect effects of host nutrition on ruminant gastrointestinal nematodes. In: Proceedings of the Fourth International Symposium on the Nutritionof Herbivores, Mérida, Mexico, pp. 213–236 [t' Mannetje L; Ramirez-Aviles L; Sandoval-Castro CA, Ku-Vera JC,editors], University of Yucatan press: Merida.

Houdijk JGM (2008) Influence of periparturient nutritional demand on resistance to parasites in livestock. *Parasite Immunol* **30**, 113-121.

Houdijk JGM, Jackson F & Kyriazakis I (2009) Nutritional sensitivity of resistance to *Trichostrongylus colubriformis* in lactating ewes. *Vet Parasitol* **160,** 258-266.

Houdijk JGM, Jackson F, Coop RL & Kyriazakis I (2006) Rapid improvement of immunity to *Teladorsagia circumcincta* is achieved through a reduction in the demand for protein in lactating ewes. *Int J Parasitol* **36**, 219-227.

Houdijk JGM, Jessop NS & Kyriazakis I (2001a) Nutrient partitioning between reproductive and immune functions in animals. *Proc Nutr Soc* **60**, 515-525.

Houdijk JGM, Jessop NS, Knox DP & Kyriazakis I (2003a) Breakdown of immunity to *Nippostrongylus brasiliensis* in lactating rats. *Br J Nutr* **90**, 809-814.

Houdijk JGM, Jessop NS, Knox DP & Kyriazakis I (2005a) Secondary infection of *Nippostrongylus brasiliensis* in lactating rats is sensitive to dietary protein content. *Br J Nutr* **93**, 493-499.

Houdijk JGM, Kyriazakis I, Coop RL & Jackson F (2001b) The expression of immunity to *Teladorsagia circumcincta* in ewes and its relationship to protein nutrition depend on body protein reserves. *Parasitology* **122**, 661-672.

Houdijk JGM, Kyriazakis I, Jackson F, Coop RL (2001c) The relationship between protein nutrition, reproductive effort and breakdown in immunity to *Teladorsagia circumcincta* in periparturient ewes. *Anim Sci* **72**, 595-606.

Houdijk JGM, Kyriazakis I, Jackson F, Huntley JF & Coop RL (2005b) Effects of protein supply and reproductive status on local and systemic immune responses to *Teladorsagia circumcincta* in sheep. *Vet Parasitol* **129**, 105-117.

Houdijk JGM, Kyriazakis I, Jackson F, Huntley JF & Coop RL (2000) Can an increased intake of metabolizable protein affect the periparturient relaxation in immunity against *Teladorsagia circumcincta* in sheep? *Vet Parasitol* **91**, 43-62.

Houdijk JGM, Kyriazakis I, Jackson F, Huntley JF & Coop RL (2003b) Is the allocation of metabolisable protein prioritised to milk production rather than to immune functions in *Teladorsagia circumcincta*-infected lactating ewes? *Int J Parasitol* **33**, 327-338.

Hristov AN, Etter RP, Ropp JK & Grandeen KL (2004) Effect of dietary crude protein level and degradability on ruminal fermentation and nitrogen utilization in lactating dairy cows. *J Anim Sci* **82**, 3219-3229.

Ihler F (2010) Anthelmintic resistance. An overview of the situation in the Nordic countries. *Acta Vet Scand* **52**, S1-S24.

Iikuni N, Lam QL, Lu L, Matarese G & La Cava A (2008) Leptin and Inflammation. *Curr Immunol Rev* **4**, 70-79.

Ing R, Su Z, Scott ME & Koski KG (2000) Suppressed T helper 2 immunity and prolonged survival of a nematode parasite in protein-malnourished mice. *Proc Natl Acad Sci USA* **90**, 7078-7083.

Jackson F & Miller J (2006) Alternative approaches to control-Quo vadit? *Vet Parasitol* **139**, 371-384.

Jarrett EEE, Jarrett WFH & Urquhart GM (1968) Quantitative studies on the kinetics of establishment and expulsion of intestinal nematode populations in susceptible and immune hosts. *Nippostrongylus brasiliensis* in the rat. *Parasitology* **58**, 625-639.
Jessop NS (1997) Protein metabolism during lactation. *Proc Nutr Soc* **56**, 169-175.

DeMan JM (1999) Principles of Food Chemistry, 3rd edition, pp.111-162 Aspen Publishers, Maryland.

Jones LA, Houdijk JGM, Knox DP & Kyriazakis I (2009) Immunomodulatory effects of dietary protein during *Nippostrongylus brasiliensis* re-infection in lactating rats. *Parasite Immunol* **31**, 412-421.

Jones LA, Houdijk JGM, Sakkas P, Bruce AD, Mitchell M, Knox DP & Kyriazakis I (2011) Dissecting the impact of protein versus energy host nutrition on the expression of immunity to gastrointestinal parasites during lactation. *Int J Parasitol* **41**, 711-719.

Kahn LP, Knox MR, Gray GD, Lea JM & Walkden-Brown SW (2003a) Enhancing immunity to nematode parasites in single-bearing Merino ewes through nutrition and genetic selection. *Vet Parasitol* **112**, 211–225.

Kahn LP, Knox MR, Walden-Brown SW & Lea JM (2003b) Regulation of the resistance to nematode parasites of single- and twin-bearing Merino ewes through nutrition and genetic selection. *Vet Parasitol* **114**, 15-31.

Kalesnikoff J & Galli SJ (2008) New developments in mast cell biology. *Nat Immunol* **9**, 1215-1223.

Kambara T, McFarlane RG, Abell TJ, McAnulty RW & Sykes AR (1993) The effect of age and dietary protein on immunity and resistance in lambs vaccinated with *Trichostrongylus colubriformis*. *Int J Parasitol* **23**, 471-476.

Kambara T & McFarlane RG (1996) Changes in T cell subpopulations of sheep due to age and dietary protein intake; Association with protective immunity to *Trichostrongylus colubriformis*. *Vet Immunol Immunopathol* **51**, 127-135.

Kaminsky R, Ducray P, Jung M, Clover R, Rufener L, Bouvier J, Schorderet Weber S, Wenger A, Wieland-Berghausen S, Goebel T, Gauvry N, Pautrat F, Skripsky T, Froelich O, Komoin-Oka C, Westlund B, Sluder A & Mäser P (2008) A new class of anthelmintics effective against drug-resistant nematodes. *Nature* **452**,176-180.

Kaplan RM (2004) Drug resistance in nematodes of veterinary importance: a status report. *Trends Parasitol* **20**, 477-481.

Karasuyama H, Wada T, Yoshikawa S & Obata K (2011) Emerging roles of basophils in protective immunity against parasites. *Trends Immunol* **32**, 125-130.

Kassai T (1982) Handbook of *Nippostrongylus brasiliensis* (nematode). Budapest: Commonwealth Agricultural Bureaux.

Keatinge R, Houdijk JGM, Jackson F & Kyriazakis I (2003) The effect of nutrition on nematode faecal egg output in lactating, organically managed ewes. In: Proceedings of the 119th Meeting of the British Society of Animal Science, p. 29.

Kenyon F, Greer AW, Coles GC, Cringoli G, Papadopoulos E, Cabaret J, Berrag B, Varady M, Van Wyk JA, Thomas E, Vercruysse J & Jackson F (2009) The role of targeted selective treatments in the development of refugia-based approaches to the control of gastrointestinal nematodes of small ruminants. *Vet Parasitol* **164,** 3-11.

Kepka-Lenhart D, Mistry SK, Wu G & Morris SM Jr (2000) Arginase I: a limiting factor for nitric oxide and polyamine synthesis by activated macrophages? *Am J Physiol Regul Integr Comp Physiol* **279**, R2237-R2242.

Ketzis JK, Vercruysse J, Stromberg BE, Larsen M, Athanasiadou S & Houdijk JGM (2006) Evaluation of efficacy expectations for novel and non-chemical helminth control strategies in ruminants. *Vet Parasitol* **139**, 321-335.

Keulen J & Young BA (1977) Evaluation of the acidinsoluble ash as a natural marker in ruminant digestibility studies. *J Anim Sci* **44**, 282-287.

Khan WI, Abe T, Ishikawa N, Nawa Y & Yoshimura K (1995) Reduced amount of intestinal mucus by treatment with anti-Cd4 antibody interferes with the spontaneous cure of *Nippostrongylus brasiliensis* infection in mice. *Parasite Immunol* **17**, 485-491.

Kidane A, Houdijk JGM, Tolkamp, Athanasiadou S & Kyriazakis I (2009) Consequences of infection pressure and protein nutrition on periparturient resistance to *Teladorsagia circumcincta* and performance in ewes. *Vet Parasitol* **165**, 78-87.

Kim SW & Easter RA (2001) Nutrient mobilization from body tissues as influenced by litter size in lactating sows. *Anim Sci* **79**, 2179-2186.

Kim SW, Mateo RD, Yin YL & Wu G (2007) Functional amino acids and fatty acids for enhancing production performance of sows and piglets. *Asian-Aust J Anim Sci* **20**, 295-306.

Kim SW, Osaka I, Hurley WL & Easter RA (1999) Mammary gland growth as influenced by litter size in lactating sows: Impact on lysine requirement. *Anim Sci* **77**, 3316-3321.

Kim YS & Ho SB (2010) Intestinal goblet cells and mucins in health and disease. *Curr Gastroenterol Rep* **12**, 319-330.

Kliewer RL & Rasmussen KM (1987) Malnutrition during the reproductive cycle effects on galactopoietic hormones and lactational performance in the rat. *Am J Clin Nutr* **46**, 926-935.

Knox M & Steel J (1996) Nutritional enhancement of parasite control in small ruminant production systems in developing countries of south-east Asia and the Pacific. *Int J Parasitol* **26**, 963-970.

Knox MR & Steel JW (1999) The effects of urea supplementation on production and parasitological responses of sheep infected with *Haemonchus contortus* and *Trichostrongylus colubriformis*. *Vet Parasitol* **83**, 123-135.

Knox MR, Torres-Acosta JFJ & Aguilar-Caballero AJ (2006) Exploiting the effect of dietary supplementation of small ruminants on resilience and resistance against gastrointestinal nematodes. *Vet Parasitol* **139**, 385-393.

Konashi S, Takahashi K & Akiba Y (2000) Effects of dietary essential amino acid deficiencies on immunological variables in broiler chickens. *Br J Nutr* **83**, 449-456.

Koski KG & Scott ME (2001) Gastrointestinal nematodes, nutrition and immunity: Breaking the negative spiral. *Annu Rev Nutr* **21**, 297-321.

Koski KG & Scott ME (2003) Gastrointestinal nematodes, trace elements, and immunity. *J Trace Elem Exp Med* **16**, 237-252.

Koski KG, Su Z & Scott ME (1999) Energy deficits suppress both systemic and gut immunity during infection. *Biochem Biophys Res Commun* **264**, 796-801.

Kristan DM & Hammond KA (2000) Combined effects of cold exposure and sub-lethal intestinal parasites on host morphology and physiology. *J Exp Biol* **203**, 3495-3504.

Kristan DM (2002) Effects of intestinal nematodes during lactation: consequences for host morphology, physiology and offsprings mass. *J Exp Biol* **205**, 3955-3965.

Kristan DM (2007) Chronic calorie restriction increases susceptibility of laboratory mice (*Mus musculus*) to a primary intestinal parasite infection. *Aging Cell* **6**, 817-825.

Kyriazakis I & Emmans GC (1992a) The effects of varying protein and energy intakes on the growth and body composition of pigs. 1. The effects of energy intake at constant, high protein intake. *Br J Nutr* **68**, 603-613.

Kyriazakis I & Emmans GC (1992b) The effects of varying protein and energy intakes on the growth and body composition of pigs. 2. The effects of varying both energy and protein intake. *Br J Nutr* **68**, 615-625.

Kyriazakis I & Houdijk J (2006) Immunonutrition: Nutritional control of parasites. *Small Rumin Res* **62**, 79-82.

Kyriazakis I (2010) Is anorexia during infection in animals affected by food composition? *Anim Feed Sci Technol* **156**, 1-9.

Kyriazakis I, Anderson DH, Coop RL & Jackson F (1996) The pathophysiology and development of immunity during long-term subclinical infection with

Trichostrongylus colubriformis of sheep receiving different nutritional treatments. *Vet Parasitol* **65**, 41-54.

Lawrence CE (2003) Is there a common mechanism of gastrointestinal nematode expulsion? *Parasite Immunol* **25**, 271-281.

Le Floc'h N, Melchior D & Obled C (2004) Modifications of protein and amino acid metabolism during inflammation and immune system activation. *Livest Prod Sci* **87**, 37-45.

Li P, Knabe DA, Kim SW, Lynch CJ, Hutson SM & Wu G (2009) Lactating porcine mammary tissue catabolizes branched-chain amino acids for glutamine and aspartate synthesis *J Nutr* **139**, 1502-1509.

Li P, Yin YL, Li DF, Kim SW & Wu G (2007) Amino acids and immune function. *Br J Nutr* **98**, 237-252.

Liu Q, Kreider T, Bowdridge S, Liu Z, Song Y, Gaydo AG, Urban JF Jr & Gause WC (2010) B cells have distinct roles in host protection against different nematode parasites. *J Immunol* **184**, 5213-5223.

Liu SM, Masters DG & Adams NR (2003) Potential impact of nematode parasitism on nutrient partitioning for wool production, growth and reproduction in sheep. *Aust J Exp Agr* **43**, 1409-1417.

Liu SM, Smith TL, Briegel J, Murray A, Masters DG, Karlsson LJE, Palmer DG, Greeff JC, Besier RB & Gao SB (2005) Comparing productive performance of nematode resistant Merino sheep with non-selected control. *Livest Prod Science* **97**, 117-129.

Liu SM, Smith TL, Briegel J, Gao SB & Peng WK (2007) Fractional protein synthesis rate and polyamine concentrations in tissues of Merino sheep selected for gastrointestinal nematode resistance. *Livest Sci* **106**, 65-75.

Lloyd S (1983) Effect of pregnancy and lactation upon infection. *Vet Immunol Immunopathol* **4**, 153-176.

Lobley GE, Connell A, Lomax MA, Brown DS, Ilne E, Calder AG & Farningham DAH (1995) Hepatic detoxification of ammonia in the ovine liver: possible consequences for amino acid catabolism. *Br J Nutr* **73,** 667-685.

López N, Sánchez J, Picó C, Palou A & Serra F (2010) Dietary l-leucine supplementation of lactating rats results in a tendency to increase lean/fat ratio associated to lower orexigenic neuropeptide expression in hypothalamus. *Peptides* **31**, 1361-1367

Lund P & Williamson DH (1985) Inter-tissue nitrogen fluxes. *Br Med Bull* **41**, 251-256.

Lunn PG, Northrop CA & Wainwright M (1988) Hypoalbuminemia in energy malnourished rats infected with *Nippostrongylus-Brasiliensis* (Nematoda). *J Nutr* **118**, 121-127.

Lynch CJ, Gern B, Lloyd C, Hutson SM, Eicher R & Vary TC (2006). Leucine in food mediates some of the postprandial rise in plasma leptin concentrations. *Am J Physiol Endocrinol Metab* **291**, E621–E630.

MacPherson A, Gray D, Mitchell GBB & Taylor CN (1987). Ostertagia infection and neutrophil function in cobalt-deficient and cobalt-supplemented cattle. *Br Vet J* **143**, 348-353.

Mahida YR (2003) Host-parasite interactions in rodent nematode infections. *J Helminthol* **77**, 125-131.

Maizels RM & Holland MJ (1998) Parasite immunity: Pathways for expelling intestinal helminths. *Curr Biol* **8**, R711-R714.

Maizels RM, Balic A, Gomez-Escobar N, Nair M, Taylor MD & Allen JE (2004) Helminth parasites-masters of regulation. *Immunol Rev* **201**, 89-116.

Maizels RM, Pearce EJ, Artis D, Yazdanbakhsh M & Wynn TA (2009) Regulation of pathogenesis and immunity in helminth infections. *J Exp Med* **206**, 2059-2066.

Marsland BJ, Kurrer M, Reissmann R, Harris NL & Kopf M (2008) *Nippostrongylus brasiliensis* infection leads to the development of emphysema associated with the induction of alternatively activated macrophages. *Eur J Immunol* **38,** 479-488.

Malmezat T, Breuille´ D, Pouyet C, Buffiere C, Denis P, Mirand PP & Obled C (2000) Methionine transulfuration is increased during sepsis in rats. *Am J Physiol* **279**, 1391-1397.

McClure SJ (2008) How minerals may influence the development and expression of immunity to endoparasites in livestock. *Parasite Immunol* **30**, 89-100.

McClure SJ, McClure TJ & Emery DL (1999) Effects of molybdenum intake on primary infection and subsequent challenge by the nematode parasite *Trichostrongylus colubriformis* in Merino lambs. *Res Vet Sci*; **67**, 17-22.

McNeill DM, Slepetis R, Ehrhardt RA, Smith DM & Bell AW (1997) Protein requirements of sheep in late pregnancy: partitioning of nitrogen between gravid uterus and maternal tissues *J Anim Sci* **75**, 809-816.

Mepham TB (1982) Amino acid utilization by lactating mammary gland. *J Dairy Sci* **65**, 287-298.

Mezl VA & Knox WE (1977) Metabolism of arginine in lactating rat mammary gland. *Biochem J* **166**, 105-113.

Michail S & Abernathy F (2004) A new model for studying eosinophil migration across cultured intestinal epithelial monolayers. *J Pediatric Gastroenterol Nutr* **39,** 56-63.

Mikolayunas-Sandrock C, Armentano LE, Thomas DL & Berger YM (2009) Effect of protein degradability on milk production of dairy ewes. *J Dairy Sci* **92,** 4507-4513.

Miller HRP (1980) Expulsion of *Nippostrongylus brasiliensis* from rats protected with serum. I. The efficacy of sera from singly and multiply infected donors related to time of administration and volume of serum injected. *Immunology* **40**, 325-334.

Miller HRP (1987) Gastrointestinal mucus, a medium for survival and for elimination of parasitic nematodes and protozoa. *Parasitology* **94**, S77-S100.

Miller HR, (1996) Mucosal mast cells and the allergic response against nematode parasites. *Vet Immunol Immunopathol* **54**, 331-336.

Min BR & Hart SP (2003) Tannins for suppression of internal parasites *J Anim Sci* **81,** E102–E109.

Mitre E & Nutman TB (2006) Basophils, basophilia and helminth infections. *Chem Immunol Allergy* **90**, 141-156.

Morag M, Popliker F & Yagil R (1975) Effect of litter size on milk yield in the rat. *Lab Anim* **9**, 43-47.

Murray DM & Slezacek O (1994) Effects of a 50-day period of slow live weight loss on the body composition of crossbred wethers. *Proc Aust Soc Anim Prod* **20**, 397.

Naomoto Y, Yamatsuji T, Shigemitsu K, Ban H, Nakajo T, Shirakawa Y, Motok T, Kobayashi M, Gunduz M & Tanaka N (2005) Rational role of amino acids in intestinal epithelial cells (Review). *Int J Mol Med* **16**, 201-204.

Nawa Y, Ishikawa N, Tsuchiya K, Horii Y, Abe T, Khan AI, Bing-Shi, Itoh H, Ide H & Uchiyama F (1994) Selective effector mechanisms for the expulsion of intestinal Helminths. *Parasite Immunol* **16**, 333-338.

Nkabyo YS, Gu LH, Jones DP & Ziegler TR (2006) Thiol/disulfide redox status is oxidized in plasma and small intestinal and colonic mucosa of rats with inadequate sulfur amino acid intake. *J Nutr* **136**, 1242-1248.

Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, Bucks C, Kane CM, Fallon PG, Pannell R, Jolin HE & McKenzie AN (2010) Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* **464**, 1367-1370.

Newton SE & Munn EA (1999) The development of vaccines against gastrointestinal nematode parasites, particularly *Haemonchus contortus*. *Parasitol Today* **15**, 116-122.

Nielsen TT, NL, Stein HH, Bellaver C & Easter RA (2002) The effect of litter size and day of lactation on amino acid uptake by the porcine mammary glands. *J Anim Sci* **80**, 2402-2411.

Normanton H, Houdijk J, Jessop NS, Knox DP & Kyriazakis I (2005) The effect of changes in nutrient demand on gastrointestinal parasitism in lactating rats. *Proc Br Soc Anim Sci* **12**.

Normanton H, Houdijk JGM, Jessop NS, Knox DP & Kyriazakis I (2007) The effects of changes in nutritional demand on gastrointestinal parasitism in lactating rats. *Br J Nutr* **97**, 104-110.

O'Sullivan BM & Donald AD (1973) Responses to infection with *Haemonchus contortus* and *Trichostrongylus colubriformis* in ewes of different reproductive status. *Int J Parasitol* **3**, 521-530.

O'Connor LJ, Walkden-Brown SW & Kahn LP (2006) Ecology of the free-living stages of major trichostrongylid parasites of sheep. *Vet Parasitol* **142**, 1-15.

Odiere MR, Koski KG, Weiler HA & Scott ME (2010) Concurrent nematode infection and pregnancy induce physiological responses that impair linear growth in the murine foetus. *Parasitology* **137**, 991-1002.

Oftedal OT (2000) Use of maternal reserves as a lactation strategy in large mammals. *Proc Nutr Soc* **59**, 99-106.

Ogilvie BM (1965) Role of adult worms in immunity of rats to *Nippostrongylus brasiliensis*. *Parasitology* **55**, 325-335.

Ohnmacht C & Voehringer D (2010) Basophils protect against reinfection with hookworms independently of mast cells and memory Th2 cells. *J Immunol* **184**, 344-350.

Ohnmacht C, Schwartz C, Panzer M, Schiedewitz I, Naumann R & Voehringer D (2010) Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. *Immunity* **33**, 364–374

Oldham JD & Friggens NC (1989) Sources of variability in lactational performance. *Proc Nutr Soc* **48**, 33-43.

Padigel UM, Stein L, Redding K, Lee JJ, Nolan TJ, Schad GA, Birnbaumer L & Abraham D (2007) Signalling through Galphai2 protein is required for recruitment of neutrophils for antibody-mediated elimination of larval *Strongyloides stercoralis* in mice. *J Leukoc Biol* **81**, 1120-1126.

Parkin J & Cohen B (2001) An overview of the immune system. *Lancet* **357**, 1777-1789.

Parkins JJ & Holmes PH (1989) Effects of gastrointestinal helminth parasites on ruminant nutrition. *Nutr Res Rev* **2**, 227-246.

Parmley KLS, Machado CR & McNamara JP (1996) Rates of lipid metabolism in adipose tissue of pigs adapt to lactational state and dietary energy restriction. *J Nutr* **126**, 1644-1656.

Passos MCF, Ramos CF & Moura EG (2000) Short and long term effects of malnutrition in rats during lactation on the body weight of offspring. *Nutr Res* **20**, 1603-1612.

Patel N, Kreider T, Urban JF & Gause WC (2009) Characterisation of effector mechanisms at the host:parasite interface during the immune response to tissue-dwelling intestinal nematode parasites. *Int J Parasitol* **39**, 13-21.

Pesce J, Kaviratne M, Ramalingam TR, Thompson RW, Urban Jr JF, Cheever AW, Young DA, Collins M, Grusby MJ & Wynn TA (2006) The IL-21 receptor augments Th2 effector function and alternative macrophage activation. *J Clin Invest* **116**, 2044-2055.

Peterson AL, Qureshi MA, Ferket PR & Fuller JC (1999) In vitro exposure with b-hydroxy-b-methylbutyrate enhances chicken macrophage growth and function. *Vet Immunol Immunopathol* **67**, 67-78.

Petkevicius S, Nansen P, Bach KKE & Skjoth F (1999) The effect of increasing levels of insoluble dietary fibre on the establishment and persistence of *Oesophagostomum dentatumin* pigs. *Parasite* **6**, 17-26.

Pilarczyk B, Doligalska MJ, Donskow-Schmelter K, Balicka-Ramisz A & Ramisz A (2008) Selenium supplementation enhances the protective response to *Toxocara canis* larvae in mice. *Parasite Immunol* **30**, 394-402.

Pine AP, Jessop NS & Oldham JD (1994) Maternal protein reserves and their influence on lactational performance in rats. *Br J Nutr* **71**, 13-27.

Poppi DP, MacRae JC, Brewer A & Coop RL (1986) Nitrogen transactions in the digestive tract of lambs exposed to the intestinal parasite *Trichostrongylus colubriformis*. *Br J Nutr* **55**, 593-602.

Prentice A (2003) Micronutrients and the bone mineral content of the mother, fetus and newborn. *J Nutr* **133**, 1693S-1699S.

O'Quinn PR, Knabe DA & Wu G (2002) Arginine catabolism in lactating porcine mammary tissue. *J Anim Sci* **80**, 467-474.

Ramos Morales E, de la Torre Adarve G, Molina Alcaide E & Sanz Sampelayo MR (2010) Nitrogen and energy utilization in lactating dairy goats fed diets with different legume seeds *J Anim Physiol Anim Nutr (Berl)*. **94,** 659-664.

Riedijk MA, Stoll B, Chacko S, Schierbeek H, Sunehag AL, van Goudoever JB & Burrin DG (2007) Methionine transmethylation and transsulfuration in the piglet gastrointestinal tract. *Proc Natl Acad Sci USA* **104**, 3408-3413.

Roberts HC, Hardie LJ, Chappell LH & Mercer JG (1999) Parasite-induced anorexia: leptin, insulin and corticosterone responses to infection with the nematode, *Nippostrongylus brasiliensis*. *Parasitology* **118,** 117-123.

Roberts LS & Janovy J (2000) In Foundations of Parasitology [Schmidt GD & Roberts LS, editors] 6th ed. McGraw -Hill International Editions.

Romjali E, Dorny P, Batubara A, Pandey VS & Gatenby RM (1997) Periparturient rise in faecal strongyle egg counts of different genotypes of sheep in North Sumatra, Indonesia. *Vet Parasitol* **68,** 191-196.

Rothenberg ME & Hogan SP (2006) The eosinophil. *Annu Rev Immunol* **24**, 147-174.

Rothwell TL (1989) Immune expulsion of parasitic nematodes from the alimentary tract. *Int J Parasitol* **19**, 139-168.

Russel A (1984) Body condition scoring of sheep. *Parasitology* **6**, 91–93.

Sakkas P, Houdijk JGM, Athanasiadou S & Kyriazakis I (2010) Use of European grown protein sources to control gastrointestinal parasitism in small ruminants. *61st Annual meeting of the European Association of animal production.* 23-27 August, Heraklion, Greece, **16**, pp199

Sakkas P, Houdijk JGM, Jones A, Knox DP & Kyriazakis I (2011) Dietary protein and energy supplies differentially affect resistance to parasites in lactating mammals. *Br J Nutr* (in press) doi:10.1017/S0007114511001565.

Scott I, Khalaf S, Simcock DC, Knight CG, Reynolds GW, Pomroy WE & Simpson HV (2000) A sequential study of the pathology associated with the infection of sheep with adult and larval *Ostertagia circumcincta*. *Vet Parasitol* **89**, 79-94.

Shaw KL, Nolan JV, Lyneh JJ, Coverdale OR, Gill HS (1995) Effect of weaning, supplementation and gender on acquired immunity to *Haemonchus contortus* in lambs. Int J Parasitol **25**, 381-387.

Shea-Donohue T & Urban JF (2004) Gastrointestinal parasite and host interactions. *Curr Opin Gastroenterol* **20**, 3-9.

Sheibak VM, Kravchuk RI, Matsyuk YR & Goretskaya MV (2007) Morphological changes in the liver, thymus, spleen, and small intestine of animals after leucine treatment. *Bull Exp Biol Med* **143**, 268-271.

Shi HN, Koski KG, Stevenson MM & Scott ME (1997) Zinc deficiency and energy restriction modify immune responses in mice during both primary and challenge infection with *Heligmosomoides polygyrus* (Nematoda). *Parasite Immunol* **19,** 363-373.

Simpson HV (2000) Pathophysiology of abomasal parasitism: is the host or parasite responsible? *Vet J* **160**, 177-191.

Smith WD & Zarlenga DS (2006) Developments and hurdles in generating vaccines for controlling helminth parasites of grazing ruminants. *Vet Parasitol* **139,** 347-459.

Stear MJ, Bairden K, Mckeller QA, Scott I, Strain S & Bishop SC (1999) The relationship between the number and size of nematodes in the abomasum and the concentration of pepsinogen in ovine plasma. *Res Vet Sci* **67**, 89-92.

Stear MJ, Boag B, Cattadori I & Murphy L (2009) Genetic variation in resistance to mixed, predominantly *Teladorsagia circumcincta* nematode infections of sheep: from heritabilities to gene identification. *Parasite Immunol* **31,** 274-282.

Stear MJ, Doligalska M & Donskow-Schmelter K (2007) Alternatives to anthelmintics for the control of nematodes in livestock. *Parasitology* **134**, 139-51.

Stear MJ, Strain S & Bishop SC (1999) How lambs control infection with *Ostertagia circumcincta*. *Vet Immunol Immunopathol* **72**, 213-218.

Stepek G, Lowe AE, Buttle DJ, Duce IR & Behnke JM (2007) The anthelmintic efficacy of plant-derived cysteine proteinases against the rodent gastrointestinal nematode, *Heligmosomoides polygyrus*, in vivo. *Parasitology* **134**, 1409-1419.

Stipanuk MH (2004) Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. *Annu Rev Nutr* **24**, 539-577.

Stoll B, Henry J, Reeds PJ, Yu H, Jahoor F & Burrin DG (1998) Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. *J Nutr* **128**, 606-614.

Strain SAJ & Stear MJ (2001) The influence of protein supplementation on the immune response to *Haemonchus contortus*. *Parasite Immunol* **23**, 527-531.

Sukhotnik I, Mogilner J, Krausz MM, Lurie M, Hirsh M, Coran AG & Shiloni E (2004) Oral arginine reduces gut mucosal injury caused by lipolysaccharide end toxemia in rat. *J Surg Res* **122**, 256-262

Suttle NF, Knox KW, Angus KW, Jackson F & Coop RL (1992) Effects of dietary molybdenum on nematode and host during *Haemonchus contortus* infection in lambs. *Res Vet Sci*; **52**, 230-235.

Sykes AR & Kyriazakis I (2007) Opportunities to control herbivore nematodes through manipulation of the grazing environment. In Herbivore Nutrition for the Development of Efficient, Safe and Sustainable Livestock Production, pp. 329–353 [QX Ming, LP Ren and ZJ Gao, editors]. Beijing: China Agricultural University Press.

Sykes AR (1994) Parasitism and production in farm animals. *Anim Prod* **59**, 155-172.

Sykes AR (2008) Manipulating host immunity to improve nematode parasite control--Quo vadit. *Parasite Immunol* **30**, 71-77.

Tesseraud S, Coustard SM, Collin A & Seiliez I (2009) Role of sulfur amino acids in controlling nutrient metabolism and cell functions: implications for nutrition *Br J Nutr* **101**, 1132-1139.

Thomas PC, Robertson S, Chamberlain DG, Levingstone RM, Garthwaite PH, Dewey PJS, Smart R & Whyte C (1988) Predicting the metabolizable energy (ME) content of compound feeds for ruminants. In Recent Advances in Animal Nutrition, pp. 127–146 [W Haresign and DJA Cole, editors]. London: Butterworths.

Trommenschlager JM, Thénard V, Faurie´ F & Dupont D (2003) Effets de différentes sources de complémentation azotée sur les performances de vaches laitières Holstein et Montbéliardes et les aptitudes à la coagulation des laits. *Renc Rech Ruminants* **10**, 382.

Trottier NL, Shipley CF, & Easter RA (1997) Plasma amino acid uptake by the mammary gland of the lactating sow. *J Anim Sci* **75**, 1266-1278.

Tsubokawa D, Nakamura T, Goso Y, Takano Y, Kurihara M & Ishihara K (2009) *Nippostrongylus brasiliensis*: increase of sialomucins reacting with anti-mucin monoclonal antibody HCM31 in rat small intestinal mucosa with primary infection and reinfection. *Exp Parasitol* **123**, 319-325.

Tu T, Koski KG, Wykes LJ & Scott ME (2007) Re-feeding rapidly restores protection against *Heligmosomoides bakeri* (Nematoda) in protein-deficient mice. *Parasitology* **134**, 899-909.

Tu T, Scott ME, Sabally K & Koski KG (2009) Tissue mineral distributions are differentially modified by dietary protein deficiency and a murine nematode infection. *Biol Trace Elem Res* **127**, 234-244.

Urquhart GM, Armour J, Duncan JL, Dunn JL & Jennings FW (1996) Veterinary Parasitology, 2nd edition) ed.: Blackwell Science.

Vagenas D, Bishop SC & Kyriazakis I (2007) A model to account for the consequences of host nutrition on the outcome of gastrointestinal parasitism in sheep: model evaluation. *Parasitology* **134**, 1279-1289.

Van Houtert M & Sykes AR (1996) Implications of nutrition for the ability of ruminants to withstand gastrointestinal nematode infections. *Int J Parasitol* **26**, 1151-1167.

Van Houtert MFJ, Barger IA & Steel JW (1995a) Dietary protein for young grazing sheep: interactions with gastrointestinal parasitism. *Vet Parasitol* **60**, 283-295.

Van Houtert MFJ, Barger IA, Steel JW, Windon RG & Emery DL (1995b) Effects of dietary protein intake on responses of young sheep to infection with *Trichostrongylus colubriformis*. *Vet Parasitol* **56**, 163-180.

Van Klinken BJ, Einerhand AW, Buller HA & Dekker J (1998) Strategic biochemical analysis of mucins. *Anal Biochem* **265**, 103-116.

Vina JR & Williamson DH (1981) Effects of lactation on L-leucine metabolism in the rat. *Biochem J* **194,** 941-947.

VSN International Ltd (2008) GenStat Release 11.1 (PC/Windows). Hemel Hempstead, UK: VSN International.

Wakelin D (1996) Immunity to parasites: How parasitic infections are controlled, 2 ed.: Cambridge.

Wallace DS, Bairden K, Duncan JL, Eckersall PD, Fishwick G, Gill M, Holmes PH, McKellar QA, Murray M, Parkins JJ & Stear MJ (1998) The influence of dietary supplementation with urea on resilience and resistance to infection with *Haemonchus contortus*. *Parasitology* **116**, 67-72.

Waller PJ (1997) Nematode parasite control of livestock in the tropics/subtropics: the need for novel approaches. *Int J Parasitol* **27**, 1193-1201.

Wang JJ, Chen LX, Li P, Li XL, Zhou HJ, Wang FL, Li DF, Yin YL & Wu G (2008) Gene expression is altered in piglet small intestine by weaning and dietary glutamine supplementation. *J Nutr* **138,** 1025-1032.

Wang WW, Qiao SY & Li DF (2009) Amino acids and gut function. *Amino Acids* **37**, 105-110.

Wei LH, Wu G, Morris SM Jr & Ignarro LJ (2001) Elevated arginase I expression in rat aortic smooth muscle cells increases cell proliferation. *Proc Natl Acad Sci USA* , 9260-9264.

Willis C & Poulin R (1999) Effects of the tapeworm *Hymenolepis diminuta* on maternal investments in rats. *Can J Zool* **77**, 1001-1005.

Woodward B & Miller RG (1991) Depression of thymus-dependent immunity in wasting protein-energy malnutrition does not depend on an altered ratio of helper (CD4+) to suppressor (CD8+) T cells or on a disproportionately large atrophy of the T-cell relative to the B-cell pool. *Am J Clin Nutr* **53**, 1329-1335.

Wu G (2009) Amino acids: metabolism, functions, and nutrition. *Amino Acids* **37**, 1-17.

Wu G, Bazer FW, Davis TA, Kim SW, Li P, Marc Rhoads J, Carey Satterfield M, Smith SB, Spencer TE & Yin Y (2009) Arginine metabolism and nutrition in growth, health and disease. *Amino Acids* **37**, 153-168.

Wu G (1998) Intestinal mucosal amino acid catabolism. *J Nutr* **128**, 1249-1252.

Wu GY, Fang YZ, Yang S, Lupton JR & Turner ND (2004) Glutathione metabolism and its implications for health. *J Nutr* **134**, 489-492.

Wu GY, Flynn NE, Flynn SP, Jolly CA & Davis PK (1999) Dietary protein or arginine deficiency impairs constitutive and inducible nitric oxide synthesis by young rats. *J Nutr* **129**, 1347-1354.

Xie HL, Stankiewicz M, Huntley JF, Sedcole JR, McAnulty RW, Green RS & Sykes AR (2004) The effects of cold exposure, food allowance and litter size on immunity of periparturient sheep to *Teladorsagia circumcincta* and *Trichostrongylus colubriformis*. *Anim Sci* **78**, 149-158.

Yamauchi J, Kawai Y, Yamada M, Uchikawa R, Tegoshi T & Arizono N (2006) Altered expression of goblet cell- and mucin glycosylation-related genes in the intestinal epithelium during infection with the nematode *Nippostrongylus brasiliensis* in rat. *APMIS* **114,** 70-78**.**

Yarandi SS, Hebbar G, Sauer CG, Cole CR & Ziegler TR (2011) Diverse roles of leptin in the gastrointestinal tract: modulation of motility, absorption, growth, and inflammation. *Nutrition* **27**, 269-275.

Yu F, Bruce LA, Calder AG, Milne E, Coop RL, Jackson F, Horgan GW & MacRae JC (2000) Subclinical infection with the nematode *Trichostrongylus colubriformis* increases gastrointestinal tract leucine metabolism and reduces availability of leucine for other tissues. *J Anim Sci* **78**, 380-390.

Zaralis K, Tolkamp BJ, Houdijk JGM, Wylie ARG & Kyriazakis I (2009) Consequences of protein supplementation for anorexia, expression of immunity and plasma leptin concentrations in parasitized ewes of two breeds. *Br J Nutr* **101**, 499-509.

Zhao A, Urban JF Jr, Anthony RM, Sun R, Stiltz J, van Rooijen N, Wynn TA, Gause WC & Shea-Donohue T (2008) Th2 cytokine-induced alterations in intestinal smooth

muscle function depend on alternatively activated macrophages. *Gastroenterology* **135**, 217-225.