

**Does Selection for Production
Traits Affect the Ability to Cope
with Pathogens?**

by

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Abstract

Phenotypic selection for production traits causes changes in the underlying genetics of the animal. As such, intensive selection on one trait may have consequences on other traits. Indeed alterations to traits seemingly unrelated to the desirable trait under selection have been documented, although the strength and direction have been inconsistent in livestock species. This leads to the question of how selection for growth may alter the ability to cope with pathogens, and whether because of its associated increase in nutrient requirements, improved nutrition could ameliorate any loss of resilience or resistance arising from this selection (Chapter One). This thesis uses a unique mouse line, divergently selected for low (Roslin low: ROL) and high (Roslin high: ROH) body weight and a chronic gastrointestinal nematode infection, *Heligmosomoides bakeri*, to address this question.

Chapter 2 investigates the effects of dietary crude protein contents ranging from scarce to more than adequate on resilience and resistance traits of uninfected and primary infected ROH and ROL mice, using a fixed level of 250L₃ as infection pressure. The data suggest that ROH mice had a greater penalty of infection on resilience, which was overcome by increased protein nutrition, and showed higher worm burdens and egg counts. Chapter 3 goes on to investigate the existence of a minimum parasite dose for the observation of loss of resilience and resistance during infection. Over a range of primary infection pressure from 0 to 250L₃, it was found that an incoming parasite dose of 150L₃ and over was required to reduce weight gain in ROH mice fed a low protein diet and that this loss in weight gain was ameliorated by increased protein nutrition. Resilience of ROL mice was not affected. It was also observed that worm burdens and egg counts of all mice reached a plateau at 150L₃. Samples were taken for cytokine and chemokine analysis (Chapter 4) and data showed that the parasite infection did not polarise a Th2 type immune response as

expected, whilst infection in ROL mice and ROH mice on low protein diets resulted in inflammatory immune response. Chapter 5 compares primary and secondary infection in ROH and ROL mice, finding that not only do ROH reduce weight gain in response to a primary and secondary infection during protein scarcity, they also show the greatest reduction in worm burdens and egg counts due to previous exposure.

The data from this thesis, discussed in Chapter 6, suggests that intensive selection for high body weight can cause a loss of resilience that is sensitive to protein nutrition but that this may be due to a prioritisation of immunity over growth. Intensive selection for low body weight can cause a greater degree of resilience but cause a reduction in resistance to a pathogen challenge. Whilst this thesis therefore provides evidence that intensive selection, in either direction, can alter an animal's ability to cope with a pathogen challenge, future work using a non-selected control line is required to advance this hypothesis.

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

Jennifer Coltherd

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Publications

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Chapter 1. General Introduction

Phenotypic selection of livestock for improved production traits causes the co-selection of a generally unknown underlying genetic architecture (Dekkers & Hospital, 2002). As a consequence selection for desired traits can have positive but also detrimental effects on other traits (Williams, 2005). For example, long-term selection for high growth in mice has produced large mice with changes in body composition, seemingly shorter lifespan and reduced overall fertility (Bünger et al., 2001b). This leads to questions of which other traits may also be changed or compromised especially if the ability of the host to cope with pathogens is influenced. Disease incidences and metabolic disorders in livestock have increased over the past 50 years in tandem with increased selection for improved production and longevity (Reviewed by Aggrey, 2010)

The genetic mechanism behind the change in correlated traits could be through pleiotropy and/or genetic linkage and the consequences would be seen under all environmental conditions (Rauw et al., 1998). However, an alternative mechanism to account for an observed loss in expression of traits in limiting environments could arise from the allocation of scarce resources primarily towards the selected trait leaving fewer resources for other functions such as immunity (Beilharz et al., 1993; Beilharz, 1998a; 1998b; Glazier, 2002). Improved protein nutrition has been shown to enhance immunity to parasites at times of increased nutrient demand, for instance as a consequence of lactation (reviewed in Houdijk et al., 2001a). As the immune system is highly proteinaceous in nature, it follows that protein malnutrition could have a severe impact on an individuals' ability to cope with a pathogen challenge, i.e. mount an immune response. Selection for high growth can also be expected to increase nutrient demand and thus reduce disease resistance, however results in farm

animals have been inconsistent for the strength and direction of the genetic correlation between growth and resistance (Broughan and Wall, 2007).

Studies in livestock species involving interactions between growth and immunity to gastrointestinal (GI) nematodes are limited by the shortage of truly comparable breeds, as breeds have been selected from different founder populations and also selected for different and changing breeding goals. Here, I have been able to account for this through the use of a unique mouse model involving two lines derived from the same base population (implying an identical initial genetic makeup), divergently selected solely for high and low body weight at 42 days of age for more than 20 generations under similar environmental conditions. I have used these lines, which will be described below, to further elucidate interactions between selection for growth traits and immunity, by asking the question: does selection for growth potential affect the ability to cope with parasite infections.

The following literature review will discuss the observed phenotypic and genetic correlations between growth and disease resistance traits and the possible mechanisms behind this observation, i.e. genetic correlations and allocation of scarce resources. The allocation of scarce resources, with respect to the implications for nutritional control of parasites and immunonutrition, will then be discussed in detail. After these aspects have been considered, the last sections of the literature review will present my PhD objectives in context with existing published knowledge.

1.1 Phenotypic and genetic correlations between growth and resistance

The selection for desired traits based purely on phenotypic observation has successfully established a diverse range of breeds in different species displaying the

selected traits and being adapted for different environments (Andersson, 2001). However, phenotypic selection focused on production traits only (i.e. a narrow breeding goal) can have a detrimental effect on fitness traits, such as fertility and resistance to disease in livestock species, presumably from creating an imbalance between the genetic gains in the selected traits and the overall fitness traits (Williams, 2005). Rauw et al (1998) reviewed literature on poultry, pigs and dairy cattle, and concluded that selection for high production in dairy cattle and poultry (for milk and meat respectively) has led to an increase in disease incidences and decreased adaptive immune responses. In support, more recent studies also show selection for high production in dairy herds can decrease fertility and health traits (Oltenucu and Algers, 2005) while selection for lean carcass traits in pigs can reduce immunity which is not seen in 'fat' porcine breeds (Galina-Pantoja et al., 2006).

In studies concerning body weight, Bayyari et al (1997) found that turkeys selected for increased body weight and egg production showed a decrease in primary antibody responses when compared to random bred lines, whilst they also suggest that conversely selection for increased adaptive immune traits can produce decreases in body weight. Parmentier et al (1996) and Siwek et al (2004) also found that selection for increased antibody responses towards sheep red blood cells in poultry resulted in a reduction in body weight when compared with low antibody response lines.

The observation of selection for one trait producing changes in other traits is due to underlying genetic correlations and may result in scarce resources for other traits, which can have substantial effects on the animals, especially in constrained environments. The genetic correlation could arise from genetic linkage, created by

loci controlling different traits lying close together on a chromosome, or through pleiotropy which involves a single gene controlling multiple traits (Rauw et al., 1998). If the genetic correlation is caused by genetic linkage there are opportunities to use recombinant animals to break this correlation over time. However if the effects are pleiotropic then the chances of overcoming the genetic correlation by selection are minimal.

1.2 Possible explanation for selection of one trait causing changes in other traits

1.2.1 Genetic correlations between growth and resistance traits

Research on genetic correlations between growth and resistance (low faecal egg count, FEC) have shown little consensus in livestock species (reviewed in Bisset et al., 2001). This section will detail unfavourable correlations (e.g. an increase in body weight leads to a decrease in resistance) followed by favourable or neutral correlations (e.g. an increase in body weight has no effect on resistance or an increase in resistance).

In New Zealand and Australia, breeders have successfully bred sheep that show reduced FEC in response to GI nematode infection proving that resistance has a heritable genetic component (Douch et al., 1995). Unfavourable correlations between growth and resistance have been observed in naturally infected sheep breeds in New Zealand studies (Eady et al., 1998; Bisset et al., 2001; Morris et al., 2005). Studies involving Romney and Perendale sheep breeds selected for either 100 day live weight or low FEC respectively, saw a reduction in resistance (higher FEC) or in body weight and weight gain, respectively, when exposed to natural infection conditions and compared to control lines (McEwan et al., 1992; Morris et al., 2005).

Eady et al (1998) used Merino sheep selected for reduced FEC in Australia under artificial infection with *Haemonchus contortus*, an economically important GI parasite. These studies have reported favourable correlations or correlations close to zero between growth and immunity (Eady et al., 1994; 1998). Bishop et al (1996) and Bouix et al (1998) performed studies in Europe (Scotland and Poland respectively). The breeds used in these studies had been initially bred for production traits and then selected for high and low FEC, the genetic correlation between growth and resistance was found to be strongly favourable but with a small phenotypic correlation which may indicate a large environmental effect.

The inconsistent correlations observed between growth and resistance traits may be due to the diverse range of breeding goals and environmental conditions the breeds used have been selected for (Eady et al., 1998). Eady et al (1998) also suggest that a more intensive selection pressure applied to traits may produce a partitioning of resources towards the selected trait to the detriment of other traits, such as immunity. The allocation of scarce resources can be seen as a consequence of the selection of traits and the mechanism by which the phenotypic outcome of selection may differ between breeds and between environments.

1.2.2 Allocation of scarce resources: nutrient partitioning

Selection for production traits diverts the allocation of environmental resources away from the 'optimal' pattern achieved through natural selection. One of the nutrition allocation theories states that an animal will aim to obtain maximal fitness in its particular environment and any deviation away from the 'optimum' will reduce overall fitness at times of environmental resource scarcity (Beilharz et al., 1993; Beilharz, 1998a; 1998b; Glazier, 2002). This reduction in fitness is illustrated by, for

example, American Holstein dairy cattle having fertility difficulties and lower production when reared in Australia and New Zealand, where nutrition is poorer, compared to when reared in the USA where very high levels of concentrate are used (Beilharz, 1998b). This example shows that phenotypic traits can be limited by environmental resources and, as a consequence of metabolic disturbance (i.e. a disturbance of the normal integrated pathways of the metabolism of ingested resources), production diseases are observed in resource limiting environments (Beilharz, 1998a).

Coop and Kyriazakis (1999) formulated a nutrient partitioning framework, suggesting that allocation of scarce resources to maintaining body protein and reproduction (i.e. functions enabling survival and passing on of genes) is prioritised over immune function (see Table 1.1).

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 and Kyriazakis, 1999)

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

This framework suggests that during periods of increased nutrient demand, such as lactation, allocation of nutrients will be prioritised towards maintenance of body protein and reproduction which in turn could aid in the explanation for the observation of periparturient relaxation of immunity (Coop and Kyriazakis, 1999; 2001). From this framework it would then follow that increasing the available

protein (often considered to be the first limiting nutrient) should counteract this effect, and has been shown to be the case in protein supplemented periparturient ewes and lactating rats and mice (Houdijk *et al.*, 2004; Houdijk *et al.*, 2005; Odier *et al.*, 2010, respectively). Under this same partitioning framework growing animals are thought to prioritise scarce nutrients to maintenance of body protein and protein gain over expression of immune function during pathogen challenge. However, in the case of a naïve animal, scarce nutrient allocation to protein gain takes a lower priority than acquiring immunity (Coop and Kyriazakis, 1999). In studies involving poultry diseases (mainly viral and bacterial pathogens) it is suggested that the immune system is prioritised during resource allocation, over productivity (Koutsos and Klasing, 2005). The cellular immune response creates the most severe pathology for the host due to the nature of the pathogen (intracellular) and the response required to effectively eliminate the infection (Klasing, 2004). It may be that the severity of the invading pathogen causes the immune system to be prioritised to avoid the death of the individual, where chances of mortality are high without immune intervention.

Nutrient partitioning suggests that enabling more resources to be available for the animal will aid in overcoming observed losses in traits. Research in lactating ewes, rats and mice, with regard to the periparturient breakdown of immunity to GI nematodes, supports the nutrient partitioning framework proposed by Coop and Kyriazakis (1999) and shows that protein supplementation can overcome this loss of immunity (Houdijk *et al.*, 2004; Houdijk *et al.*, 2005; Odier *et al.*, 2010). Improved nutrition could become part of a non-chemical multi-faceted approach to reduce the use of anthelmintics in parasite control and slow down rate of parasitic resistance to anthelmintics (Houdijk and Athanasiadou, 2003; Kyriazakis and Houdijk, 2006; Torres-Acosta & Hoste, 2008).

1.3 Nutrient partitioning and implications for immunity to parasites

1.3.1 Nutritional control of resistance to parasites

The economic costs of parasite infection through increased anthelmintic use and reduced productivity are of major concern to the livestock industry (Koski and Scott, 2001; Knox *et al.*, 2006; Odoi *et al.*, 2008). The use of improved nutrition is receiving increased attention as a possible alternative method of parasite control due to the increasing presence of anthelmintic resistance in parasite populations (Jackson and Miller, 2006; Torres-Acosta & Hoste, 2008).

Anorexia, a reduction in voluntary intake, is a common characteristic of gastrointestinal nematode infection (Kyriazakis *et al.*, 1998) and is thought to cause nutrient scarcity due to reduced intake (Colditz, 2008). This reduction in food intake has been shown to be due to the host immune response (Adamo *et al.*, 2010) and to convey a protective function; Murray and Murray (1979) observed increased mortality in mice due to *Listeria monocytogenes* infection when force-fed so that their food intake was that of *ad libitum* fed uninfected mice. This provides the strong suggestion that improving the nutritional quality of feed rather than aiming to achieve higher intakes will improve the nutritional resources available to an anorexic infected animal, as reduction in voluntary food intake may occur in any nutritional environment (Kyriazakis, 2010).

A re-feeding experiment using mice showed that, during a primary or challenge infection with *Heligmosomoides bakeri* (= *Heligmosomoides polygyrus bakeri*, *Nematospiroides dubius* (see Cable *et al.*, 2006)), switching food types from protein deficient (30g/kg) to protein sufficient (240g/kg) rapidly restored body weights and reduced worm burdens and faecal egg counts to that of individuals that had been on

the protein sufficient diet for the entire study (Tu et al., 2007). Similar observations have been seen in livestock studies (reviewed by Koski and Scott, 2001). Bown *et al* (1991) found that naïve growing sheep that received an infusion of casein were able to maintain normal weight gain and reduce their worm burden and FEC by as much as 50% when compared to un-supplemented or glucose-supplemented controls. In lactating ewe studies increased metabolisable protein (MP), either through supplementation with dietary protein (Houdijk et al., 2004), or through a reduction in reproductive effort (i.e. removal of one twin lamb) caused significant decrease in FEC and worm burdens within a week, as much as 50% in the lamb removal study (Houdijk et al., 2006). Lactating rats also show increased worm burdens and number of eggs in colon when fed low protein (100 g/kg) compared to medium and high protein diets (200 and 300 g/kg respectively) (Houdijk *et al.*, 2005).

During growth, and especially under selection for high growth, it is expected that an increase in nutrient requirement occurs when individuals are compared to unselected counterparts. This is supported by an enhanced development of immunity observed in growing animals fed high protein diets compared to their low protein counterparts (Koski and Scott, 2001; Houdijk and Athanasiadou, 2003). The hosts' protein reserves can act as a source of metabolizable protein during protein scarcity and as such show that the nutritional status of the host prior to exposure to pathogens is important to consider when protein scarcity is to be induced (Pine et al., 1994; Houdijk et al., 2001b; Houdijk and Athanasiadou, 2003). In further support of the importance of body protein reserves, supplementation of animals post-weaning with protein has been shown to confer an increased ability to control parasite infection in later life (Koski and Scott, 2001).

The role for adequate protein nutrition has been observed in experimental studies using GI nematode infections in livestock species and rodents (Kyriazakis and Houdijk, 2006; Tu et al., 2007). The observed increase in resistance during protein supplementation is most likely due to enhanced immune responses, the characteristic immune response produced during a GI nematode infection and the effects of protein scarcity will be discussed in the following section.

1.3.2 Immune responses during GI nematode infection: effects of protein scarcity

GI nematode infections induce a highly polarized T helper subset 2 (Th2) protective immune response across host species (Garside et al., 2000; Rzepecka et al., 2006; Saunders et al., 2007; Terefe et al., 2007). The Th2 response is characterised by distinct patterns of cytokines (see Figure 1.1) which cross-regulate the opposing subset (Th1) and upregulate proliferation of naïve T cells to their own subset (Abbas et al., 1996). Cytokines (messenger protein) and chemokines (chemotactic cytokines that mobilise cells) are proteins that affect every leukocyte and immune cell in the body via interactions with receptors on these cells (Luster, 1998; Jankovic *et al.*, 2001; Karupiah, 2003). The effector functions of the Th2 response involve the stimulation of B cells by Interleukin-4 (IL-4) and IL-13 to produce Immunoglobulin G1 (IgG1) and mast cell degranulation (through involvement of IgE), while IL-5 stimulates the activation and differentiation of eosinophils (Abbas et al., 1996). More recently, the discovery of a new proinflammatory subset, Th17, has enabled further elucidation of the mechanisms behind some autoimmune diseases that were previously not sufficiently explained by the presence of the Th1 phenotype (Korn *et al.*, 2009). A subset of lymphocytes called regulatory T (Treg) cells have the ability

to dampen the effects of Th1, Th2 and Th17 phenotypes and inhibit autoimmune tissue injury (Bettelli *et al.*, 2006; Oldenhove *et al.*, 2009).

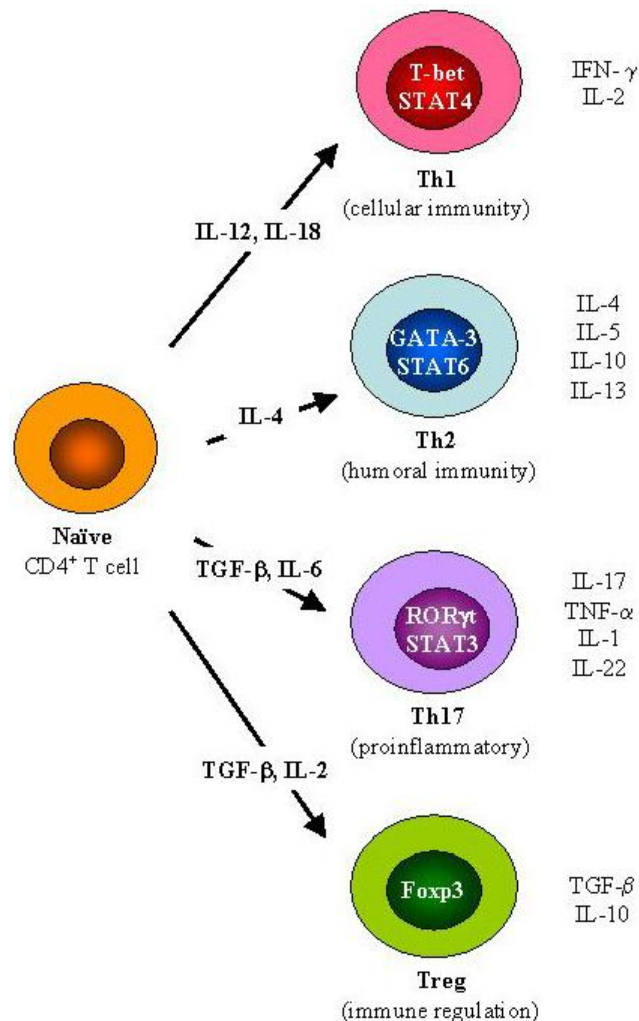


Figure 1.1 – Specific cytokines released by Th1, Th2, Th17 and Treg lymphocytes. Cytokines in the arrows are required to be present for naïve CD4⁺ T cells to differentiate into the T-helper cell subset.

Specific transcription factors involved in the differentiation of T-helper subsets are found inside the “cell”. (Diagram from La Cava, 2009)

Abbreviations used – Interleukin (IL), Interferon – γ (IFN- γ), Tumour-Necrosis factor – α (TNF- α), Tumour Growth Factor – β (TGF- β)

Rodent GI nematode infections are the most immunologically defined and are used as models to try and better understand the underlying immune mechanisms of both human and livestock infections (Wakelin, 1996; Shea-Donohue and Urban, 2004). The species of nematode used most frequently in model systems are *Strongyloides ratti* and *Trichuris muris*, which are related to species found in man and livestock. Species found only in rodents, such as *Nippostrongylus brasiliensis* and *H. bakeri* are

also used as they approximate to important economical or clinical species (Wakelin, 1996). Each of these parasitic infections creates a different pathology and type of infection with only *H. bakeri* being a truly chronic infection (Else, 2005).

The nutritional status of the host has effects on the intensity and spread of parasitic infection through affecting the immune function or through effects on the parasite and its' environment (Boulay et al., 1998). Protein deficiency can affect the ability of a host to expel parasites such as *N. brasiliensis* and *T. muris* while curtailing the hosts ability to inhibit the survival and fecundity of *H. bakeri* (Boulay et al., 1998; Koski and Scott, 2001). 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 and expel the parasite. It has also been shown that the ability of the host to develop acquired immunity is impaired through protein malnutrition (Slater and Keymer, 1988). This reduced immune response is thought to be due to less protein being available for immunity during nutrient partitioning (Coop & Kyriazakis, 1999). The extent of nutrient partitioning during intensive selection for growth and the ability of protein supplementation to overcome any effects of a pathogen challenge remains to be sufficiently established.

1.4 Context of the PhD objectives

1.4.1 Justification and use of a mouse model

The basis of this PhD is to investigate whether (and how) selection for body weight affects the ability to cope with pathogen challenges. It will then be investigated as to whether nutrient partitioning is the cause of observed reductions in immunity during intensive selection for growth in livestock research by investigating the possibility of increased dietary protein contents to overcome the penalty of infection and possible reduction in immunity.

When using livestock during experiments the results can be highly variable between studies. This variability could be due to differences between the breeds used and/or environmental conditions. Livestock breeds are rarely produced from direct selection for one specific trait and can be found in a range of environments ranging from low to high infection risks and extensive to intensive management strategies. A model animal (such as a mouse) selected divergently from the same genetic background for a single selected trait would enable a better comparison of genetic and environmental factors determining the outcome of infection. The highly controlled environmental conditions afforded to laboratory animals will also allow the effects of protein supplementation to be seen without other environmental variables confounding the data.

1.4.2 The mouse model: 'Roslin' lines

In this case the "Roslin lines" of mice, (described in detail by Heath et al., 1995; Bünger et al., 2001a), produced from a C57BL/6J x DBA/2J inbred over 20 generations and divergently selected for high (ROH) or low (ROL) body weight (BW) at 42 days of age, will enable a direct comparison between animals which have

been produced by divergent selection on one trait. This intensive selection has resulted in the ROH line able to reach body weights of approximately 50g and the ROL line reaching approximately 18g at 70 days of age (Bünger et al., 2001a). Genetic studies on the ‘Roslin’ lines have been limited to the identification of growth quantitative trait loci (QTL) in the C57BL/6J x DBA/2J F₂ cross (Morris *et al.*, 1999), which were found to be of relatively small magnitude and fairly evenly spaced across the genome. Mapping of these growth QTL in relation to resistance QTL would enable a more thorough knowledge of the underlying genetics in the ‘Roslin’ mouse lines but unfortunately this data is not available at this time. To my knowledge there is no literature on the effects of an *H. bakeri* infection on an F1 cross of C57BL/6J and DBA/2J mice. However, previous studies using *H. bakeri* infections in the founder mouse lines found that C57BL/6J mice have a weak response phenotype that is stable across environments, whilst DBA/2J mice have an intermediate response phenotype that is unstable across environments and individuals (Behnke *et al.*, 2006). My prediction is that the “Roslin” mice may inherit a phenotype that would be closer to an intermediate responder as the DBA/2J mice are more variable than the C57BL/6J mice. Using the ‘Roslin’ mouse lines, the effect of a chronic pathogen challenge on growth and the ability of protein supplementation to overcome losses in growth will be investigated following a number of experimental objectives that are outlined in the following section.

1.4.3 Objectives and hypotheses

The overall objectives of this research will be to:

- (1) Use widely divergent genotypes to investigate the consequences of protein nutrition on performance and resistance to *Heligmosomoides bakeri* using

nutritional conditions that range in several increments from scarce to more than adequate. This will be addressed in Chapter 2.

- (2) Use widely divergent genotypes to investigate the consequences of infection pressure on performance and resistance to *Heligmosomoides bakeri* using larval pressures that range in several increments from 0 L₃ to 250 L₃ in different nutritional environments. This will be addressed in Chapter 3.
- (3) Investigate whether genetic selection for growth potential and nutritional environment interactively affect different cellular and humoral components of the immune response to *H. bakeri*. This will be addressed in Chapters 3 and 4.
- (4) Use widely divergent genotypes to address the question of whether intensive genetic selection for growth has led to reduced resistance to primary infection and to re-infection with *H. bakeri* in the growing host under conditions of protein scarcity. This will be addressed in Chapter 5.

The hypotheses of the objectives are as follows:

- (1) Increasing protein nutrition above a certain level will reduce the penalty of infection, this level will be higher in mice selected for high body weight than in mice selected for low body weight.
- (2) Increasing larval dose above a certain level will increase the penalty of infection, this level will be lower in mice selected for high body weight and than in mice selected for low body weight. Increased protein nutrition is expected to increase the larval dose required to produce this effect in both lines of mice.
- (3) Immune responses to GI nematodes are expected to be impaired (reduced) in mice selected for high body weight compared to mice selected for low body

weight. Increased protein nutrition is expected to enhance immune responses towards GI nematodes.

- (4) Secondary infections with GI nematodes are expected to increase the penalty of infection on mice selected for high body weight compared to their low body weight counterparts. Increased protein nutrition is expected to ameliorate this penalty.

Each of the following chapters deals with a separate objective and is presented as a self-contained paper including a brief introduction, and a comprehensive discussion. The chapters will then be summarized in the general discussion which will link the findings of all the experiments with the overall context of the PhD (i.e. what does extreme and narrow selection do to an animal in terms of resilience and resistance to infection). The general discussion will also critically evaluate the research and suggest how future research using the model could be directed.

Chapter 2. Genetic growth potential interacts with nutrition on the ability of mice to cope with *Heligmosomoides bakeri* infection.

Coltherd J.C, Bünger, L, Kyriazakis I and Houdijk J.G.M (2009). *Parasitology*, **136** (9), 1043-1055.

2.1 Summary

Artificial selection for improved productivity may reduce animals' ability to cope with pathogens. Here, we used Roslin mice, uniquely divergently selected for high (ROH) and low (ROL) body weight, to assess interactive effects of differing growth potential and protein nutrition on host resilience and resistance. In a 2x2x6 factorial design, ROH and ROL mice were either sham-infected or infected with 250 L₃ *Heligmosomoides bakeri* and fed diets with 30, 80, 130, 180, 230 and 280 g crude protein per kg. The infected ROL-30 treatment resulted in clinical disease and was discontinued. In the remaining ROL mice, infection and feeding treatments did not affect growth but infection reduced weight gain in ROH-30, ROH-80 and ROH-130 mice. Although infection resulted in temporarily reduced food intake (anorexia) in both mouse lines, mean food intake over the whole experiment was reduced in ROH mice only. ROH mice excreted more worm eggs and had higher worm burdens, with relatively fewer female worms, than ROL mice. However, these resistance traits were not sensitive to dietary protein. These results support the view that selection for high growth may reduce the ability to cope with pathogens, and that improved protein nutrition may to some extent ameliorate this penalty.

Key words: *Heligmosomoides bakeri*, protein nutrition, anorexia, resilience, resistance, growth potential

2.2 Introduction

The phenotypic selection to improve performance traits, e.g. growth or milk yield, causes the co-selection of a generally unknown underlying genetic architecture (Dekkers & Hospital, 2002). As a consequence, selection for desired traits can have detrimental effects on other traits (Williams, 2005). For example, long-term selection for growth in mice has produced individuals with seemingly shorter lifespans and reduced fertility (Bünger *et al.*, 2001b). A review by Rauw *et al.* (1998) concluded that selection for high body weight in poultry decreased fertility and immunocompetence whilst also increasing the occurrence of defective eggs and chromosomal abnormalities. This review also suggested that selection for high milk yield in dairy herds increased incidence of diseases such as ketosis or mastitis and general leg problems. The observation of these correlations may be due to various mechanisms.

It has been suggested that the genetic mechanism behind changes in correlated traits could be due to pleiotropy and/or genetic linkage and that consequently these would be seen under all environmental conditions (Rauw *et al.*, 1998). Alternatively, observed losses in traits could be explained by a change in the prioritisation of allocation of scarce resources. In selectively bred hosts, relatively more of the available scarce resources may be allocated towards the selected trait because of its increased nutrient demand, when compared to unselected counterparts. As a consequence, fewer resources would be allocated to other bodily functions, including immunity (Beilharz *et al.*, 1993; Beilharz, 1998a; 1998b; Glazier, 2002). In support of this view, protein scarcity has been shown to reduce immunity (resistance) to parasites to a greater extent in fast growing and multiple-rearing hosts compared to

their slower growing and single-rearing counterparts (reviewed in Houdijk *et al.*, 2001a; Houdijk & Athanasiadou, 2003).

During gastrointestinal (GI) parasitic infection, anorexia (or the reduction in voluntary food intake) is a common observation, which may be considered as a functional host response and substantially contributes to observed losses in production (Kyriazakis *et al.*, 1998). It has been suggested that selected animals may have an altered degree of anorexia due to the observation that intensely selected animals show reduced resistance and the assumption that anorexia diminishes as immunity increases (Sandberg *et al.*, 2006; Vagenas *et al.*, 2007). Selection for high growth can be expected to increase nutrient demand and thus reduce disease resistance, however results have been inconsistent for the strength and direction of the genetic correlation between growth and resistance (Broughan and Wall, 2007). Studies in livestock species involving interactions between genetic selection for growth and immunity to GI nematodes are limited by the shortage of truly comparable breeds, as breeds have been selected often from different founder populations and have been selected for different and changing breeding goals in different environments. The use of a unique mouse model involving two lines derived from the same base population (implying an identical initial genetic makeup) divergently selected purely on the basis of high and low body weight at 42 days of age may enable the elucidation of further interactions between selecting for growth traits and immunity.

Using this mouse model our objectives were to assess the effects of selection for high body weight on the ability to cope with a pathogen challenge, on the degree of anorexia observed and the response to increasing protein nutrition during infection.

We hypothesized that mice selected for high body weight experience a greater penalty to the resilience (performance under infection) and resistance to infection than their low body weight counterparts, that anorexia would be more severe in mice selected for high body weight, and that these penalties would be reduced at higher dietary protein contents.

2.3 Materials and Methods

2.3.1 *Animals and housing*

72 Roslin High (ROH) and 72 Roslin Low (ROL), newly weaned male mice aged 21 to 23 days were housed in a room with an ambient temperature of $21\pm 1^{\circ}\text{C}$ and a 12h light cycle (0600 to 1800 hours). The “Roslin lines” of mice were produced from a cross of two inbred lines (C57BL/6J x DBA/2J), which were subsequently divergently selected for high (ROH) or low (ROL) body weight (BW) at 42 days of age and afterwards inbred for over 38 generations (described in detail by Heath *et al.* 1995; Bünger *et al.* 2001a). ROH mice reach body weights of 36g and 41g (at 42 and 70 days of age respectively) with ROL mice reaching 16g and 19g (at 42 and 70 days of age respectively) when offered a standard diet (see below) and kept under standard maintenance conditions. ROH and ROL mice entered the adaptation phase (see below) of the experiment with a mean (\pm s.e.) body weight of $16.7\pm 0.48\text{g}$ and $6.66\pm 0.17\text{g}$, respectively. Mice were individually housed in solid bottomed cages with fresh sawdust and bedding material provided weekly. The experimental details described below were approved by the Animal Experiment Committee of Scottish Agricultural College (ED AE 05/2007) and carried out under Home Office regulations (PPL 60/3626).

2.3.2 *Diets*

All mice were fed *ad libitum* a standard pelleted expanded breeding diet [Rat and Mouse No. 3, Special Diet Services, Witham, UK; digestible crude oil: 39 g/kg; digestible crude protein: 209 g/kg, starch: 273 g/kg; sugars: 112 g/kg; digestible energy: 12.1 MJ/kg] for 3 days after arrival. A total of 6 isoenergetic (Digestible Energy, 15 MJ/kg) pelleted experimental diets with a fixed amino acid to crude protein (CP) ratio were used at differing levels of CP; 30, 80, 130, 180, 230 and 280

g/kg (Table 2.1). These CP levels were chosen to range from scarce to more than adequate in gradually increasing increments (NRC, 1995), where most standard diets contain 180-200 g/kg, and to capture the low dietary protein contents used elsewhere in studies on nutritional sensitivity of parasitism in mice (Slater & Keymer, 1988; Boulay *et al.*, 1998; Ing *et al.*, 2000; Tu *et al.*, 2007). As casein was used as the protein source, 15 g cysteine was added to each kg of casein to account for the relative scarcity of this amino acid. The 30 and 280 g/kg CP diets were formulated and the 80, 130, 180 and 230 g/kg CP diets were then produced using an appropriate mixture of the 30 and 280 g/kg CP diets (Table 2.1).

Table 2.1: Chemical and analysed composition of the 6 experimental diets

Ingredients (% inclusion)	Dietary Crude Protein (g/kg)					
	30	80	130	180	230	280
Casein	3.0	8.86	14.7	20.6	26.4	32.3
Rice starch	57.0	51.1	45.1	39.2	33.2	27.26
Maltodextrin	13.2	13.2	13.2	13.2	13.2	13.2
Sucrose	10.0	10.0	10.0	10.0	10.0	10.0
Soya oil	7.0	7.0	7.0	7.0	7.0	7.0
Cellulose	5.0	5.0	5.0	5.0	5.0	5.0
Vitamins, minerals and amino acids	4.80	4.88	4.97	5.05	5.14	5.23
Analysed Composition						
Dry matter (g/kg)	919	918	912	909	904	906
Crude protein (g/kg dry matter)	31.0	85.7	144	197	230	292
Acid detergent fibre (g/kg dry matter)	32.7	35.3	34.9	40.3	42.3	40.6
Ash (g/kg dry matter)	28.7	27.0	27.8	28.4	32.3	31.5

2.3.3 Infection protocol and experimental design

At day 0 of the experiment mice either received a single infection of 250 *Heligmosomoides bakeri* infective larvae (L₃) suspended in 0.2ml water (I) or a sham infection of 0.2ml water (C) via oral gavage (Houdijk and Bünger, 2007). The *H. bakeri*, formerly known as *Heligmosomoides polygyrus bakeri* and *Nematospiroides dubius* (Cable *et al.*, 2006), were donated by Professor Jerzy Behnke, University of Nottingham, UK (see Jenkins and Behnke, 1977 for full origin details). The dose of

H. bakeri was chosen to produce a sub-clinical level of infection that is known to affect the growth of the mice (Houdijk & Bünger, 2006; 2007). Previous studies using higher levels of infection with *H. bakeri* found that 400 L₃ caused a 10% mortality rate in heterozygous mice (Ehrenford, 1954a) whereas in NZB mice 100% mortality was achieved at 300 L₃ (Mitchell & Prowse, 1979). Although it is known that variation in the genetic background of mice used in infection studies substantially affects the response to larval dose (Liu, 1966; Mitchell & Prowse SJ, 1979; Behnke *et al.*, 2003), it is not known whether this is also the case for the ROH/ROL parent lines.

I and C mice of the ROL and ROH line were fed *ad libitum* on one of the six experimental diets (referred to as 30, 80, 130, 180, 230 and 280) with 6 replicates in each group, resulting in 24 treatment combinations. Figure 2.1 shows the experimental design and time table. Mice entered the adaptation phase at approximately 3 weeks of age (day -10 of experiment). This consisted of a period where a 50:50 mix of experimental and standard diet was offered to acclimatise the mice to the experimental diet (day -7 until day -4) with infection occurring at day 0; between days -3 to 0 mice were only offered the experimental diets. Mice were humanely killed (aged between 49 and 53 days) on day 28 post primary infection (p.i.), for the assessment of worm burdens, colon egg count and body fat percentage.

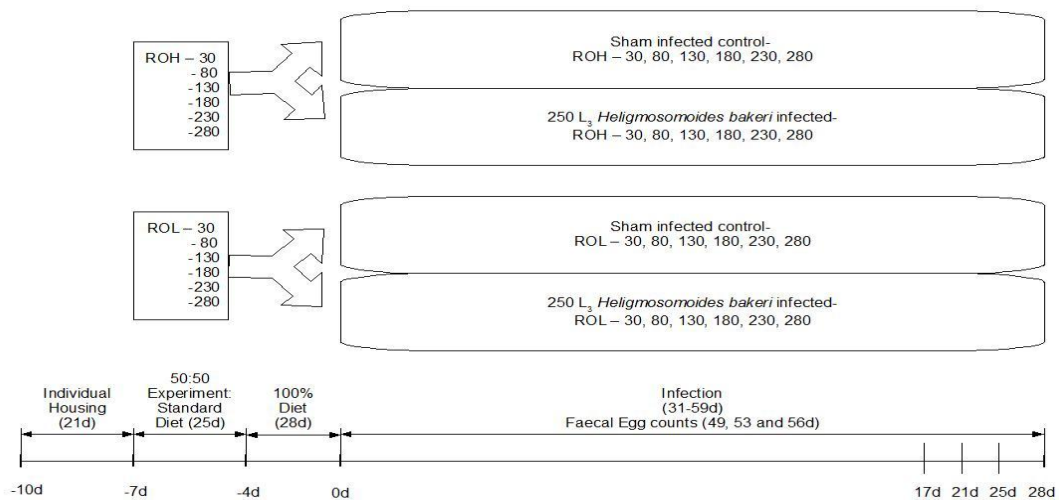


Figure 2.1 - Diagram of experimental design. Timeline in experimental days shown along the bottom and mean age of mice between brackets. Allocation of mice to diets occurred when 50:50 experimental:standard diet was offered, mice were then further allocated to infection groups within these diets at day 0 of the experiment.

2.3.4 Sample measurements and collection

Body weight and food intake. Mice and food refusals were weighed twice weekly (Tuesday and Friday) throughout the experiment resulting in 8 experimental periods for food intake and 8 observations on post infection body weight until day 28. On each of these days food refusals were weighed out and fresh food weighed in, around 30g was offered to ROH and 15g to ROL mice, this was sufficient for *ad libitum* feeding. Food intake was calculated per individual per day within each of the 8 experimental periods. Body weight data was used to calculate body weight gain over the post infection (p.i.) period.

Nematode egg counts. Mice were placed onto wire-bottomed cages overnight and faecal samples collected on days 17, 21 and 25 p.i. to assess faecal egg counts (eggs per g faeces). This was carried out using a modified flotation technique (Christie and

Jackson, 1982). The total period of faecal collection (finish time – start time) was recorded to estimate faeces volume per 12 hours, assuming even production of faeces during the collection period. This faeces volume was used to standardise egg output per 12 hours (EO, eggs per 12 h) to eliminate variation between achieved collection times as well as to account for the dilution effect on faecal egg counts expected for ROH mice due to their larger volumes of faeces.

Colon contents and worm burden. Mice were humanely killed on day 28 via CO₂ inhalation and dissected to obtain the small intestine and the colon. The small intestine was weighed, opened up and placed in a gauze pouch suspended in Hanks' solution, this was then incubated at 37°C for 3h to collect the adult worms (Wahid and Behnke 1992). Formalin at 5% solution was added to the recovered worms, and the intestine and gauze checked for remaining worms. Male and female worms were separated and counted. The colon contents were weighed and a colon egg count (eggs per g) performed. The colon egg count was then multiplied by colon contents weight to account for dilution effects arising from the expected larger colon content volume of the ROH mice. Resultant data were therefore expressed as number of eggs in colon (EIC, number of eggs). The EIC was divided by the number of females counted to obtain an estimate for the *per capita* fecundity (PCF, eggs per female).

Fat percentage. The carcasses were then weighed and bagged for subsequent freeze-drying to allow prediction of fat percentage. To prepare the carcass for the freeze-drying process incisions were made in the back, tail and head of the animal to allow maximum water loss. The carcasses were then placed onto individual labelled trays and the freeze-drier turned on. After approximately 7 days (or weight loss ceased) at

-70°C the carcasses were removed and re-weighed. To calculate fat percentage the following equation was used (Hastings and Hill, 1989):

$$\text{Fat percentage} = [(\text{freeze-dried weight} \times 1.13) / \text{carcass wet weight} - 0.302] \times 100$$

2.3.5 Statistical Analysis

Due to the skewed nature of the data, EO, EIC and PCF were $\log_{10}(n+1)$ transformed. These are reported as backtransformed least-square means, accompanied by a lower and upper confidence interval, calculated from backtransforming least-square mean of transformed data (μ), $\mu - \text{s.e.}$ and $\mu + \text{s.e.}$ respectively. To adequately account for the relatively large differences in performance data between the mouse lines, arising from the difference in body weights *per se* between ROL and ROH mice, body weight and food intake data were also $\log_{10}(n+1)$ transformed before analysis (Falconer & MacKay, 1996). Repeated measures Restricted Maximum Likelihood (REML) was used to assess interactive effects of genetic growth potential, dietary protein content, infection status and time on body weight, food intake and EO. The full repeated measures model is as follows: parameter of interest = genetic potential (G) + crude protein content (CP) + infection status (I) + time (t) + all possible six 2-way interaction + all possible three 3-way interaction + the 4-way interaction (G.CP.I.t). REML analysis was also used to assess interactive effects between genetic growth potential, dietary protein content and infection status on average daily weight gain, average food intake and body fat percentage. The full REML model is as follows: parameter of interest = G + CP + I + all possible three 2-way interaction + the 3-way interaction (G.CP.I). Where significant, litter was included as a random effect. To account for a possible overestimation of effect size arising from the unbalanced nature of the data (see

results), each p-value reported was calculated conservatively through including first all other terms into the final REML model. Interaction terms that did not approach significant at $p < 0.05$ were omitted from the final REML models for each parameter. F-statistics reported are followed by the numerator then the denominator degrees of freedom in subscript. Effects with p-values less than 0.05 are considered significant whilst those with p-values between 0.05 and 0.10 are described as tendencies or trends. All statistical analyses were performed using Genstat 11 for Windows release 11.1, 2008 (VSN international, Hemel Hempstead, UK).

Table 2.2: Summary of mean body weights (BW in g) and pooled standard errors (SE) over time and at each time point^{a, b}

Line	Infection	Protein nutrition	d0 BW	d3 BW	d7 BW	d10 BW	d14 BW	d17 BW	d21 BW	d25 BW	d28 BW	Pooled SE	
ROH	C	30	23.96	24.14	24.56	24.97	25.71	26.17	26.64	26.71	27.64	0.89	
		80	28.21	31.33	34.66	38.26	41.13	44.65	46.13	47.55	49.77	2.52	
		130	28.82	32.11	34.89	38.19	41.38	45.02	46.75	48.21	50.20	1.51	
		180	28.21	31.20	34.34	36.91	39.11	41.68	43.17	43.74	44.27	1.61	
		230	31.36	34.70	38.39	41.33	43.91	46.76	48.69	48.95	50.48	2.44	
		280	28.01	30.89	33.87	37.68	40.16	42.76	44.24	45.59	47.33	0.69	
		I	30	23.07	22.97	22.37	22.87	23.26	24.14	24.14	23.34	22.42	1.05
	80	27.41	29.77	33.56	35.15	37.50	40.13	40.21	40.71	41.66	41.66	1.92	
	130	29.60	31.86	34.92	37.96	39.57	42.10	44.02	45.00	47.12	47.12	0.92	
	180	29.82	32.24	35.74	37.82	40.30	41.74	43.32	44.31	46.26	46.26	2.04	
	230	28.02	31.42	34.40	36.34	38.29	40.52	41.10	41.65	43.10	43.10	1.23	
	280	28.02	30.71	34.12	36.41	38.96	41.18	42.46	44.12	45.69	45.69	1.22	
	Pooled SE for ROH			0.48	0.41	0.39	0.36	0.42	0.42	0.45	0.46	0.49	
	ROL	C	30	11.38	11.95	12.60	13.39	13.70	14.31	14.80	15.18	15.52	0.50
80			12.26	13.29	14.38	15.30	15.88	16.55	16.93	16.98	17.69	0.31	
130			10.21	11.81	13.28	13.99	15.09	15.61	16.21	16.46	17.12	0.45	
180			11.81	13.01	14.37	15.12	15.80	16.22	17.05	17.64	17.74	0.36	
230			11.13	12.21	13.63	14.30	15.07	15.42	15.84	16.46	16.60	0.65	
280			10.67	12.39	13.74	14.60	15.57	16.12	16.60	16.65	17.33	0.52	
I			30 ^c	-	-	-	-	-	-	-	-	-	-
80		10.79	12.10	13.16	13.82	14.67	15.27	15.82	16.56	16.95	16.95	0.52	
130		10.96	12.04	13.63	14.24	15.00	15.79	16.49	16.96	17.65	17.65	0.42	
180		11.77	12.82	13.98	14.62	15.33	15.98	16.35	17.12	17.69	17.69	0.33	
230		11.01	12.51	14.06	15.06	15.75	16.37	16.65	17.10	18.32	18.32	0.44	
280		12.08	12.91	14.60	14.95	15.55	16.48	17.03	17.59	18.42	18.42	0.61	
Pooled SE for ROL			0.15	0.13	0.13	0.12	0.12	0.14	0.13	0.14	0.14		

^a raw data shown. ^b 'Roslin' mice divergently selected for high (ROH) and low (ROL) body weight were either sham-infected (C) or infected with 250L₃ *Heligmosomoides bakeri* (I). Experimental diets contained 30, 80, 130, 180, 230 or 280 g crude protein per kg.

^c ROL-30-I showed clinical signs of infection and were discontinued.

Table 2.3: Summary of mean average daily food intakes (DFI in g) and pooled standard errors (SE) over time and at each time period^{a, b}

Line	Infection	Protein nutrition	d0-d3 DFI	d3-d7 DFI	d7-d10 DFI	d10-d14 DFI	d14-d17 DFI	d17-d21 DFI	d21-d25 DFI	d25-d28 DFI	Pooled SE		
ROH	C	30	4.47	3.86	3.57	3.64	3.31	3.38	3.17	3.32	0.15		
		80	5.25	5.19	5.52	5.27	5.52	5.04	4.93	4.90	0.09		
		130	4.88	4.97	5.13	5.41	5.69	5.39	5.23	5.00	0.07		
		180	4.88	5.03	4.84	5.19	4.87	4.66	4.51	3.98	0.09		
		230	5.20	5.71	5.36	5.66	5.47	5.21	4.77	4.81	0.11		
		280	4.70	4.97	5.23	5.17	5.06	5.08	4.62	4.51	0.08		
	I	30	4.32	3.28	3.33	3.93	3.70	3.09	2.37	1.96	0.11		
		80	4.94	4.71	4.39	4.62	4.92	4.48	4.21	3.87	0.09		
		130	4.79	4.72	4.77	4.88	4.80	5.15	4.71	4.84	0.09		
		180	4.73	4.91	4.57	4.72	4.70	4.71	4.60	4.71	0.08		
		230	5.12	4.84	4.24	4.68	4.98	4.60	4.44	4.72	0.07		
		280	4.73	4.79	4.41	4.79	4.81	4.74	4.62	4.65	0.07		
		Pooled SE for ROH			0.03	0.02	0.02	0.03	0.02	0.03	0.03	0.03	
		ROL	C	30	2.91	2.96	3.02	2.95	2.94	3.14	3.01	2.96	0.05
80	2.64			2.65	2.60	2.49	2.54	2.69	2.51	2.49	0.03		
130	2.55			2.51	2.52	2.57	2.47	2.52	2.56	2.54	0.03		
180	2.67			2.67	2.51	2.50	2.48	2.74	2.58	2.62	0.03		
230	2.56			2.52	2.44	2.41	2.46	2.50	2.53	2.27	0.04		
280	2.55			2.44	2.50	2.48	2.38	2.49	2.41	2.43	0.04		
I	30 ^c		-	-	-	-	-	-	-	-	-	-	
	80		2.48	2.24	2.27	2.37	2.42	2.48	2.58	2.38	0.03		
	130		2.46	2.36	2.32	2.45	2.58	2.54	2.60	2.59	0.03		
	180		2.52	2.30	2.29	2.46	2.40	2.47	2.63	2.44	0.03		
	230		2.66	2.47	2.47	2.57	2.56	2.69	2.54	2.68	0.04		
	280		2.47	2.39	2.19	2.47	2.53	2.59	2.47	2.53	0.04		
	Pooled SE for ROL			0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		

^a raw data shown. ^b 'Roslin' mice divergently selected for high (ROH) and low (ROL) body weight were either sham-infected (C) or infected with 250L₃ *Heligmosomoides bakeri* (I). Experimental diets contained 30, 80, 130, 180, 230 or 280 g crude protein per kg.

^c ROL-30-I showed clinical signs of infection and were discontinued

Table 2.4: Summary showing means and pooled standard errors (SE) of performance data^{a,b}

Line	Infection	Protein nutrition	Daily Weight Gain (g)	Daily Food Intake (g)	Fat (%)	
ROH	C	30	0.13	3.59	18.63	
		80	0.66	5.20	25.87	
		130	0.64	5.21	22.39	
		180	0.47	4.75	20.30	
		230	0.56	5.27	25.26	
		280	0.59	4.92	21.68	
	I	30	-0.02	3.16	14.20	
		80	0.42	4.51	16.50	
		130	0.54	4.83	19.20	
		180	0.50	4.71	18.80	
		230	0.42	4.70	16.90	
		280	0.54	4.68	19.20	
		Pooled SE		0.15	0.04	1.50
		<hr/>				
ROL	C	30	0.13	2.99	16.00	
		80	0.16	2.58	11.24	
		130	0.19	2.53	11.82	
		180	0.17	2.60	13.50	
		230	0.16	2.46	12.66	
		280	0.18	2.46	12.45	
	I	30 ^c	-	-	-	
		80	0.17	2.40	11.30	
		130	0.20	2.49	9.94	
		180	0.17	2.44	10.70	
		230	0.21	2.58	9.74	
		280	0.20	2.46	8.95	
		Pooled SE		0.06	0.02	0.92

^a data were log transformed for statistical analysis

^b 'Roslin' mice divergently selected for high (ROH) and low (ROL) body weight were either sham-infected (C) or infected with 250L₃ *Heligmosomoides bakeri* (I). Experimental diets contained 30, 80, 130, 180, 230 or 280 g crude protein per kg.

^c ROL-30-I showed clinical signs of infection and were discontinued

2.4 Results

2.4.1 Loss of the infected ROL-30 group

ROL-30-I mice were found to show severe clinical signs of infection at day 7 p.i. such as starry coat, unsteadiness and disorientation. These mice were euthanized and this treatment group discontinued in accordance with previously defined end points. Thus, the resulting dataset was characterized as an incomplete 2 x 2 x 6 factorial design, which consequently was appropriately analysed through REML.

2.4.2 Body weight gain and food intake

Mean observed body weight and food intake are summarised in Tables 2.2 and 2.3, respectively, whilst mean performance data for each line x infection x feeding treatment is summarised in Table 2.4. Figure 2.2 shows the log transformed mean of daily weight gain and food intake over the experiment. ROH mice, with the exception of ROH-30-I mice, gained more weight over the experiment than ROL mice (mean = 0.497 ± 0.202 g per day and 0.175 ± 0.005 g per day respectively; $F_{1,114}=529.55$, $p < 0.001$). However, on average ROH-30-I mice did not gain weight across the experiment (mean = -0.02 ± 0.04 g per day).

A three-way interaction between genetic growth potential, protein nutrition and infection status was observed for body weight gain ($F_{4,114}=3.01$, $p=0.021$). This was due to ROH mice showing a reduction in weight gain on the 30g CP per kg diet and also in response to infection on 30, 80 and 130g CP per kg diets whilst ROL mice maintained a relatively stable weight gain regardless of experimental group. ROH mice gained more weight than ROL mice, with the exception of ROL-30-C gaining more than ROH-30-I ($F_{1,114}=529.55$, $p < 0.001$). The random effect of litter was not significant ($p > 0.10$).

Genetic growth potential interacted with dietary protein content and with infection status for average daily (log transformed) food intake (Figure 2.2). The interaction with dietary protein content resulted from decreased food intake of ROH-30 mice whereas ROL-30 mice increased their intake on this diet when compared to other diets ($F_{5,103}=29.44$, $p<0.001$). The interaction with infection status was reflected in a reduced intake following infection in ROH mice but not in ROL mice ($F_{1,99}= 6.70$, $p=0.011$). Across feeding and infection treatments, ROH mice consumed more food than ROL mice ($F_{1,36}= 1188.36$, $p<0.001$). The random effect of litter was significant (deviance ratio = 6.53, d.f. = 1, $p=0.01$).

Figure 2.3 shows the log transformed mean of daily food intake over time. Time and infection status interacted for average daily food intake ($F_{7,811} = 3.33$, $p = 0.002$); infection caused a temporary decrease in voluntary intake between day 3 and day 14 p.i. This anorexia was not affected by genetic growth potential. However, the analysis of food intake over time showed two three-way interactions. Firstly, infection status interacted with protein and time as evidenced by the variable presence of anorexia over the six levels of dietary CP ($F_{35,860} = 2.6$, $p<0.001$). Anorexia was not observed for three of the remaining eleven mice line - feeding treatment combinations, i.e. ROH-130, ROH-180 and ROL-230. Secondly, genetic growth potential interacted with protein and time as evidenced by a larger decline in intake over time on the 30 g/kg CP in ROH mice compared to ROL mice ($F_{35,860} = 1.62$, $p = 0.014$). In addition and in comparison with the other experimental diets, ROH-30 mice had a lower intake whilst for ROL-30 mice intake on this diet was the highest.

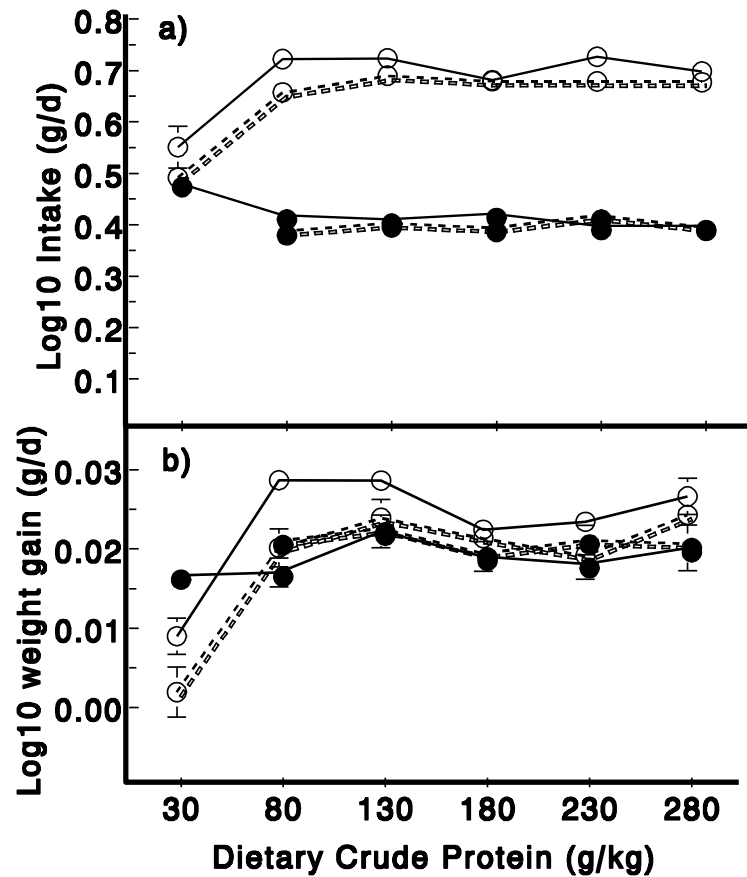


Figure 2.2 - a) Log_{10} transformed daily food intake and b) body weight gain of high (ROH – open circle) and low (ROL – closed circle) body weight mice averaged across 28 days of infection with *H. bakeri* (dashed line) or sham infection with water (solid line) at different levels of dietary CP.

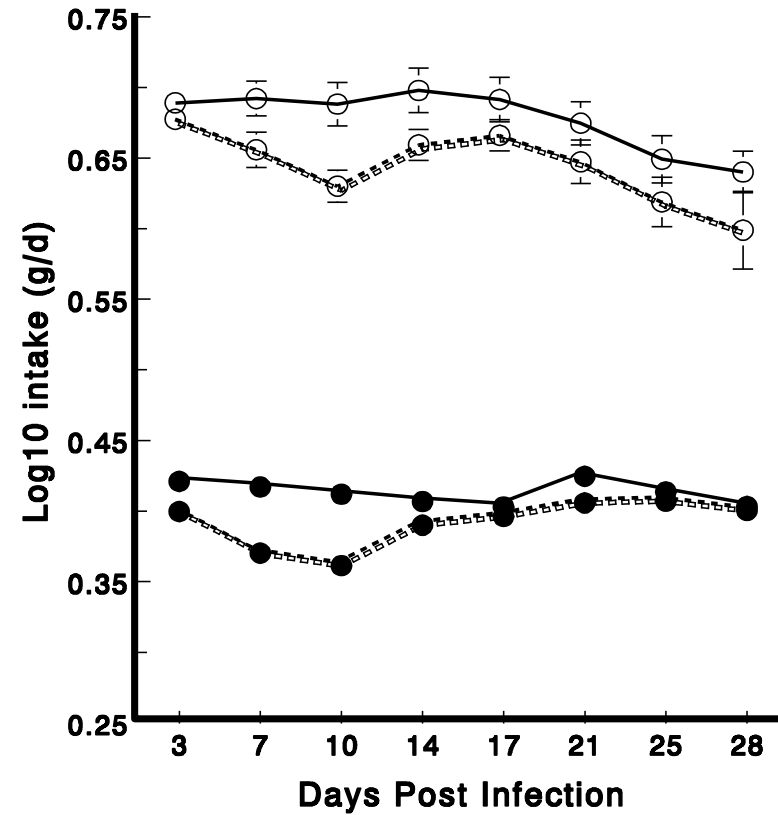


Figure 2.3 - Log_{10} transformed daily food intake of high (ROH – open circle) and low (ROL – closed circle) body weight mice following infection with *H. bakeri* (dashed line) or sham infection with water (solid line) over the 28 day experimental period.

Table 2.5: Summary showing means and pooled standard errors (SE) of parasitism data^{a,b}

Line	Protein	Eggs in Colon (n)	Egg Output (n/12hr)	Total Worms (n)	Female Worms (n)	Male Worms (n)	<i>Per Capita</i> Fecundity (n/female)
ROH	30	2032	9937	214	106	108	23.88
	80	7309	21582	268	140	128	52.69
	130	4154	21139	126	60	66	77.26
	180	7186	12347	191	100	91	75.37
	230	6813	13147	229	114	115	71.80
	280	6170	19217	146	75	71	82.58
	Pooled SE	1104	4314	31	21	21	12.38
ROL	30 ^c	-	-	-	-	-	-
	80	6028	5956	143	84	59	64.51
	130	2735	13604	139	73	67	39.42
	180	4586	6587	129	68	62	69.99
	230	2808	6828	152	78	74	45.07
	280	2916	11787	173	94	79	35.56
	Pooled SE	978	1306	15	12	12	17.36

^a egg count and fecundity data were log transformed for statistical analysis

^b 'Roslin' mice divergently selected for high (ROH) and low (ROL) body weight were fed either 30, 80, 130, 180, 230 or 280 g crude protein per kg diets

^c ROL-30-I showed clinical signs of infection and were discontinued

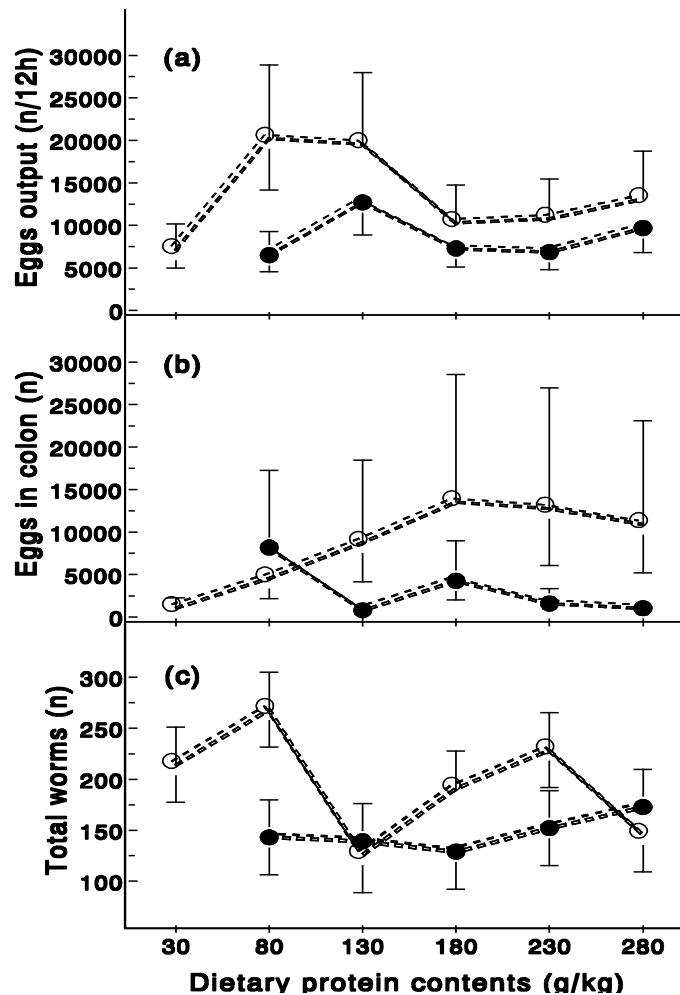


Figure 2.4 - a) Backtransformed 12 hour egg output averaged over days 17, 21 and 24 post infection, b) backtransformed number of eggs in the colon on day 28 post infection and c) total worm burden on day 28 post infection for high (ROH – open circle) and low (ROL – closed circle) body weight mice, infected with *H. bakeri* and fed different levels of dietary crude protein (CP) for 28 days.

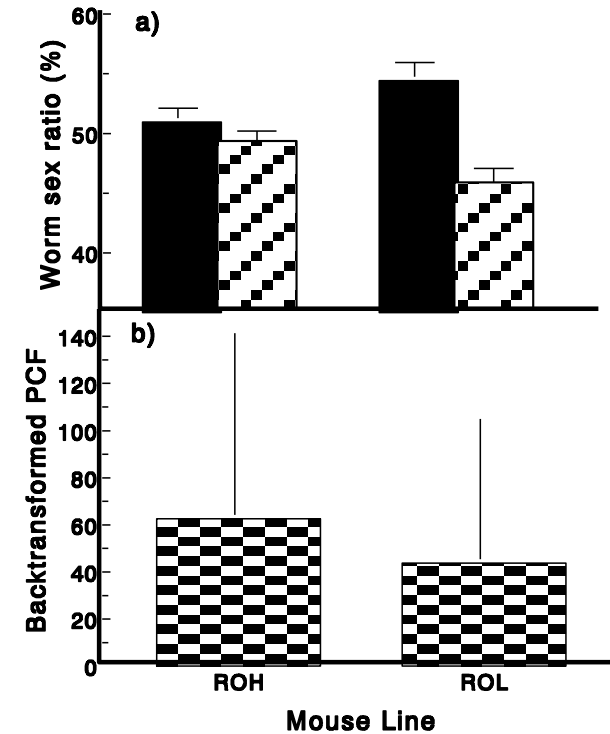


Figure 2.5 - a) Sex ratio of worm burdens (% female worms-solid bar and % male worms-patterned bar) and b) mean backtransformed *per capita* fecundity (PCF) for high (ROH) and low (ROL) body weight mice taken 28 days after infection with *H. bakeri*.

2.4.3 Egg output, eggs in colon and worm burden

Mean parasitism data for each line x infection x feeding treatment combination is summarised in Table 2.5. Figure 2.4 shows the backtransformed mean EO, EIC and mean total worm burden. 12 hour faecal production was higher in ROH mice than in ROL mice (0.55 vs 0.34 g; s.e.d: 0.025 g; $F_{1,54} = 38.49$, $p < 0.001$), which justified the need to account for potential dilution effects on faecal egg counts. Genetic growth potential and feeding treatment did not significantly interact with time for EO. Therefore, the mean EO averaged over days 17, 21 and 25 p.i. was analysed. Backtransformed EO are shown in Figure 4. Genetic growth potential and dietary protein contents interacted for EO ($F_{4,29} = 2.95$, $p = 0.036$); EO tended to be consistently higher in ROH than in ROL mice ($F_{1,32} = 2.94$, $p = 0.096$) but was significantly higher on 80 g/kg CP diets only. Increasing dietary protein content also increased 12 hour faecal production in both lines ($F_{5,27} = 5.65$, $p < 0.001$). The random effect of litter was significant (deviance ratio = 19.19, d.f. = 1, $p < 0.001$).

Genetic growth potential and feeding treatment interacted for colon contents weight ($F_{5,54} = 23.01$, $p < 0.001$); increasing dietary protein content increased colon content weight in ROH mice but decreased colon contents weight in ROL mice. Across feeding treatments, weight of colon contents was higher in ROH mice than in ROL mice (0.21 vs 0.16 g; s.e.d. 0.014 g; $F_{1,54} = 13.00$, $p = 0.001$). As with faecal output, these observations justified accounting for effect of colon contents volume on worm egg concentrations. Genetic growth potential and feeding treatment did not interact for EIC. However, EIC was significantly higher in ROH mice than ROL mice ($F_{1,58} = 5.97$, $p = 0.018$; Figure 2.4). The random effect of litter was not significant ($p > 0.10$).

Genetic growth potential and feeding treatment did not interact for total worm counts (Figure 2.4). However, genetic growth potential affected male worm numbers, as ROH mice had significantly higher numbers of male worms than ROL mice (96 vs 68; s.e.d 10.9; $F_{1,54} = 6.10$, $p = 0.017$). Figure 5 shows the sex composition of the worm burdens and *per capita* fecundity. Genetic growth potential significantly affected worm burden sex composition; ROL mice had a higher percentage of female worms than ROH mice (54.43 vs 49.72 %; s.e.d. 1.889 %; $F_{1,63} = 6.21$, $p = 0.021$). *Per capita* fecundity tended to be higher in ROH mice than in ROL mice ($F_{1,62} = 3.37$, $p = 0.071$) but was not affected by dietary CP contents. The random effect of litter was not significant for worm numbers or *per capita* fecundity ($p > 0.10$).

2.4.4 Body fat percentage

A three-way interaction between genetic growth potential, dietary protein and infection status was significant for body fat percentage ($F_{4,109} = 2.78$, $p = 0.030$; Figure 2.6). ROH mice had higher body fat percentage than ROL mice ($F_{1,35} = 134.95$, $p < 0.001$) and infection reduced body fat percentage in both ROH and ROL mice ($F_{1,92} = 65.88$, $p < 0.001$). However, body fat percentage seem to increase with higher levels of dietary CP contents for infected ROH mice, whilst it decreased for infected ROL mice. ROL mice had their highest body fat percentage at 30 g CP per kg. The random effect of litter was significant (deviance ratio = 9.78, d.f. = 1, $p = 0.002$).

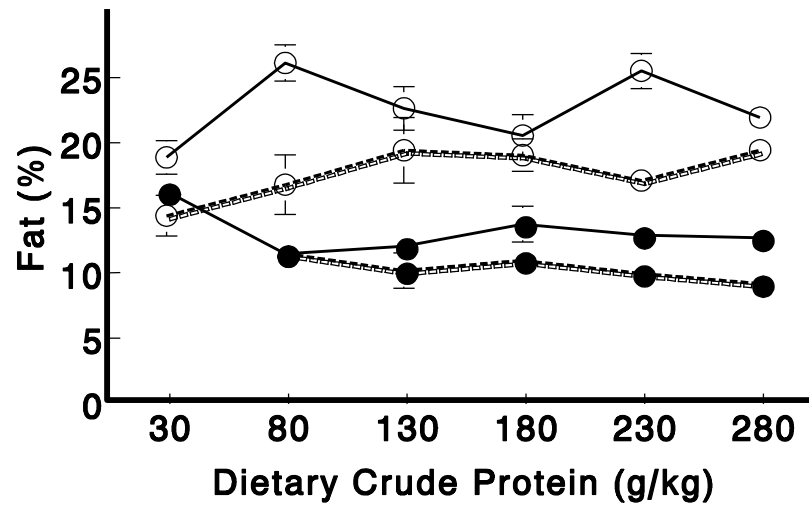


Figure 2.6 - Average body fat percentage of high (ROH – open circle) and low (ROL – closed circle) body weight mice either infected with *H. bakeri* (dashed line) or sham infected with water (solid line) at different levels of dietary CP, taken 28 days after infection.

2.5 Discussion

The results of this study supported our hypothesis that selection for high body weight in mice imposes a greater penalty on resistance and resilience to parasite infection than selection for low body weight, and that improved protein nutrition could ameliorate the penalty on host resilience. However, in contrast to our other hypotheses, our results also showed that this difference in genetic growth potential did not affect anorexia, and that increased protein nutrition did not affect host resistance.

2.5.1 *Body weight and food intake*

It was found that ROH mice had a greater reduction in average daily intake and daily weight gain, but a similar reduction in body fat percentage in response to infection relative to ROL mice. Although this effect has not been addressed in divergently selected mouse lines, infections have been shown to produce reductions in both food intake and body weight gain in mice (Brailsford & Mapes, 1987; Tu *et al.*, 2007). Moreover, Kristan and Hammond (2001; 2006) investigated the effect of *H. bakeri* infection on body fat in Swiss-Webster mice and found a reduction of 20% on average, which corresponds very well to the reduction found in the current study in both the ROH and ROL mice (an average of 21.5% and 21.8% respectively). This reduction of body fat appears to suggest that the two genetic lines utilised energy similarly in response to infection and suggests a possible role for energy nutrition during gastrointestinal nematode infections. Indeed, caloric restriction, via restrictive feeding protocols (causing mainly energy restriction but in addition also some degree of protein restriction), may increase susceptibility to parasite infection and also worm burdens and parasite fecundity (Koski *et al.*, 1999; Kristan, 2008). However, the sham-infected animals showed a difference in their allocation rules, with ROH-80-

280 mice having increased body fat compared to ROH-30 whilst ROL-30 mice have the highest body fat compared to ROL-80-280.

Increasing dietary CP contents to 130 g CP per kg and above resulted in increased host resilience in ROH mice, as illustrated by reduced penalty of infection on food intake and body weight gain. A preliminary study using ROH and ROL mice did find that feeding a 250 g CP per kg diet similarly reduced the penalty of infection on body weight gain in ROH mice when compared to ROH mice fed 50 g CP per kg (Houdijk & Bünger, 2007). This is consistent with our findings, and suggests that moderate protein nutrition using highly digestible protein sources (130 g CP per kg diet) may ameliorate losses in production during a primary infection with gastrointestinal nematode parasites.

Anorexia, characterised as a temporary reduction in food intake following infection, is a common outcome of exposure to pathogens, including gastrointestinal parasites (Kyriazakis *et al.*, 1998; Mercer *et al.*, 2000). Its biological relevance is evident from force-feeding studies. For example, Murray and Murray (1979) observed increased host mortality following force-feeding mice during a *Listeria monocytogenes* infection. However, the time course of anorexia has not been described in detail during *H. bakeri* infection; to date, studies have only reported a decrease in food intake over the entire experimental period (Brailsford & Mapes, 1987; Shi *et al.*, 1997; Boulay *et al.*, 1998; Tu *et al.*, 2007). This study, therefore, is the first to describe the time course of anorexia during a primary infection of *H. bakeri*. Anorexia in both lines was found to occur between day 3 and day 14 post primary infection. Although genetic growth potential was not found to affect anorexia overall, whether anorexia differed between lines depended on dietary

protein content. ROH mice did not show anorexia at 130 or 180 g CP per kg whilst ROL mice did not show anorexia at 230 g CP per kg only. This apparent lack of systematic effect of dietary CP content on anorexia in both lines may be due to variation in food intake at different levels of dietary CP in the sham infected mice. Although a lack of systematic research in this area makes it difficult to reach a consensus on the effect of diet composition on anorexia (Kyriazakis, 2010), the data from this study would support the view that the degree of anorexia does not depend on dietary CP contents. The latter is consistent with earlier work done on *H. bakeri* (Brailsford & Mapes, 1987) but also on sheep infected with *T. colubriformis* (Kyriazakis *et al.*, 1996) and *Haemonchus contortus* (Datta *et al.*, 1998).

2.5.2 Egg output, eggs in colon and worm burden

Compared to ROL mice, infection in ROH mice produced greater 12 hour egg output, total eggs in the colon contents and *per capita* fecundity. This suggests that ROH mice had an impaired resistance to *H. bakeri*, which in turn implies that selection for high body weight may produce a loss of immunity towards pathogen challenge. Since this reduced immunocompetence was observed at times of apparently adequate CP nutrition, it may not necessarily have arisen from reduced allocation of scarce protein to host immune functions. Influences of host genetics on *H. bakeri* infection have long been considered and some commonly used mouse strains have now been categorized as high or low responders based on LD₅₀ experiments and also according to time taken to clear out the infection (Liu, 1966; Iraqi *et al.*, 2003). Given that genetic differences in immunocompetence between the ROH and ROL mice exist they could potentially be the result of a true genetic correlation or drift. As selection for high production in lambs can also impair the ability to cope with pathogens, as lambs with a high growth potential had higher

faecal egg counts than their low growth potential counterparts (Zaralis *et al.*, 2008) it is suggested that it is more likely a genetic correlation than drift.

Consistent with our results, Slater and Keymer (1988) also did not find an effect of increased protein nutrition on worm burdens or egg counts during a primary infection. However, Boulay *et al.* (1998) did report a significant reduction in worm burdens in mice fed 240 g CP per kg when compared to 30 and 70 g CP per kg diets. These mice were older than the ones in the current study, which may have led to stronger immune response *per se* (Goff *et al.*, 2001; Miller *et al.*, 2005). In addition, it remains to be elucidated whether the differences between Boulay's findings and our own may have arisen from differences in feeding treatment regime, i.e. our study introduced the experimental diets one week before infection whilst in Boulay *et al.* (1998), mice were fed the experimental diets several weeks before infection. During secondary infections, protein scarcity, arising from feeding 20 or 30 g CP per kg diets, decreased weight gain and increased worm burdens and egg counts when compared with diets with 70 or more g CP per kg (Slater & Keymer, 1988; Boulay *et al.*, 1998). In addition, it was shown that switching food types from protein deficient (30g/kg) to protein sufficient (240g/kg) during primary or secondary infection with *H. bakeri* was also shown to rapidly restore body weights in mice and resulted in a reduction of worm burdens and faecal egg counts during secondary challenge to levels achieved on the protein sufficient diet for the entire study (Tu *et al.*, 2007). Taken together, these and our findings support the view that protein scarcity affects expression of immunity to a larger extent than acquisition of immunity (Coop & Kyriazakis, 1999).

The lower level of EO in ROH mice on diet 30 compared to diet 80 was an unexpected result but in agreement with similar findings in sheep, primary infected with the small intestinal nematode *Trichostrongylus colubriformis* (Athanasiadou *et al.*, 2001). These observations may suggest that very low levels of nutrient supply can limit the parasite as well as the responses of the host (Houdijk & Athanasiadou, 2003). In the case of *H. bakeri*, these limitations may be incurred through villus atrophy that occurs under protein scarce environments (Tu *et al.*, 2007). This in turn may reduce availability of epithelial cells as a food resource to *H. bakeri* (Bansemir & Sukhdeo, 1994; Bansemir & Sukhdeo, 1996)

The worm burden results were more subtly altered than egg count parameters in this current study with ROL mice showing a female worm bias. Such a bias is commonly reported in studies involving wild mice (Keymer & Dobson, 1987; Gregory *et al.*, 1992). This difference in worm sex ratios between ROH and ROL may also be a product of potential genetic drift or inherent genetic correlation. Alternatively, differences in parasite sex ratios may be influenced by factors such as mating probabilities and disproportional survival between the sexes during larval stages in the host (Stien *et al.*, 2005).

2.5.3 Loss of ROL-30-I group

The loss of the ROL-30-I group made comparison of the treatment groups incomplete. Had this group been present, then the effect of protein on scarcity on resilience and resistance to *H. bakeri* infection may have been better understood. Clinical symptoms of infection were observed in this group from day 7 p.i. which corresponds to the time when larvae migrate from the intestinal mucosa to the lumen

for their final moult to adult worms (Ehrenford, 1954b). This process is probably associated with 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). Because such losses would interfere with host maintenance, parasitized hosts would be expected to attempt to replenish them (Coop & Kyriazakis, 1999). Moreover, replenishment of these losses is the largest contributor to elevated protein requirements during gastrointestinal nematode infections (Houdijk *et al.*, 2001a). The clinical signs observed in the ROL-30-I mice may have arisen from their inability to respond to this temporary increased protein requirement. ROH-30-I mice did not show any clinical signs but displayed a small, temporal reduction in body weight on day 7 p.i. relative to day 4 and day 10 p.i. at similar levels of intake (Table 2). This may suggest they were more able than their ROL counterparts to utilize body reserves to cope with the assumed temporal elevation of protein requirements at times of dietary protein scarcity. ROL mice infected with 250 L₃ *H. bakeri* and fed *ad libitum* a 50 g CP per kg food did not have any clinical signs of parasitism as observed in this study (Houdijk & Bünger, 2007). In addition, a single infection with 150 L₃ in three spare ROL-30-I mice also resulted in no clinical signs but a small temporal drop in body weight and a ~20% reduction in body weight gain over 28 days p.i. was observed, relative to ROL-30-C mice (data not shown). Taken together, these observations suggest that the inability of ROL-30-I mice to cope with the experimental treatment was likely a combination of the low dietary CP contents, the relatively high level of infection and their small body size.

The growth performance data obtained in the current experiment support the view that protein scarcity may only have been achieved in the ROH-30 mice; Figure 2 suggests that growth performance did not differ between 80-280 g CP per kg in both

lines whilst 30 g CP per kg causes a significant loss of performance in ROH mice when mice were not infected. This is in agreement with earlier findings, where BALB-c mice fed diets with 70 and 240 g CP per kg had similar growth performance, which was higher than that of mice fed diets with 30 g CP per kg (Boulay *et al.*, 1998). The food intake data (Figure 2) suggest ROL attempted to compensate for protein scarcity on the 30 g CP per kg diet through increasing their food intake. Such an increased intake would have been associated with a higher intake of energy, which in our experiment is reflected in the higher body fat percentage observed for ROL-30 mice compared to ROL mice on higher CP diets (Figure 6). In contrast, ROH mice were apparently unable to compensate for protein scarcity through an increased food intake; in fact, their intake on the 30 g CP per kg diet was significantly lower compared to ROH mice on higher CP diets (Figure 2). This result, is in accordance with the findings of Boulay *et al* (1998) where food intake on the 30 g CP per kg diet was greatly reduced and the highest intake was observed on 70 g CP per kg diet. The significantly reduced intake of ROH-30 mice coincided with them having the lowest body fat percentage (Figure 6). It remains unclear why ROL and not ROH were able to overcome protein scarcity through displaying increased food intake but the data support the view that the protein requirement in ROL mice may be considerably lower than that in ROH mice. As such, the conditions for studying the effects of protein scarcity on genetic growth potential have not been met, which consequently confounds effects of protein scarcity with effects of genetic growth potential at the lowest levels of protein used in our study.

In conclusion, this study supports the view that narrow selection for a performance trait, body weight in this instance, may penalise immunocompetence and thus host

ability to cope with a primary pathogen challenge and that, at least in terms of resilience, ensuring sufficient protein nutrition could minimise this penalty. Resistance *per se* may be due to genetic linkage or pleiotropy or alternatively through genetic drift during a primary infection, as this study found a lack of evidence supporting the allocation theory. Whether host protein nutrition affects the consequences of narrow selection for production traits on ability to cope with secondary infections remains to be addressed.

Chapter 3. Interactive effects of protein nutrition, genetic growth potential and *Heligmosomoides bakeri* infection pressure on resilience and resistance in mice

Coltherd, J.C., Babayan, S.A., Bünger, L., Kyriazakis, I., Allen, J.E. and Houdijk, J.G.M (2011) *Parasitology*, *Under Review*

3.1 Summary

The ability of animals to cope with an increasing parasite load, in terms of resilience and resistance, may be affected by both nutrient supply and demand. Here, we hypothesised that host nutrition and growth potential interact and influence the ability of mice to cope with different parasite doses. Mice selected for high (ROH) or low (ROL) body weight were fed a low (40 g/kg; LP) or high (230 g/kg; HP) protein diet and infected with 0, 50, 100, 150, 200 or 250 L₃ infective *Heligmosomoides bakeri* larvae. ROH-LP mice grew less at doses of 150 L₃ and above, whilst growth of ROH-HP and of ROL mice was not affected by infection pressure. Total worm burdens reached a plateau at doses of 150L₃, whilst ROH mice excreted fewer worm eggs than ROL mice. Serum antibodies increased with infection dose and ROH mice were found to have higher parasite-specific IgG1 titres than ROL mice. In contrast, ROL had higher total IgE titres than ROH mice, only on HP diets. The interaction between host nutrition and growth potential appears to differentially affect resilience and resistance in mice. However, the results support the view that parasitism penalises performance in animals selected for higher growth.

KEYWORDS

Threshold level of parasitism; *Heligmosomoides bakeri*, protein nutrition; growth potential; resilience; resistance; serum antibody; mice

3.2 Introduction

Exposure to parasites is ubiquitous in both livestock and wildlife species (Holmes, 1993). In most cases this exposure leads to subclinical infection and immunity is gradually acquired. Development of immunity to gastrointestinal nematodes usually coincides with periods of high growth rates and thus high nutrient requirements (Coop and Kyriazakis, 2001). In addition, parasitic infection can be accompanied by variable pathology (Garside *et al.*, 2000) and anorexia (Kyriazakis, 2010), which can readily lead to nutrient scarcity even when animals are fed on good quality foods. Under these conditions, hosts may be forced to allocate scarce nutrient resources between the competing traits of growth and parasite control (Coop and Kyriazakis, 2001). Here, we assess whether this allocation is sensitive to infection pressure and genetic potential for growth.

It has long been proposed that a minimum level of antigenic stimulation or infection pressure may be necessary for stimulation of an immune response upon parasitic infection (Dineen, 1963). In agreement, a certain level of parasite infection pressure is also required to produce clinical infection (Vercruyssen and Claerebout, 2001) and this has long been known as the case for *Heligmosomoides bakeri* infections (Ehrenford, 1954). It has been suggested that such ‘thresholds’ may be lower during periods of nutrient scarcity (Bransby, 1993). In addition, Keymer and Tarlton (1991) observed that the accumulation of *H. bakeri* was proportional to the infection pressure at times of protein scarcity, whereas this pattern of accumulation was not observed at high protein levels.

Rauw *et al* (1998) suggested that artificial selection for enhanced production traits can lead to detrimental changes in host behaviour, physiology and immunity. These

changes may arise from allocation of a higher proportion of scarce environmental resources towards the trait selected for, leaving fewer resources available for investment in other physiological processes that contribute to overall fitness (Rauw *et al.*, 1998; Coop and Kyriazakis, 1999; Doeschl-Wilson *et al.*, 2008). Consequently, we predict that increased resource availability would minimise these negative consequences of artificial selection for enhanced production traits.

We recently demonstrated that mice selected for high body weight (Roslin-high mice, ROH) were less resilient (measured as performance under infection [described by Albers *et al.*, 1987]) and less resistant to a single primary infection of 250 L₃ *H. bakeri* than their low growth counterparts (Roslin low growth mice, ROL), especially when given access to low protein foods (Coltherd *et al.*, 2009). This suggests that hosts with a higher potential for productive functions may allocate more nutrients to such functions when nutrients are scarce, at the expense of functions involved with limiting parasitism, including anti-helminth immune responses (Wahid *et al.*, 1994). Here, we hypothesise that this penalty on resilience in ROH mice will decrease with lower infection levels and that ROL mice will tolerate higher infection level. We also hypothesise that the levels of infection, which would be tolerated are lower at times of protein scarcity, and that this nutritional sensitivity would be more pronounced in mice genetically selected for high growth (ROH).

3.3 Materials and Methods

3.3.1 Animals and housing

A cross of two inbred lines (C57BL/6J x DBA/2J) was used as foundation population for a selection experiment in which divergent selection for high and low body weight (BW) at day 42 of age took place over 20 generations resulting in due course in ROH and ROL lines, respectively (Heath *et al.*, 1995; Bünger *et al.*, 2001a). Under maintenance conditions and when offered a standard diet, at 42 and 70 days of age respectively, ROH mice would be expected to reach weights of 36g and 41g, with ROL mice reaching 16g and 19g respectively (Bünger *et al.*, 2001b). For this experiment, we obtained 72 ROH and 72 ROL inbred male mice, weaned at 21 to 23 days and housed individually in a controlled environment with an ambient temperature of $21\pm 1^{\circ}\text{C}$ and a 12h light cycle. At weaning, our ROH mice had a body weight of $15.4\pm 0.25\text{g}$, whereas ROL mice weighed $7.4\pm 0.26\text{g}$. Mice were housed in solid bottomed cages with fresh sawdust and bedding material was provided twice weekly. The experiment details described below were approved by the Animal Experiment Committee of Scottish Agricultural College (ED AE 26/2007) and carried out under Home Office regulations (PPL 60/3626).

3.3.2 Diets

All mice were fed *ad libitum* standard expanded breeding diet [RM3(P), Special Diet Services, Witham, UK; digestible crude oil: 38 g/kg; digestible crude protein (CP): 202 g/kg; starch: 339 g/kg; sugars: 44 g/kg; digestible energy (DE), 12.2 MJ/kg] for one week after arrival. Two iso-energetic experimental diets (15 MJ DE/kg) with a fixed amino acid to CP ratio were used, formulated to provide 40 (LP) and 230 (HP) g CP per kg (Table 3.1). These CP levels were expected to result in protein scarcity

or protein adequacy, respectively, relative to expected requirements (NRC, 1995) and as informed by our previous work using a wide range of dietary CP levels (Coltherd *et al.*, 2009; Chapter 2). As casein was used as the protein source, 15 g cysteine was added to each kg of casein to account for the scarcity of sulphur containing amino acids.

Table 3.1: Ingredients and analysis of the experimental diets

Ingredients (g/kg)	Experimental diets	
	LP	HP
Rice starch	556.8	332.0
Maltodextrin	132.0	132.0
Sucrose	100.0	100.0
Soya oil	70.0	70.0
Cellulose	50.0	50.0
Vitamins, minerals and amino acids	48.2	51.4
Casein edible acid	43.0	264.0
Analysis		
Dry matter (g/kg)	902.00	878.40
Crude protein (g/kg dry matter)	46.70	219.00

3.3.3 Infection protocol and experimental design

The strain of *H. bakeri* used was obtained from Professor Jerzy Behnke (University of Nottingham, UK), and maintained in our lab through passages in C57BL mice (See Jenkins & Behnke, 1977 for full origin details). At day 0 of the experiment (see below) all mice either received a single infection of 0, 50, 100, 150, 200 or 250 *H. bakeri* infective larvae (L3) suspended in 0.2ml water or a sham infection of 0.2ml water via oral gavage. The doses of *H. bakeri* were chosen to produce a range of sub-clinical infections that were expected to include levels where growth performance would be reduced (Houdijk & Bünger, 2006; 2007; Coltherd *et al.*, 2009; Chapter 2).

Mice within each line, infected at one of the six experimental infection levels (referred to as 0, 50, 100, 150, 200 and 250), were given *ad libitum* access to either LP or HP foods. The experiment was thus 2 lines x 6 levels of infection x 2 protein foods; each cell within this design contained 6 replicates, thus a total of 144 mice were used. Figure 3.1 illustrates the experimental design and timing of procedures. Mice entered the experiment (day 0) through an adaptation phase (day -14 to day 0), which included a period where a 50:50 mix of experimental and standard diet was offered to acclimatise the mice to the experimental diet (day -7 to day -4) followed by feeding the experimental diets alone (day -4 onwards). Mice were humanely killed on day 28 post infection (p.i.), for the assessment of worm burdens, colon egg count, body fat percentage and serum antibodies.

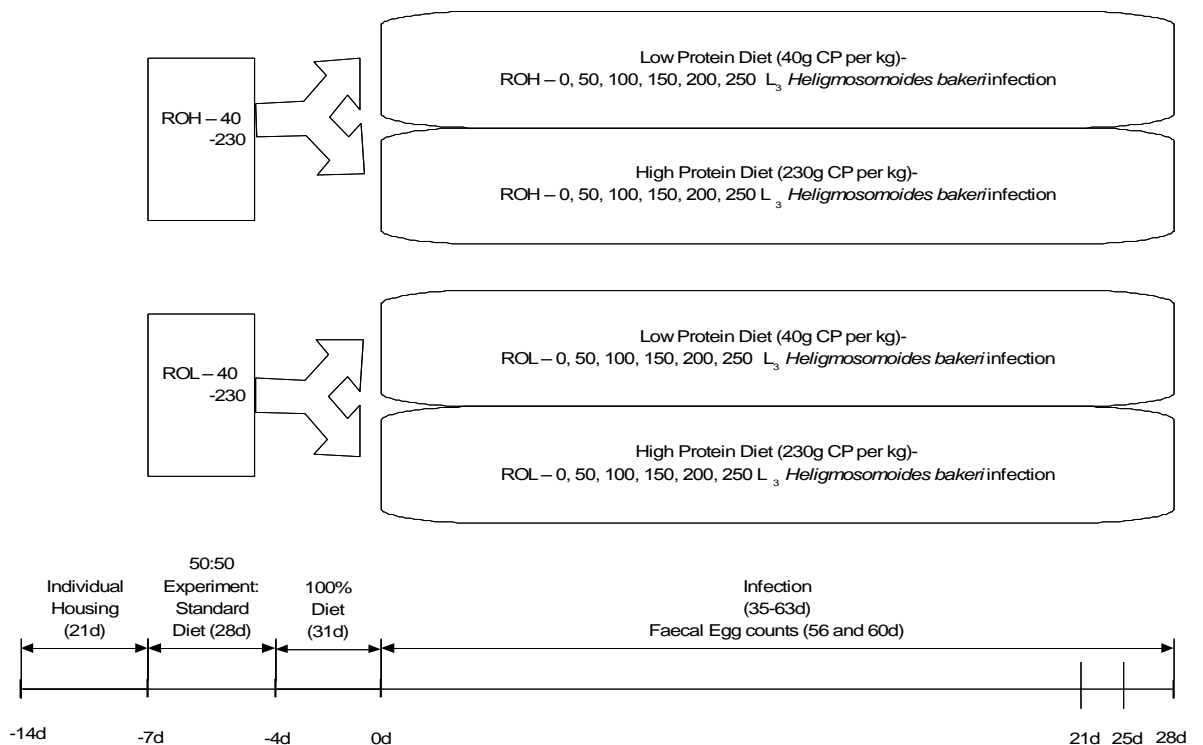


Figure 3.1. Diagram of experimental design. Timeline in experimental days shown along the bottom and age of mice between brackets under the experimental procedure description above the line.

3.3.4 Sample measurements and collection

Body weight and food intake. Between day 0 and 28 p.i., mice and food refusals were weighed twice weekly (Tuesday and Friday) resulting in 8 experimental periods for food intake. On each of these days food refusals were weighed out and fresh food weighed in. Around 30g was offered to ROH and 15g to ROL mice to ensure *ad libitum* feeding (Coltherd *et al.*, 2009; Chapter 2). Average daily food intake was calculated per day for each of the 8 experimental periods for each mouse, as well as mean food intake over the total 8 experimental periods.

Nematode egg count in faeces. Mice were placed onto wire-bottomed cages overnight for faecal sample collection on days 21 and 25 p.i. A modified flotation technique (Christie & Jackson, 1982) was used to assess the concentration of nematode eggs per g faeces. The total period of faecal collection (finish time – start time) was recorded to calculate an estimated faeces volume per 12 hours per mouse. This value was used to standardise egg output (EO, eggs excreted per 12 h) to eliminate the dilution effect expected for ROH mice due to their larger size, food intake and thus larger volumes of faeces (Coltherd *et al.*, 2009; Chapter 2).

Worm burden and nematode egg count in colon contents. Mice were killed humanely on day 28 via CO₂ inhalation and dissected to obtain the small intestine and the colon. The small intestine was weighed, opened and placed directly into a 5% formaldehyde solution pending assessment of the number of male and female worms. The colon contents were weighed and a colon egg count (eggs/g) performed for every mouse. The resulting colon egg count was then multiplied by the colon contents weight to eliminate any dilution effects and obtain a final number of eggs in

colon (EIC) value. The EIC was divided by the number of females counted to estimate *per capita* fecundity (eggs per female).

Fat percentage. To predict percentage of carcass fat, the mouse carcasses were weighed and bagged upon dissection for subsequent freeze-drying. In preparation for the freeze-drying process, incisions were made, to allow maximal water loss, in the back, tail and head of the animal. When weight loss ceased (approximately 7 days later) the dried carcasses were re-weighed. Fat percentage was calculated as $[(\text{freeze-dried weight} \times 1.13)/\text{carcass weight}] - 0.302 \times 100$ (Hastings & Hill, 1989)

Enzyme-Linked Immunosorbent Assays – ELISAs. At dissection, blood was collected from the chest cavity into a 2ml tube containing Serasieve® (Hughes & Hughes Ltd., Wellington, UK) and serum collected through centrifugation at 3000 rpm. In the sera, parasite-specific IgG1 and IgG2a was measured as key antibodies associated with Th2 and Th1 responses, respectively, whilst total IgE was measured as an antibody specifically implicated in protective immunity against parasites like *H. bakeri* (Urban *et al.*, 1991; 1992; Wahid *et al.*, 1994; Negrao-Correa *et al.*, 1999) and specifically regulated by the Th2 cytokine IL-4 (Silva *et al.*, 2006). Antibody levels were used as read-outs for an indirect measurement of immune costs to indicate possible trade-offs. The concentration of anti-*H. bakeri* antibody IgG1 and IgG2a was measured by an indirect ELISA. 50µl *H. bakeri* soluble extract in carbonate buffer (5µg/ml) was added to each well of a 96 well plate and left to incubate over night at 4°C. Plates were then washed with PBS-Tween and blocked using 100µl of PBS + BSA 4% for one hour at 37°C in the dark. Plates were again washed with PBS-Tween, 50µl of serum, serial-diluted 11 times from 1/400 in PBS-BSA 1%, added and the plates incubated over night at 4°C. Plates were washed with PBS-Tween and 50µl HRP-

conjugated detection antibody was used. The plates were finally incubated in 50% TMB - 50% H₂O₂ (KPL) under silver foil to protect from the light until the top two standards had saturated. Colour development was then stopped using 25µl 1N HCl, and plates were read at 450nm.

The concentration of total IgE antibody was measured by a capture ELISA. 96-well plates were coated with 50µl IgE (2µg/ml) capture antibody and left at 4°C overnight. Capture antibody was flicked off and 100µl Marvel solution:carbonate buffer (5%) added and left for 2 hours incubation at 37°C. Plates were then washed in TBST, a row of standards was created from a top standard of 5µg/ml and doubling down across the columns, the sera wells were diluted 1/10 and 1/20 and the plates were incubated at 37°C for 2 hours. Plates were again washed in TBST, 50µl biotinylated 2° detection IgE (2µg/ml) added then incubated for a further hour at 37°C. Plates were washed in TBST, 100µl conjugate Extravidin Peroxidase (1/8000) added and the plates incubated at 37°C for 30 minutes. Plates were finally washed in TBST, then in H₂O before 100µl substrate TMB was added to each well, and the plate developed in darkness until saturation had occurred and stopped using 100µl 1M HCl. The plate was then read at 450nm on a spectrophotometer.

3.3.5 Statistical Analysis

Due to the skewed nature of the data, Egg Output (EO), Eggs In Colon (EIC) and worm burdens were Log₁₀ (n+1) transformed. To account for the relatively large differences in performance data between the mouse lines, arising from *a priori* differences in body weight, feed intake and body weight gain data (both in mg per day) were also Log₁₀ transformed before analysis (Falconer and MacKay, 1996; Coltherd *et al.*, 2009). Repeated measures through Restricted Maximal Likelihood

(REML) was used to assess interactive effects of line, dietary CP content, infection dose and time on food intake. The interactive effects of line, dietary protein content and infection pressure on mean food intake, body fat percentage, EO, worm burden data and serum antibody titres were analysed with REML to allow inclusion of litter origin as a random effect. Interactions that did not approach significance were omitted from the final model. EO, EIC and total worm burdens were checked for non-linearity by fitting a quadratic regression using infection pressure as an explanatory variable. Significance is indicated by a p-value less than 0.05 and where appropriate trends are indicated by a p-value between 0.051 and 0.1. All reported error values are standard errors of the mean. All statistical analyses were performed using Genstat 11 for Windows release 11.1, 2008 (Lawes Agricultural Trust, Rothamsted, UK).

3.4 Results

3.4.1 Food intake and body weight gain

Resilience was measured by assessing food intake and weight gain under infection. Figure 3.2 shows \log_{10} -transformed averaged daily food intake and average daily weight gain. Mouse line and dietary protein content interacted significantly for average daily food intake ($F_{1,96}=16.28$, $p<0.001$; Figure 3.2a). This interaction arose from ROH-0 mice on the LP food consuming less food than their HP counterparts, whilst in contrast ROL-0, ROL-50 and ROL-100 mice on the LP food consumed more food than their HP counterparts. As expected, ROH mice consumed more food than ROL mice ($F_{1,32}=260.20$, $p<0.001$). In addition, there were no 2, 3 or 4 way interactions containing time and infection pressure ($p>0.10$) indicating the absence of observable anorexia. However, time did interact with mouse line and dietary protein ($F_{8,936}=5.25$, $p<0.001$) for food intake; ROL-LP mice ate more than ROL-HP mice in each period, whilst ROH-LP ate more than ROH-HP during periods 1 and 2 only, but ate less than ROH-HP from period 4 onwards (data not shown).

All mice gained weight during the experiment, ROH mice gained an average of $0.36\pm 0.019\text{g/d}$ while ROL mice gained on average $0.15\pm 0.005\text{g/d}$. Genetic growth and dietary protein interacted for weight gain ($F_{1,117}=50.12$, $p<0.001$). This was due to ROH mice reducing their weight gain on the LP diet whilst ROL mice were unaffected. A trend was observed for the three-way interaction between genetic growth, dietary protein content and infection pressure for mean daily weight gain ($F_{5,117}=1.96$, $p=0.09$; Figure 3.2b). Weight gain of ROH-LP mice was similar as infection pressure increased from 0 to 100 L_3 , but significantly decreased as infection pressure further increased 150 to 200 and 250 L_3 , whilst infection pressure did not affect weight gain of ROH-HP and ROL mice. However, in the absence of infection,

the effect of dietary protein on weight gain differed between ROH and ROL mice; weight gain of ROL-LP mice was the same as that of ROL-HP mice, whilst weight gain was lower for ROH-LP mice compared to ROH-HP mice ($F_{1,117}=86.10$, $p<0.001$).

3.4.2 Body fat percentage

Body fat percentage was measured to assess the extent to which excess energy was stored by mice. The interaction between mouse line and dietary protein content was highly significant for body fat percentage (Figure 3.3; $F_{1,97} = 14.41$, $p<0.001$); independent of infection pressure, ROL-HP mice had a lower body fat percentage than ROL-LP mice, whilst feeding treatment did not affect fat percentage in ROH mice, causing this interaction to be observed. Parasitism did not impose an energetic requirement that could not be met by the experimental diets fed in this experiment.

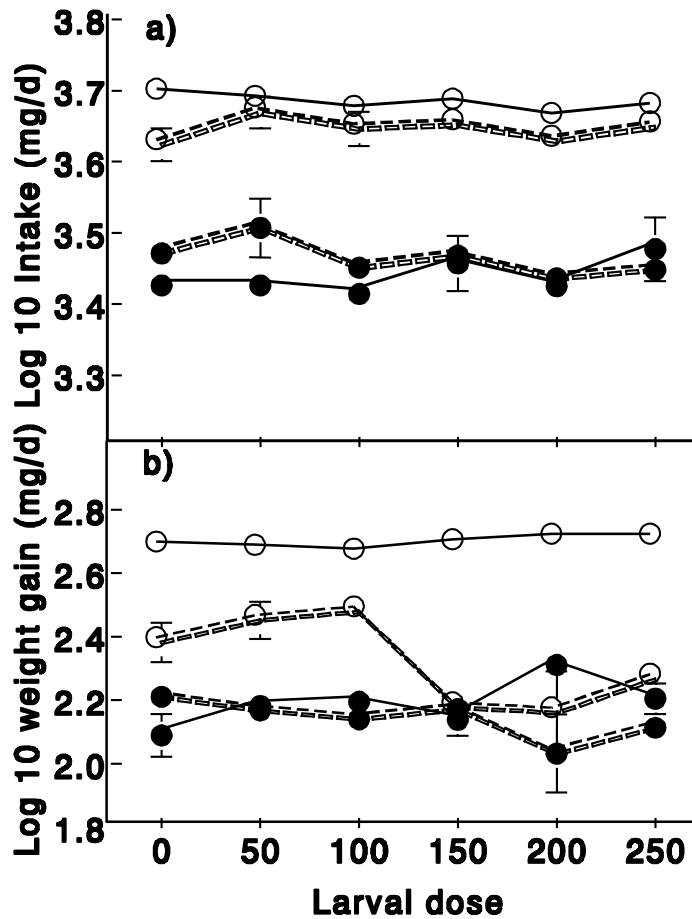


Figure 3.2. a) Log₁₀ transformed daily food intake and b) body weight gain of high (ROH – open circle) and low (ROL – closed circle) body weight mice averaged over 28 days of infection with *H. bakeri* or sham infection with water on a high (solid line) or low (dashed line) crude protein diet

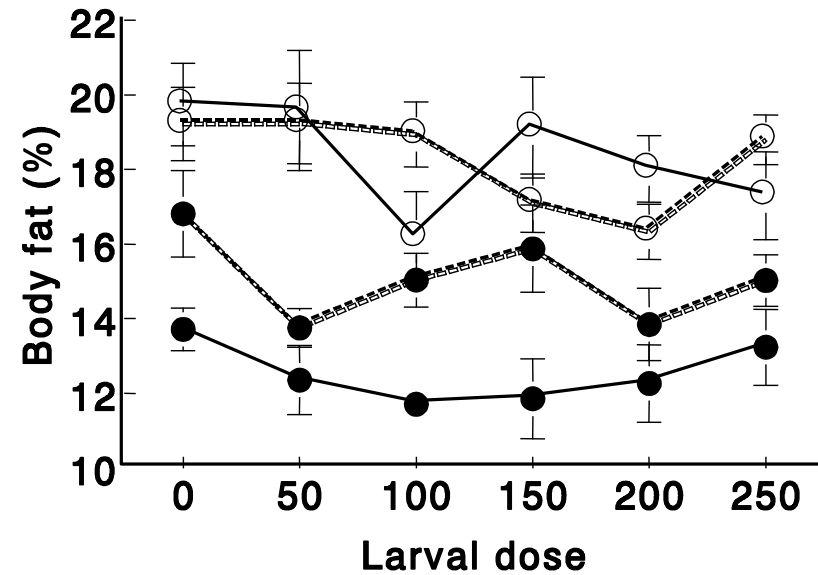


Figure 3.3. Average body fat percentage, taken 28 days after infection, of high (ROH – open circle) and low (ROL – closed circle) body weight mice, either infected with *H. bakeri* or sham infected with water and fed on a high (solid line) or low (dashed line) crude protein diet

3.4.3 Egg output (EO), eggs in the colon (EIC) and total worm burden (TWB)

Resistance to parasitism was measured by assessing worm burden and parasite egg output. Figure 3.4 shows the $\text{Log}_{10}(n+1)$ transformed mean EO, EIC and TWB. In addition, Table 3.2 provides backtransformed means with 95% confidence intervals for TWB, male and female burdens and *per capita* fecundity. The mean EO averaged over days 21 and 25 p.i. was analysed as there was no interaction between time and infection dose, dietary protein and/or genetic mouse line ($p > 0.10$). EO, EIC and TWB were found to be non-linear ($p > 0.05$). EO, EIC and TWB increased with increased infection pressure ($F_{4,83}=25.93$, $p < 0.001$, $F_{4,98}=17.72$, $p < 0.001$ and $F_{4,86}=42.3$, $p < 0.001$ respectively; Figure 3.4) until a plateau was reached at 150L₃. ROL mice had a higher EO and EIC than ROH mice ($F_{1,30}=8.21$, $p=0.008$ and $F_{1,98}=10.73$, $p=0.001$ respectively). However, mouse line and dietary protein content tended to interact for EO; ROL-HP had higher EO than ROL-LP at the lowest infection pressure only ($F_{1,83}=3.26$, $p=0.075$; Figure 3.4a). Thus, increasing infection pressure over 150 L₃ did not further increase parasite load.

Mouse line and dietary protein content interacted for number of male worms; ROH-HP mice had fewer male worms than ROH-LP mice whilst feeding treatment did not affect the number of male worms in ROL mice ($F_{1,99}=4.48$, $p=0.037$; Table 3.2). The three-way interaction between mouse line, dietary protein content and infection pressure tended towards significance for TWB composition ($F_{4,94}=2.26$, $p=0.069$). This was due to a higher percentage of female worms in ROH-HP ($62.6 \pm 3.97\%$) mice across all infection pressures, when compared to ROH-LP ($45.0 \pm 3.07\%$), whilst in contrast dietary protein content did not systematically affect TWB composition in ROL mice. *Per capita* fecundity was found to be higher in ROL mice ($F_{1,93}=8.31$, $p=0.005$) than in ROH mice but was not affected by either dietary protein contents or

infection pressure (Table 3.2). Sex ratios of *H. bakeri* were altered in ROH mice by a HP diet reducing the numbers of male worms when compared to mice on the LP diet.

3.4.4 Serum antibody titres

Figure 3.5 shows the serum IgE and IgG1 titres, which demonstrate that an immune response was induced at all infection levels. Mouse line and dietary protein content interacted for total IgE ($F_{1,101}=6.77$, $p=0.011$; Figure 3.5a); averaged IgE optical density was higher for ROL than for ROH on HP diets, but were similar for LP diets. Although Figure 3.5a suggests that these interactions were observed at the 50 and 100 L3 infection pressure only, interactions between mouse line, dietary protein contents and infection pressure were not significant ($p>0.17$). However, total IgE increased with increased infection pressure ($F_{5,102}=51.82$, $p<0.001$).

Mouse line and infection pressure interacted for anti-worm IgG1 ($F_{5,90}=7.82$, $p<0.001$; Figure 3.5b). IgG1 titres in ROL mice increased in a dose dependent manner up to 200L₃, whilst IgG1 titres were higher in ROH mice ($F_{1,31}=14.69$, $p<0.001$), and increased linearly with infection pressure up to 250L₃.

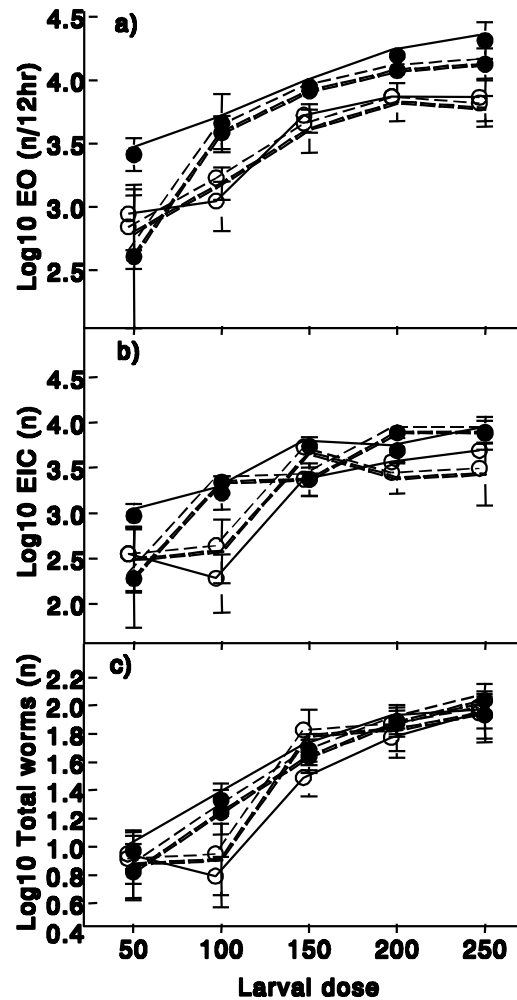


Figure 3.4 - Log₁₀ (n+1) transformed: a) 12 hour Egg Output (EO) averaged over day 21 and 25 post infection; b) Eggs in the Colon (EIC) on day 28 post infection and c) total worm burden on day 28 post infection for high (ROH – open circle) and low (ROL – closed circle) body weight mice, infected with different levels of *H. bakeri* and fed on a high (solid line) or low (dashed line) crude protein diet.

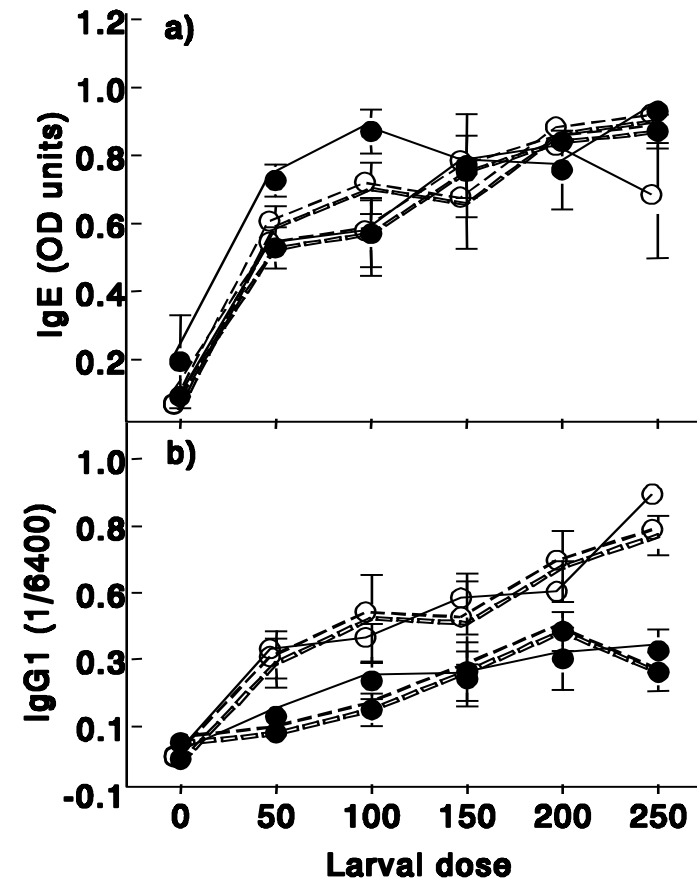


Figure 3.5 - a) Total serum IgE antibody titres, and b) Anti-*H. bakeri* IgG1 serum titre for high (ROH – open circle) and low (ROL – closed circle) body weight mice fed a high (solid line) or low (dashed line) crude protein diet.

Table 3.2. Total worm burdens in dependence of line, CP content and infection with their confidence intervals (CI)

Line	Diet	Inf	Total Mean (95% CI)	Males Mean (95% CI)	Females Mean (95% CI)	PFC Mean (95% CI)		
ROH	LP	50	10 (5-19)	5 (3-9)	5 (3-8)	127 (98-163)		
		100	12 (6-21)	6 (3-11)	6 (3-10)	151 (120-190)		
		150	76 (50-117)	38 (25-57)	38 (25-59)	169 (136-209)		
		200	78 (55-112)	37 (29-47)	44 (24-79)	113 (78-163)		
		250	97 (75-126)	54 (43-67)	46 (26-81)	105 (87-127)		
		50	9 (6-14)	5 (3-7)	4 (3-7)	143 (93-218)		
	HP	100	6 (4-10)	2 (1-3)	4 (3-7)	67 (42-108)		
		150	29 (23-36)	10 (9-12)	18 (14-24)	128 (117-140)		
		200	58 (45-74)	23 (18-30)	34 (27-44)	108 (86-135)		
		250	95 (64-142)	38 (18-30)	57 (39-82)	91 (75-110)		
		ROL	LP	50	8 (5-13)	4 (2-5)	4 (3-6)	207 (84-508)
				100	20 (13-30)	8 (5-13)	12 (8-17)	250 (196-318)
150	47 (36-62)			22 (16-30)	25 (19-33)	110 (89-136)		
200	78 (65-95)			38 (30-48)	40 (34-47)	209 (177-247)		
250	119 (90-156)			59 (44-79)	60 (47-77)	144 (129-162)		
50	10 (7-13)			3 (2-5)	6 (5-8)	190 (146-247)		
HP	100		23 (17-30)	11 (9-15)	11 (8-15)	177 (151-208)		
	150		52 (40-67)	25 (18-34)	27 (22-34)	201 (153-263)		
	200		84 (65-109)	40 (30-53)	44 (35-56)	130 (103-163)		
	250		103 (70-151)	46 (32-67)	57 (28-84)	171 (147-199)		
	P-values							
	Line			0.171	0.186	0.135	0.006	
Diet			0.245	0.026	0.886	0.629		
Inf			<0.001	<0.001	<0.001	0.913		
LxD			0.101	0.011	0.590	0.117		
LxI			0.161	0.082	0.603	0.219		
DxI			0.929	0.964	0.899	0.120		
LxDxI			0.879	0.629	0.762	0.397		

3.5 Discussion

The results of this study support our hypothesis that selection for increased body weight induces a penalty of parasitism on resilience above a certain level of infection (>150L3 *H. Bakeri*) but only when protein supply is scarce.. However, contrary to our previous findings (Coltherd *et al.*, 2009) and our hypothesis, selection for increased body weight did not detrimentally affect resistance traits, as mice selected for low body weight displayed increased nematode egg excretion. This leads to the suggestion that ROL mice may be more resilient to parasitic infection and can maintain growth despite higher levels of parasitism; On the other hand resilience in ROH mice is sensitive to nutrient scarcity. It remains to be ascertained whether this disparity in resistance between the mouse lines is due to selection for body weight inadvertently co-selecting genes associated with immunity (e.g. different MHC alleles; Behnke and Wahid, 1991). Despite our intention, this study failed to achieve protein scarcity in ROL mice meaning that some results and comparisons may be confounded by protein sufficient conditions for all ROL mice being compared with protein deficient and sufficient ROH mice. For this reason, the main comparisons between mouse lines will be between individuals fed the high protein diet to ensure a similar nutritional status.

3.5.1 Body weight, food intake and fat percentage

A reduction in body weight gain was observed in ROH mice due to reduced dietary protein content and, during protein scarcity, gain was further reduced when infection pressure increased to a dose over 150L₃. In contrast, ROL mice performance was not affected by infection, whilst uninfected ROL-LP mice gained more weight than their HP counterparts. This weight gain in ROL-0-LP was associated with higher feed intake on the LP diet compared to the HP diet, which was likely the result of

successful attempts to increase protein intake from a low protein diet (Kyriazakis *et al.*, 1991). The resulting excess energy intake would need to be lost as heat or stored as fat (Emmans and Kyriazakis, 2000); the latter is consistent with the higher body fat percentage observed for ROL-LP mice compared to ROL-HP mice in this and our earlier study (Coltherd *et al.*, 2009). There was no effect of infection on body fat percentage in this experiment in contrast to the findings of calorie restrictive and metabolic stress experiments, which show that infection decreases body fat percentage (Kristan and Hammond, 2001; 2006).

Under the theory that nutrients are partitioned towards artificially selected traits (Rauw *et al.*, 1998; Coop and Kyriazakis, 2001; Doeschl-Wilson *et al.*, 2009) and away from unselected ones, ROH growth on the low protein diet suggests that this pattern of nutrient partitioning may indeed have been the case at low levels of infection. The reduction of body weight gain at higher infection pressures (150L₃ and above) is possibly an outcome of the intensity of the infection being sufficiently high to potentially affect host fitness. Consequently, it would be expected that the host would divert nutrients away from growth functions and towards immune function to limit the pathology and/or fitness consequences caused by parasitism and increase the chances of host survival. Therefore, this suggests a novel hypothesis, i.e. that nutrient allocation is sensitive to infection pressure.

The lack of parasite-induced anorexia across the infected mice was unexpected as such a reduction had been observed in previous studies with this mouse- parasite model (Houdijk and Bünger, 2006; Coltherd *et al.*, 2009) and in other studies using *H. bakeri* (Boulay *et al.*, 1998; Ing *et al.*, 2000; Tu *et al.*, 2007). A reduction in feed intake would be expected to occur mainly during the initial stages of infection (Coltherd *et al.*, 2009; Kyriazakis, 2010), and it might be that this would only have been evident above a certain level of infection pressure (Sandberg *et al.*, 2006).

Therefore, it cannot be excluded that diluting effects of mice infected below such level of infection pressure did not allow us to statistically detect the presence of anorexia. Indeed, assessing the effect of infection on feed intake at each infection pressure separately showed that only mice infected with 250 L₃ experienced anorexia observed between days 7 and 10 post infection ($F_{8, 296} = 2.68$, $p=0.039$; data not shown). However, this effect was small, supported by the absence of a meaningful interaction between infection pressure and time for feed intake when analysing all infection pressures simultaneously ($F_{40,936}=1.46$, $p=0.102$).

3.5.2 Worm burden and egg output

As infection pressure increased, the worm burden and number of eggs released increased to a maximum level and then appeared to plateau. This maximum level was lower in ROH mice than in ROL mice, which was contrary to our previous findings (Coltherd *et al.*, 2009). The observed plateau in egg output and worm burden in ROH mice suggests an optimal parasite load controlled by the host and corresponds to the further reduction in body weight gain seen at 150L₃ and above. Indeed Paterson and Viney (2002) proposed that host immune responses reduced parasite survival to limit parasite load as they saw no effect of infection pressure on establishment of parasites, but did observe that survivorship of parasites during a heavy infection was significantly reduced as duration of infection increased. Density dependent effects on parasite numbers and egg output are commonly observed. For gastrointestinal parasites this relationship is usually negative (or inverse), i.e. as density of the parasite population increases worm survival and fecundity decrease (Bishop and Stear, 2000; Bleay *et al.*, 2007). Christensen *et al* (1995) found that egg production and *per capita* fecundity progressively reduced as infection pressure of *Oesophagostomum dentatum* increased from 2,000 to 20,000 and 200,000 L₃. This

observation of *per capita* fecundity differs from our results, where infection pressure and dietary protein content did not affect fecundity. Observations made by Keymer and Slater (1987) suggest that *per capita* fecundity is highly variable when worm burden is low, with around 78% of the worms having similar egg production to hosts with higher worm burdens. This observation may be an explanation for the lack of density dependent effects on fecundity in our model, as we also found high variability in egg counts at lower levels of infection (data not shown). There are two main proposed mechanisms for the observation of density dependent egg production (Anderson and Michel, 1977; Kerboeuf and Jolivet, 1984; Irvine *et al.*, 2001). Firstly, intra-specific competition may limit resources for the natural development of adult parasites and the production of eggs by adult females. Secondly, host immune responses may stunt worm development directly, limiting female size and thus fecundity. From the latter explanation it would be expected that infections in immune animals are more sensitive to nutritional manipulation, as has been shown in several different species (Keymer and Tarlton, 1991; Houdijk *et al.*, 2005; Ing *et al.*, 2000; Kidane *et al.*, 2009). This may account for the lack of evidence of density dependant effects on egg production during this study using a single-dose primary infection.

The lower proportion of female worms present in ROH-LP TWB when compared to ROH-HP suggests that decreased protein supply may have altered the sex ratio either through altering the gut environment, e.g. causing villi atrophy (Tu *et al.*, 2007) which in turn reduces preferred habitat and feeding opportunities (Bansemir and Sukhdeo, 1994; 1996) and may reduce mating opportunities, or alternatively through disproportionate survival of larvae (Stien *et al.*, 2005). One might expect that female worms would be most affected by reduced feeding opportunities due to their

larger size when compared to male worms (Poulin, 1997). Indeed, reduced worm egg production has been observed in animals fed very low protein diets (Athanasiadou *et al.*, 2001; Coltherd *et al.*, 2009). It follows that female worm development, and so capacity for egg production, may have been stunted due to limited feeding opportunities. This perhaps was not observed in the current study due to the low protein diet, 40g CP per kg, not being sufficiently low to produce effects on fecundity which was observed at 30g CP per kg previously (Coltherd *et al.*, 2009).

3.5.3 Antibody concentrations

The serum antibody titres, although used as an indirect measure of a resource cost born by the immune system, can also give some indication of the polarisation of the immune system. The Th2 type antibodies IgE and IgG1 were assessed due to their previously reported elevation during parasitic helminth infection (Urban *et al.*, 1991; Romagnani, 1991; Bell *et al.*, 1992), and specifically in *H. bakeri* infections (Urban *et al.*, 1991; Ben-Smith *et al.*, 1999; Negrao-Correa *et al.*, 1999). IgE levels were higher in ROL than in ROH mice, with both mice lines showing increasing IgE antibody levels with increasing infection pressure, whilst dietary protein contents did not affect IgE production. Whilst this is in agreement with Boulay *et al.* (1998), Ing *et al.* (2000) did find that at day 28 post primary infection protein malnutrition reduced IgE titres in an *H. bakeri* infection. This may be due to the different inbred mouse strain used (Behnke *et al.*, 2006) and/or a longer adaptation period to the experimental diets. Parasite-specific IgG1 levels were found to increase in response to increasing larval pressure for both mouse lines, with the exception that IgG1 concentration levelled off at 200L₃ for ROL mice whilst continuing to increase in ROH mice. ROH mice also had consistently higher IgG1 levels than ROL mice. Again, we did not observe an effect of dietary protein contents for parasite specific

IgG1, which is in accordance with Boulay et al (1998). The increasing parasite-specific antibody responses with increasing larval pressure was also observed during an early antibody response to the liver fluke *Opisthorchis viverrini* in hamsters, as the infection became more chronic (6 month infection) however, this relationship inverted and an infection with 25 metacercariae produced higher responses than 50 and 100 metacercariae (Sripa and Kaewkes, 2000). In contrast, a human study using *Necator americanus*, found that early parasite-specific IgG antibody responses were higher in subjects given 10 larvae when compared to 25 and 50 larvae, whilst later IgG (week 13) responses were lower in subjects given 10 larvae when compared to the higher doses. Furthermore early and late IgE responses were higher in subjects given 25 larvae compared to the other doses (Mortimer *et al.*, 2006). In this model, selection for increased body weight does not seem to have been detrimental to serum antibody production and, indeed, IgG1 concentrations were actually higher in ROH mice than in ROL. The differences in patterns of IgG1 vs. IgE in ROH vs. ROL mice in this study may be due to the more strict dependence of IgE on IL-4 (Silva *et al.*, 2006). Thus the polyclonal IgE response as measured here is likely to be a robust measure of ‘Th2’ bias, while the antigen-specific IgG1 responses may provide the more reliable measure of investment in parasite-specific immunity.

In conclusion, whilst the results of this study do not support our original hypothesis as far as resistance to parasites is concerned, they are consistent with the view that selection on the basis of high body weight may reduce the resilience to pathogen challenge (Rauw *et al.*, 1998). This penalty on performance may be sensitive to parasite load and can be overcome to an extent by increased dietary protein contents. Although increased dietary protein content had no effect on resistance to a primary infection consistently throughout our model (Coltherd *et al.*, 2009), it has been

demonstrated that increased protein nutrition will improve resistance to *H. bakeri* to a greater extent in a challenge infection (Boulay *et al.*, 1998; Ing *et al.*, 2000). The observation that the ROL mice showed increased resilience but also increased worm fecundity compared to ROH mice was unexpected and requires further investigation. The data potentially implies that resilient animals can be a source of infection to other animals in their environment as they are likely to be overlooked for drenching due to their lack of symptoms, thus increasing the risk to naïve animals from environmental contamination.

Chapter 4. Interactive effects of protein nutrition, genetic growth potential and *Heligmosomoides bakeri* infection pressure in resilience, resistance and *in vitro* cytokine production in mice

4.1 Summary

Host immunity towards gastrointestinal (GI) nematodes can be affected by a number of factors including genetic background and nutritional environment. Phenotypic selection for improved production traits has been shown to affect other traits such as reducing overall fertility and reducing immune responses. In the work presented in this chapter a 20-plex cytokine and chemokine assay was used (Luminex) on mesenteric lymph node (MLN) cell culture supernatants, either stimulated with *Heligmosomoides bakeri* whole worm preparation or unstimulated, to dissect the effects of mouse line, dietary protein content and *H. bakeri* infection on immune responses. These supernatants were from mice selected for high (Roslin high - ROH) or low (Roslin low - ROL) body weight fed high (HP) or low (LP) crude protein diets and infected with 200L₃ *H. bakeri* or sham infected with water. These treatments were chosen from those mice described in Chapter 3, representing a 2x2x2 factorial arrangement. The data show that the parasite infection did not polarise immune responses towards the expected Th2 phenotype, and that the LP diet and ROL mice produced an inflammatory immune response phenotype, upon infection. ROH mice did show a slight up-regulation in Th2 type cytokines, despite the lack of polarisation, and were shown to have reduced egg counts and total worm burdens as described in Chapter 3. The data from this chapter supports the view that phenotypic selection of production traits can have effects on immunity and that nutritional environment can also alter underlying immune responses.

4.2 Introduction

Parasitic infections induce host immune responses towards the invading pathogen and in the case of gastrointestinal (GI) nematodes the immune response is expected to be polarised towards a Th2 phenotype (Ing *et al.*, 2000; Ben-Smith *et al.*, 2003; Tu *et al.*, 2008). It is also known that the genetic background of the host and its current nutritional status are factors that can affect immune responses towards infectious challenges (Keymer & Tarlton, 1991; Wahid & Behnke, 1996).

It has been proposed that extreme phenotypic selection for production traits, such as increased body weight would be expected to bias the allocation of scarce resources towards the selected trait leaving fewer for others (Rauw *et al.*, 1998; Beilharz, 1998a; 2000). Indeed, it has been observed that, compared to random bred lines, selection for increased body weight and egg production in turkeys produced a decrease in primary antibody response (Bayyari *et al.*, 1997). Beilharz (1998b) illustrated the issue of limiting environments using the fact that American Holstein dairy cattle, intensively selected for high production, had lower fertility and productivity in Australia and New Zealand due to poorer nutrition than in their native America, where high levels of concentrate are fed.

In Chapters 2 and 3, obvious differences were observed between high body weight (ROH) and low body weight (ROL) mouse lines. This was characterised by a higher egg count and worm burden in ROH mice in Chapter 2 whilst the effect was reversed in Chapter 3 with ROL mice having higher worm burden and egg counts. It is well known that different mouse lines can have differences to their underlying immune responses, for example BALB/c mice are Th2 prone whilst C57BL/6 mice are Th1 prone (Peterson *et al.*, 1998), and SWR mice are classed as fast responders to

Heligmosomoides bakeri, clearing infection in 6 weeks, while CBA mice are slow responders taking several months (Menge *et al.*, 2003). The underlying immune responses of the mice used in the current set of experiments have yet to be characterised and may explain some of the differences in resistance observed.

In this chapter supernatants of mesenteric lymph node (MLN) cultures of mice from the two lines were selected for a twenty-plex Luminex assay to characterise the underlying immune responses of the lines due to infection and protein scarcity. It was hypothesised that i) infection would lead to an increase in Th2 type responses such as up-regulation of Interleukin (IL) 4 and IL-5; ii) ROL mice would produce a stronger immune response than ROH mice; and iii) protein scarcity would lead to a reduction in immune responses.

4.3 Materials and Methods

4.3.1 Animals and housing

Supernatants from mesenteric lymph node (MLN) *in vitro* restimulation assays for ROH and ROL mice fed 40 g crude protein (CP) per kg (LP) or 230 g CP per kg (HP) diets and either sham infected with water (0L₃) or infected with 200L₃ *Heligmosomoides bakeri* larvae were used. These data were obtained from mice used as previously described in Chapter 3.

4.3.2 Sample measurements and collection

Mesenteric Lymph Node Cell Proliferation Assay

Mesenteric lymph nodes (MLN) were extracted from the mice upon dissection and placed into RPMI-10%FCS with added 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine (medium). MLN were then crushed and filtered through sterile nitex (nylon mesh) and suspended in 5ml medium, this single cell suspension was then spun at 1500rpm for 5 minutes. The supernatant was discarded and 3ml RBC lysis buffer (Sigma) was added to incubate for 4 minutes. Then 10ml of medium was added and the solution again spun down for 5 minutes at 1500rpm. The supernatant was again discarded, the cells re-suspended in 10ml of medium and re-spun down as before. This was repeated again, re-suspending the cells in 5ml this time. The cells were then counted, spun down and re-suspended at a concentration of 1×10^7 cells per ml. 100µl MLN cells solution was added to round bottom 96 well plates, each individual mouse' MLN cells were cultured in triplicate with 100µl *H. bakeri* antigen (final concentration 10µg/ml) and medium alone. The cultures were incubated at 37°C 5% CO₂ for 72 hours. Cell viability and proliferation was measured by the Alamar blue assay (Ahmed *et al.*, 1994) as per the manufacturer's recommendations (AbD serotec Ltd, Oxford, UK). Briefly, 10%

Alamar blue was added to the cells for the last 48h of culture, and colour shift measured at 540nm on a spectrophotometer. The supernatant was then frozen at -20°C for later analysis as described below.

4.3.3 Luminex Analysis of MLN supernatant

Luminex protocol was followed as per manufacturer's recommendation and Luminex multiplex assay kit was used. Briefly, 10x Capture Bead stock was vortexed, sonicated and then diluted to 2.5µl in 25µl per well in Working Wash Solution. The standard and sample wells were wet with 200µl Working Wash Solution and the liquid removed from the plate with a vacuum manifold. The diluted Capture Bead Solution was then vortexed and sonicated and immediately added at 25µl along with 200µl of 1x Wash solution. The plate was then aspirated and repeated washed with 200µl of Working Wash Solution. 50µl Incubation Buffer was added to all the assay wells and 100µl of Standard was added to the designated standard wells. For the wells designated for the samples, 50µl Assay Diluents was added followed by 50µl of sample. The plate was covered and incubated for 2 hours at room temperature on an orbital plate shaker at 500-600rpm. Assay wells were aspirated and washed twice with 200µl Working Wash Solution and 100µl 1x Biotinylated Detector Antibody was added to each assay well. The plate was covered and incubated for 1 hour on a plate shaker at 500-600 rpm. The plate was again aspirated and washed twice with 200µl Working Wash Solution and 100µl 1x Streptavidin-RPE added to each assay well before the plate was covered and incubated for 30 minutes on a plate shaker at 500-600 rpm. Plates were then aspirated and washed three times with 200µl Working Wash Solution. The bottom of the plate was then dried with clean paper towels to remove residual droplets. 100µl Working Wash Solution was added to each assay well and the plate placed on a plate shaker at 500-600 rpm for 2-3

minutes. The plate was then read by the Luminex 100 instrument for Fibroblast Growth Factor (FGF), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), a series of interleukins (IL), i.e. IL-1a, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, Tumour Necrosis Factor-alpha (TNF- α) and Interferon-gamma (IFN- γ), as well as a series of chemokines, i.e. Interferon gamma induced protein (IP-10), Macrophage Inflammatory Protein 1a (MIP1a), Vascular Endothelial Growth Factor (VEGF), Keratinocyte-derived chemokine (KC) and Monokine Induced by gamma interferon (MIG). These proteins were selected for their involvement in inflammation and inflammatory disease (See Table 4.1 for functions of proteins).

4.3.4 Statistical Analysis

The Luminex multiplex data was analysed through a General Linear Mixed Model (GLMM) with a gamma distribution, and litter as a random effect. Only significant ($p < 0.05$) effects and proteins produced from the *H. bakeri* antigen (above unstimulated baseline) cultured replicates are reported in the results sections. Graphs were produced from the predicted means and standard error of deviation (S.E.D.) generated by the GLMM to enable the data to be visualised using the underlying correlations produced by the gamma distribution. Ingenuity Pathway Analysis software (IPA version 8.0, Ingenuity Systems Inc, California, USA. www.ingenuity.com) was used to investigate the expression patterns, identify biological functions and establish links between the various cytokines and chemokines measured during the Luminex analysis. The significance of the association between the data set and the pathways was determined based on a log-ratio of the number of proteins from the data set that map to the pathway divided by the total number of proteins that map to the pathway. All statistical analyses were

performed using Genstat 11 for Windows release 11.1, 2008 (Lawes Agricultural Trust, Rothamsted, UK).

Table 4.1. Ingenuity Pathway Analysis output log-ratio up-regulated (↑), down-regulated (↓) and no-change (=)

Protein	Function ^c	Line ^a (ROH vs ROL)	Diet ^b (HP vs LP)	Infection (200 vs 0 L ₃)
Interleukin 1a (IL-1a)	Innate/pro-inflammatory	↑	=	=
Interleukin 1b (IL-1b)	Innate/pro-inflammatory	↓	↓	=
Interleukin 2 (IL-2)	Adaptive pro-inflammatory	↑	↓	↑
Interleukin 4 (IL-4)	Adaptive Th2 response	=	↑	↓
Interleukin 5 (IL-5)	Adaptive Th2 response	↑	↑	=
Interleukin 6 (IL-6)	Pro-inflammatory	↓	=	↓
Interleukin 10 (IL-10)	Indicator of Treg	↓	↓	↓
Interleukin 12 (IL-12)	Adaptive Th1 response	=	↑	↑
Interleukin 13 (IL-13)	Adaptive Th2 response	↑	=	↑
Interleukin17 (IL-17)	Adaptive Th17 response	↓	↓	↑
Interferon γ (IFN γ)	Adaptive Th1 response	↓	=	↑
Tumour Necrosis Factor α (TNF α)	Innate/pro-inflammatory	↓	↑	↓
Fibroblast Growth Factor (FGF)	Innate/pro-inflammatory	↓	=	↓
Granulocyte macrophage colony stimulating factor (GM-CSF)	Innate/pro-inflammatory	↓	↓	↑
Monokine induced by gamma interferon (MIG)	Innate/pro-inflammatory	↓	↑	↓
Interferon gamma induced protein (IP-10)	Innate/pro-inflammatory	↓	=	↑
Macrophage inflammatory protein 1 (MIP1)	Innate/pro-inflammatory	=	↑	↑
Vascular endothelial growth factor (VEGF)	Innate/pro-inflammatory	↑	=	=
Keratinocyte-derived chemokine (KC)	Innate/pro-inflammatory	=	↑	↑

^aMice selected for high (ROH) and low (ROL) body weight

^bMice fed on high (HP) and low (LP) dietary protein contents

^cFunctions derived from Murphy *et al* (2008)

4.4 Results

4.4.1. Innate and pro-inflammatory responses – Luminex data

Upon analysis of the Luminex data, KC production was found to be higher in ROH mice than in ROL mice ($F_{1,16}=5.44$, $p=0.033$; Figure 4.1a). MIG and TNF- α production was higher in mice fed the LP diet than in mice fed the HP diet ($X^2_1=4.11$, $p=0.043$; Figure 4.1b, and $F_{1,27}=4.24$, $p=0.049$; Figure 4.1c respectively). Genetic growth potential, dietary protein content and infection status significantly interacted for IL-1 β production ($F_{1,35}=7.04$, $p=0.012$; Figure 4.2a). This was due to infection resulting to a down-regulation of IL-1 β in ROH-HP mice, whilst ROH-LP and all ROL mice had similar IL-1 β production regardless of infection status. For IL-12 production, infection increased production in LP mice compared to uninfected LP mice ($F_{1,30}=6.76$, $p=0.014$; Figure 4.2b), whilst infection decreased IL-12 production in HP mice. Mice fed the LP diet had higher IL-12 production than mice fed the HP diet ($F_{1,31}=5.05$, $p=0.032$) and ROH mice had higher IL-12 production than ROL mice ($F_{1,29}=11.27$, $p=0.002$).

4.4.2. Th2 immune responses – Luminex data

Genetic growth potential and infection status interacted for IL-4 production ($F_{1,35}=4.64$, $p=0.038$; Figure 4.3a). This interaction was due to infected ROL mice producing more IL-4 than uninfected ROL mice whilst infected ROH mice produced less IL-4 than uninfected ROH mice. ROH mice were found to have a higher IL-4 production than ROL mice ($F_{1,35}=5.42$, $p=0.026$). Mice fed the HP diet were found to have decreased IL-5 production when compared to mice fed the LP diet ($F_{1,27}=6.13$, $p=0.02$; Figure 4.3b).

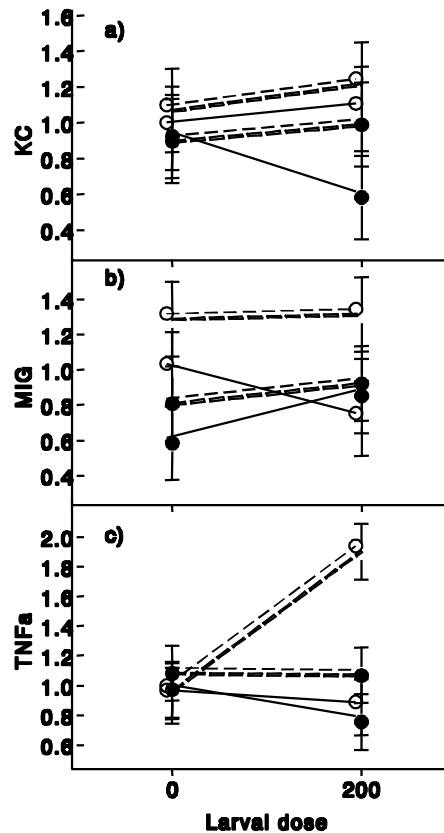


Figure 4.1 Predicted means from GLMM (with S.E.D.) of: a) Keratinocyte-derived Chemokine (KC) b) Monokine Induced by gamma interferon (MIG) and c) Tumour necrosis factor (TNF) α production, measured from supernatants collected from *in vitro* cultured mesenteric lymph node (MLN) cells. These were collected from high (ROH – open circle) and low (ROL – closed circle) body weight mice fed high (solid line) or low (dashed line) crude protein diet and infected with 200L₃ *Heligmosomoides bakeri* or sham infected with water.

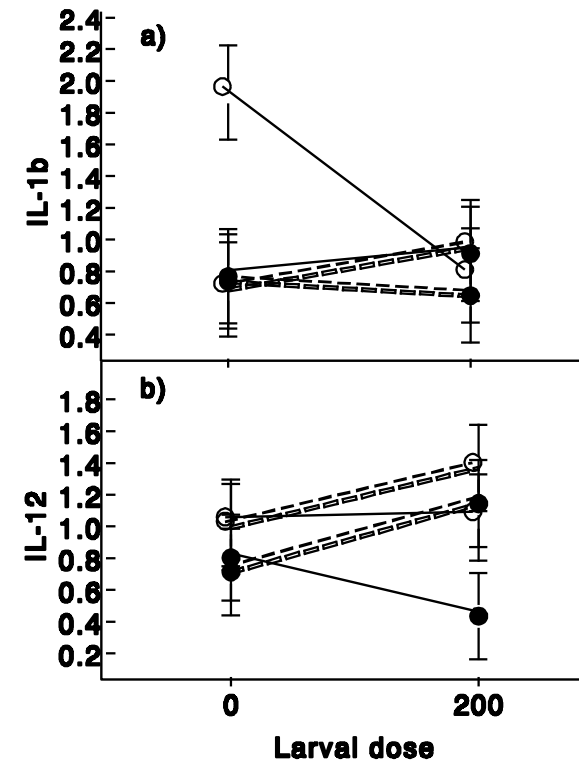


Figure 4.2 Predicted means from GLMM (with S.E.D.) of: a) Interleukin (IL) 1 β and b) IL-12 production, measured from supernatants collected from *in vitro* cultured mesenteric lymph node (MLN) cells. These were collected from high (ROH – open circle) and low (ROL – closed circle) body weight mice fed high (solid line) or low (dashed line) crude protein diet and infected with 200L₃ *Heligmosomoides bakeri* or sham infected with water.

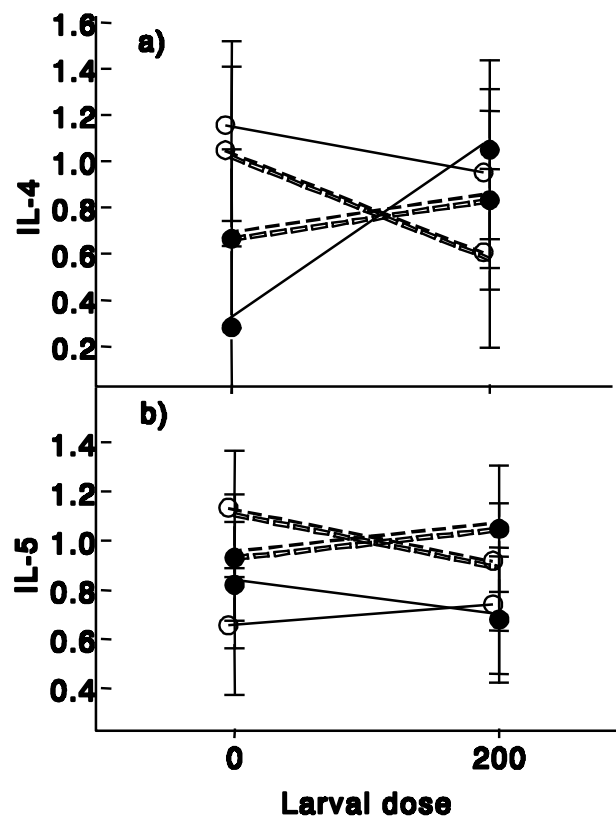
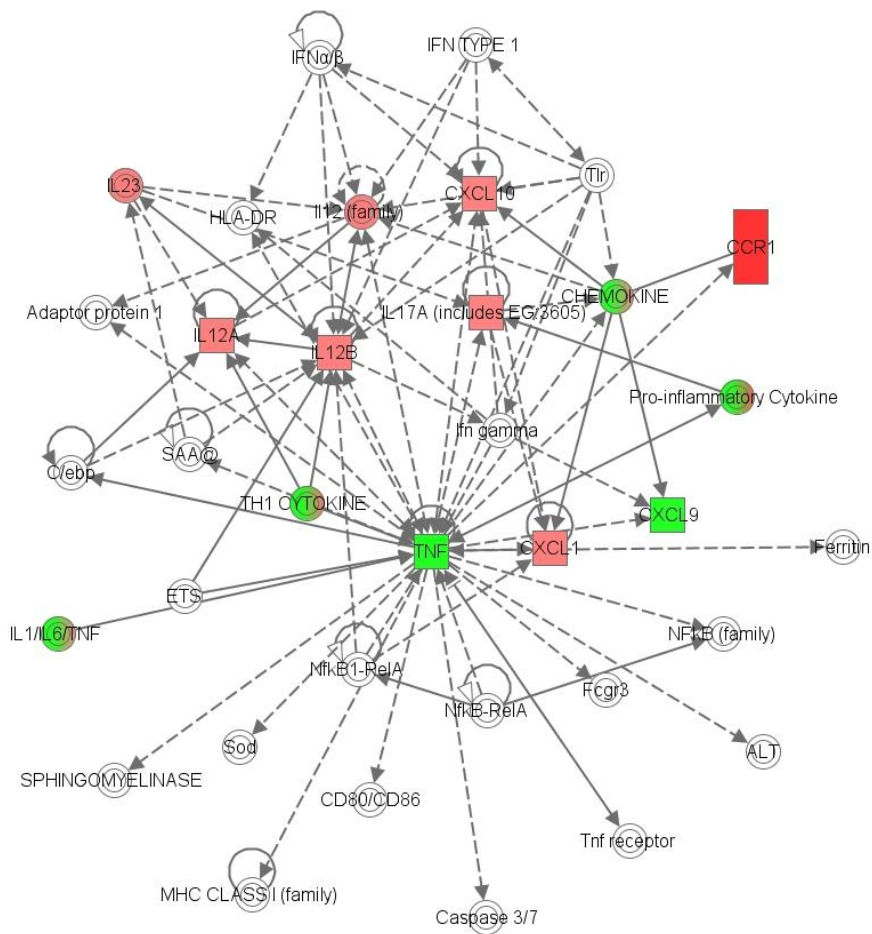


Figure 4.3 Predicted means from GLMM (with S.E.D.) of: a) Interleukin (IL) 4 and b) IL-5 production, measured from supernatants collected from *in vitro* cultured mesenteric lymph node (MLN) cells. These were collected from high (ROH – open circle) and low (ROL – closed circle) body weight mice fed high (solid line) or low (dashed line) crude protein diet and infected with 200L₃ *Heligmosomoides bakeri* or sham infected with water.

4.4.3. Ingenuity Pathway Analysis (IPA)

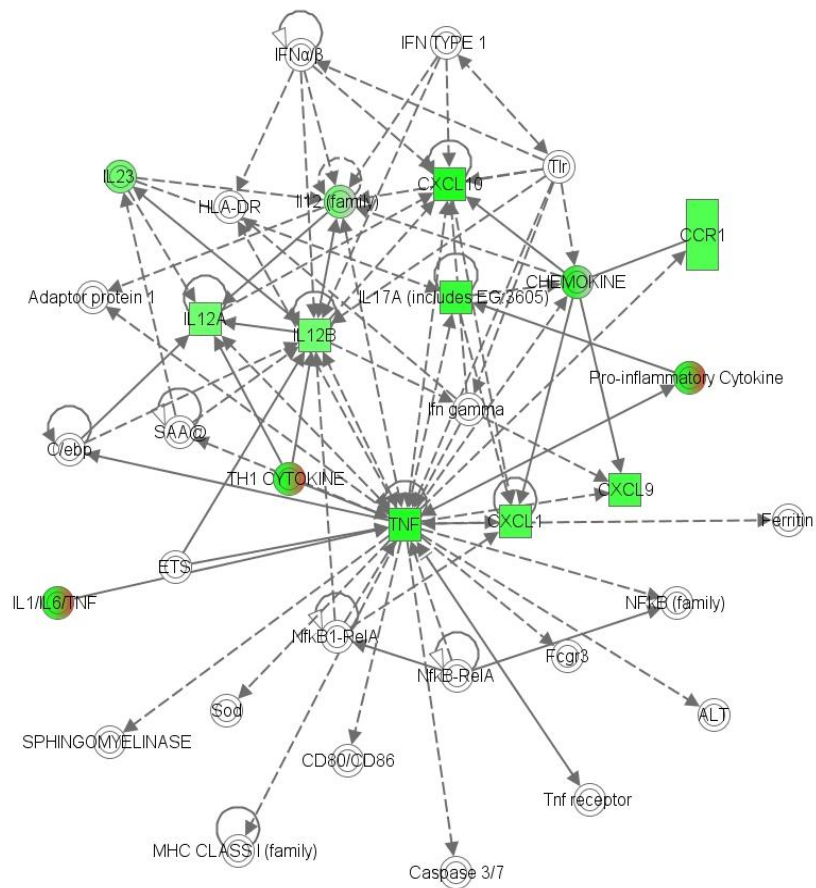
Figures 4.4, 4.5 and 4.6 illustrate the consequences of HP, parasitic infection and ROH strain, relative to LP, sham infection and ROL respectively on the network formed by the cytokines, chemokines and growth factors measured in the luminex analysis. The top biological functions overrepresented in the network, include cell-to-cell signalling, immune cell trafficking, cellular movement, growth, proliferation and development, and lipid metabolism.

Table 4.1 illustrates the IPA analysis data. Using log-ratio values, HP diet was found to increase cytokine regulation that has been associated with adaptive immune



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Figure 4.5. Diagram of the cell-to-cell signalling and interaction, and immune cell trafficking network pathways that show molecules that are up-regulated (red), down-regulated (green) and unaffected (white) in response to *Heligmosomoides bakeri* infection in comparison to sham infected mice.



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Figure 4.6. Diagram of the cell-to-cell signalling and interaction, and immune cell trafficking network pathways that show molecules that are up-regulated (red), down-regulated (green) and unaffected (white) in high (ROH) body weight mice in comparison to low (ROL) body weight mice.

4.5 Discussion

4.5.1 Infection did not polarise cytokines towards Th2 phenotype

These results indicate that, in contrast to expectation arising from the presence of increased serum IgG1 and IgE levels (Chapter 3), infection did not polarise cytokine responses towards the Th2 phenotype. This may be due to *Heligmosomoides bakeri* exerting an immunomodulatory influence over the immune system to stop the host producing a potentially damaging response to the parasite (Wakelin, 1996; Rzepecka *et al.*, 2006). Finney *et al* (2007) found that a typical Th2 polarised response occurs during early *H. bakeri* infection but this response was down-regulated by day 21 post infection. Thus, it cannot be excluded that the Th2 cytokine polarised phase was already down regulated by the 28 day primary infection protocol that was used in this model. Alternatively, the presence of *H. bakeri* antigens may have down-regulated Th2 cytokines *in vitro*.

Analysis showed that infection resulted in up-regulation of proteins related to an innate and pro-inflammatory type (MIP, GM-CSF, KC, IP-10) responses preferentially with only some Th1 (IFN γ , IL-2), Th2 (IL-13) and Th17 (IL-17) type, adaptive responses also being up-regulated. As hypothesised previously the Th2 type response may be a remnant from the earlier, now perhaps down-regulated, immune response phase, whilst KC attracts neutrophils and macrophages and can aid in the development of a Th17-derived inflammatory response (Murphy *et al.*, 2008; Wang *et al.*, 2010). The increased pro-inflammatory response may be due to altered patterns of commensal gut bacteria which are thought to drive the development of “classical” Th17 cells (Reviewed in Chow & Mazmanian, 2009). Chow and Mazmanian (2009) also hypothesise that specific classes of gut flora could be causing Th17 differentiation and that this is a highly dynamic interaction, leading

them to conclude that any changes to the commensal bacteria could have profound implications to intestinal and possibly systemic inflammatory disease. Studies involving germ-free animals have shown that commensal bacteria are essential for the morphological development of the gut and immune components such as intraepithelial lymphocytes (Hooper, 2004) as well controlling the rate of epithelial cell turnover (Creamer, 1967). During helminth infections it has been shown that “weep and sweep” motions of the gut mucosa, via increased luminal fluid and smooth muscle contractility, play an important part in parasite expulsion (Anthony *et al.*, 2007). Alongside this effect increased epithelial cell turnover has also been shown facilitate parasite expulsion (Cliffe *et al.*, 2005). Hence, commensal gut flora has the potential to influence rates of parasite expulsion as well as mucosal immunity.

4.5.2 ROL more inflammatory

Immune response (cytokine production) as measured in this study was not found to be “stronger”, i.e. increased Th2 type cytokine production, in ROL mice. Although ROL mice showed increased IL-4 production upon infection IPA analysis showed that these mice were Th1 prone and also indicated that IL-17 was up-regulated in ROL mice, which again may indicate the presence of altered commensal bacteria of the gut. The combined Th2 and Th1 up-regulation in ROH mice indicates an immune response that is slightly less prone towards an inflammatory phenotype. Chapter 3 suggests that ROL mice did not control their parasitism to the same extent as ROH mice so perhaps the lack of a “stronger” immune response was not unexpected once all the data were analysed. The intensive selection for body weight may have altered commensal gut flora composition (Chow & Mazmanian, 2009) and major-histocompatibility complex (MHC) haplotypes (Behnke & Wahid, 1991). The

Roslin mice founder strains have different MHC haplotypes, C57BL/6 have an H-2^b which is associated with a down-regulation of resistance to primary infection with *H. bakeri*, whilst DBA/2J have an H-2^d which is associated with lower worm burdens and egg counts after 5 weeks of infection with *H. bakeri* (Behnke & Wahid, 1991). It may be that these haplotypes segregated during divergent selection for body weight.

4.5.3 Protein scarcity promotes an inflammatory immune response, same as ROL line

Protein scarcity, as found in the ROH mice, was found to promote inflammatory (Th1 and Th17 type) immune responses, which is consistent with previous studies that have altered dietary protein content during *H. bakeri* infection (Ing *et al.*, 2000; Tu *et al.*, 2008). Protein malnutrition has been shown to produce a “low-grade systemic inflammation” (Ling *et al.*, 2004) and in some cases protein malnutrition has proved beneficial to combating fungal pathogens that require an inflammatory immune response (Oarada *et al.*, 2009). Perhaps, at least in the short term, protein malnutrition enables the host to combat infectious agents that would cause the most severe threat to survival. During *H. bakeri* infection Tu *et al* (2008) postulated that the increased Th1 response observed when mice were offered a protein deficient diet may have been due to increased leptin levels which in turn would stimulate the production of IFN γ . This environment is then thought to promote villus growth and thus parasite survival (Tu *et al.*, 2008). As such, this promotion of parasite survival may be an unfortunate outcome of the host attempting to maximise its’ nutrient uptake on the deficient diet by maximising the gut surface area (Ferraris & Carey, 2000). Ing *et al* (2000) found that protein deficiency during a primary infection results in an increased worm burden when compared to protein sufficient diets. Such

a protein sensitivity of worm burdens has not been seen in both Chapters 2 or 3. This inconsistency may be attributed to the different mice strains. The mice used in Ing *et al.*'s study are BALB/c which have been described as having a Th2 type immune phenotype (Peterson *et al.*, 1998). This may have enabled the protein sufficient mice to quickly produce a protective Th2 (due to increased IL-4 producing T cells) to facilitate expulsion of worms by day 28 post infection (Doherty & Coffman, 1996) or arrest the development of larvae before migration from the mucosa into the lumen of the gut. This observed protein sensitivity may also be due to the mice being a week older at the time of primary infection (6 weeks old) than the ROH and ROL (5 weeks old) mice used in Chapters 2 and 3. This theory is backed up by the dietary protein sensitivity observed in Chapter 5 (see below) where ROH mice given a primary infection at 6½ weeks showed reduced egg counts and total worm burden during protein sufficiency compared to protein deficient ROH mice. This observation may not be due to differences in immunity *per se* but to differences in the growth phase of the mice as by 6½ weeks mice should be growing more slowly than at 5 weeks (Cheverud *et al.*, 1996). This difference may mean that improved protein nutrition can be utilised for improved immunity, whilst protein deficient mice would still be attempting to utilise more nutrients towards the achievement of their optimal mature weight and reaching sexual maturity.

In conclusion, protein scarcity in ROH mice seems to be responsible for an increased inflammatory immune response which has been shown to allow persistence of this parasite, further suggesting that improved protein nutrition can aid in the alleviation of weight loss and also in the generation of an appropriate immune response towards GI nematodes. In addition, selection for body weight may have altered the immune

response to pathogen challenge, although the cause of this requires further investigation.

Chapter 5. Previous exposure to *Heligmosomoides bakeri* and protein scarcity increases the penalty of infection in mice divergently selected for body weight

5.1 Summary

The expression of immunity is considered costly and as such may be penalised during nutrient scarcity, particularly when animals have been intensively selected for increased production. Using mice divergently selected for high (ROH) and low (ROL) body weight, the interaction between genetic growth potential, parasite exposure and protein scarcity was investigated. The hypothesis was that protein scarcity increases the penalty of secondary infection with *Heligmosomoides bakeri* on resilience to infection and reduces the resistance expected during a secondary infection compared to a primary infection. This effect of protein scarcity would be more pronounced in ROH than in ROL mice. ROH and ROL mice were assigned to one of four main groups, a non-infected control group (C), a cleared primary infection group (P1, infected at d-14 only), a primary infection after ivermectin treatment group (P2, infected at d0 only) and a secondary infection group (S). These groups were further subdivided into low (30g/kg; LP) and high (230g/kg; HP) crude protein diets from d-4 onwards. Between d0 and d21, LP diet reduced weight gain in both ROH and ROL mice, ROH-P2 and ROH-S mice also showed a further reduction in weight gain on the LP diet compared to the other treatment groups. Anorexia was observed in all infected mice, and persisted throughout in ROH-P2 and ROH-S mice. Feeding HP diets reduced colon egg counts and worm burden in ROH-P2 mice whilst ROH-S mice showed the greatest reduction in worm burden and egg counts, when compared to ROL-S, with little effect of HP feeding. Against expectation, ROL mice showed the highest worm burdens and egg counts whilst showing the greatest effect of HP feeding during a secondary infection. The data supports the view that protein scarcity leads to a reduction in resilience in mice selected for high body weight during a secondary infection and that improved protein nutrition can ameliorate this penalty of infection on resilience. The data also suggest

that improved protein nutrition can lead to increased resistance during secondary infection in resilient, low body weight mice.

5.2 Introduction

Livestock and wildlife species are born naïve to parasites but over time become immune to them through repeated and continuous exposure (Holmes, 1993). The expression of immunity is considered costly due to the energetic and proteinaceous nature of immune and inflammatory responses (Boots & Bowers, 2004; Tyler *et al.*, 2006) and therefore it would be considered that protein scarcity could limit an animal's ability to mount an adaptive immune response (Coop & Kyriazakis, 1999; Houdijk *et al.*, 2001b; 2006). Consequently improved protein nutrition at times of nutrient (protein) scarcity would be expected to improve host immunity, resulting in reduced parasite burdens and parasite egg production (Ehrenford, 1954a; Brailsford & Mapes, 1987; Coop & Kyriazakis, 2001). Beilharz *et al.* (1998a; 2000) and Rauw *et al.* (1998) proposed that an extreme bias in breeding goals towards production traits (such as increased growth) would, in accordance with the nutrient partitioning framework set out by Coop and Kyriazakis (1999), be expected to prioritise the partitioning of nutrients towards the selected trait leaving fewer for other traits, such as the expression of immunity. Expression of immunity during *H. bakeri* challenge infections has been shown to produce a large decrease in body weight compared to primary infections even under protein sufficient conditions (Tu *et al.*, 2007), indicating an alteration in resource allocation.

Anthelmintic abbreviated infections have been widely used to produce immunized animals without the need for repeated infections and natural clearance of parasites, which will include a period of immunogenic larval stages and an immunosuppressant adult stage (Behnke & Robinson, 1985; Wahid *et al.*, 1994; Stankiewicz *et al.*, 1996). Since this can be achieved over a short period of time, the anthelmintic abbreviated protocol allows the stimulation of protective immunity and the induction of a

challenge infection whilst hosts are still undergoing accelerated growth associated with achieving their mature body size and reaching sexual maturity (Cheverud *et al.*, 1996).

Data reported in Chapter 3 suggested that mice selected for high body weight (ROH) were less resilient to parasitic infection than their low body weight counterparts (ROL) whilst, perhaps unexpectedly, showing lower egg counts. Though increased protein supply could overcome this penalty on resilience in ROH mice, no effect of protein supply in reducing worm numbers or parasite egg counts was observed (Coltherd *et al.*, 2009, Chapter 2 and Chapter 3). However, it should be noted that these studies employed a primary infection and that the expected effect of protein scarcity on the degree of parasitism would be more pronounced during secondary infections (Boulay *et al.*, 1998; Ing *et al.*, 2000; Houdijk *et al.*, 2005; Tu *et al.*, 2007; Kidane *et al.*, 2009). Here it is hypothesised that protein scarcity will increase the penalty of a secondary infection of *H. bakeri* on resilience whilst decreasing resistance. It is expected that this penalty will be more severe in ROH mice than in ROL mice. Improved protein nutrition is also expected to improve weight gain and overcome this penalty whilst also reducing worm numbers and egg production.

5.3 Materials and Methods

5.3.1 *Animals and housing*

A cross of C57BL/6J x DBA/2J was used as a foundation population for a divergent selection experiment in which selection for high and low body weight (BW) at day 42 of age took place over 20 generations making up the ROH and ROL lines, respectively (described in detail by Heath *et al.*, 1995; Bünger *et al.*, 2001a). For this experiment, 96 ROH and 96 ROL male mice were obtained, weaned at 21 to 23 days and housed individually in a controlled environment with an ambient temperature of $21\pm 1^{\circ}\text{C}$ and a 12h light cycle. ROH mice had a body weight of $15.94\pm 0.37\text{g}$, whereas ROL mice had a starting weight of $7.62\pm 0.19\text{g}$. Mice were housed in solid bottomed cages with fresh sawdust and bedding material provided twice weekly. The experiment details described below were approved by the Animal Experiment Committee of Scottish Agricultural College (ED AE 25/2008) and carried out under Home Office regulations (PPL 60/3626).

5.3.2 *Diets*

All mice were fed *ad libitum* standard expanded breeding diet [RM3(P), Special Diet Services, Witham, UK; digestible crude oil, 38 g/kg; digestible crude protein 202 g/kg, starch, 339 g/kg; sugars, 44 g/kg; digestible energy, 12.2 MJ/kg] for three weeks after arrival. Two isoenergetic (Digestible Energy, 15 MJ/kg) experimental diets with a fixed amino acid to crude protein (CP) ratio were used; 30 (LP) and 230 (HP) g CP per kg (Table 1). These CP levels were expected to result in protein scarcity (LP) or protein adequacy (HP) relative to expected requirements (NRC,

1995) and as informed by our previous work using a wide range of dietary CP levels (Coltherd *et al.*, 2009, Chapter 2). As casein was used as the protein source, 15 g cysteine was added to each kg of casein to account for the scarcity of sulphur containing amino acids.

Table 5.1: Ingredients and analysis of the experimental diets

Ingredients (g/kg)	Experimental diets	
	LP	HP
Rice starch	570	332
Maltodextrin	132	132
Sucrose	100	100
Soya oil	70	70
Cellulose	50	50
Vitamins, minerals and amino acids	48	52
Casein edible acid	30	265
Analysis		
Dry matter (g/kg)	909.8	901.8
Crude protein (g/kg dry matter)	34.6	235.0
Acid detergent fibre (g/kg dry matter)	42.3	58.3
Ash (g/kg dry matter)	29.4	79.0

5.3.3 Infection protocol and experimental design

The strain of *Heligmosomoides bakeri* (= *Heligmosomoides polygyrus bakeri*, *Nematospiroides dubius*) (Cable *et al.*, 2006), was obtained from Professor Jerzy Behnke, The University of Nottingham, Nottingham, UK (see Jenkins & Behnke, 1977 for full origin details), and maintained in our lab through passages in C57BL/6 mice. At day -14 and day 0 of the experiment mice either received a single infection of 200 *H. bakeri* infective larvae (L₃) suspended in 0.2ml water or 0.2ml sham infection via oral gavage (Jenkins & Behnke, 1977; Behnke & Wakelin, 1977). The sham infection was obtained by “mock culturing” non-infected mouse faeces to avoid possible confounding results due to faecal contaminants present in the larval doses. The dose of *H. bakeri* was chosen to produce a sub-clinical infection that were expected to result in sub-clinical parasitism as indicated by reduced growth performance (Houdijk & Bünger, 2006; 2007; Coltherd *et al.*, 2009, Chapter 2; Chapter 3). All mice were given ivermectin (20mg/kg) via oral gavage on day -8 of the experiment (Fakae *et al.*, 1999).

ROL and ROH mice were assigned to one of four main parasite exposure groups, a non-infected control group (C), a cleared primary infection group (P1, infected at d-14 only), a primary infection after ivermectin treatment group (P2, infected at d0 only) and a secondary infection group (S, infected at d-14 as well as on d0). Within each main group mice were subdivided in two feeding treatment groups (LP and HP), and dissection occurred on day 21. For each line-diet combination, six replicates were assigned for the C and P1 groups whilst nine replicates were assigned for the P2 and S groups. Figure 5.1 illustrates the experimental design and timings of procedures. Mice entered the experiment (day -14) through an adaptation phase (day

-24 to day -14) and first infection occurred at day -14. Mice were drenched with an Ivermectin bolus (20 mg/kg; day -8) and offered experimental LP and HP diets from day -3 onwards. The second infection occurred day 0 and mice were humanely killed on day 21, for the assessment of worm burdens, colon egg count and body fat percentage.

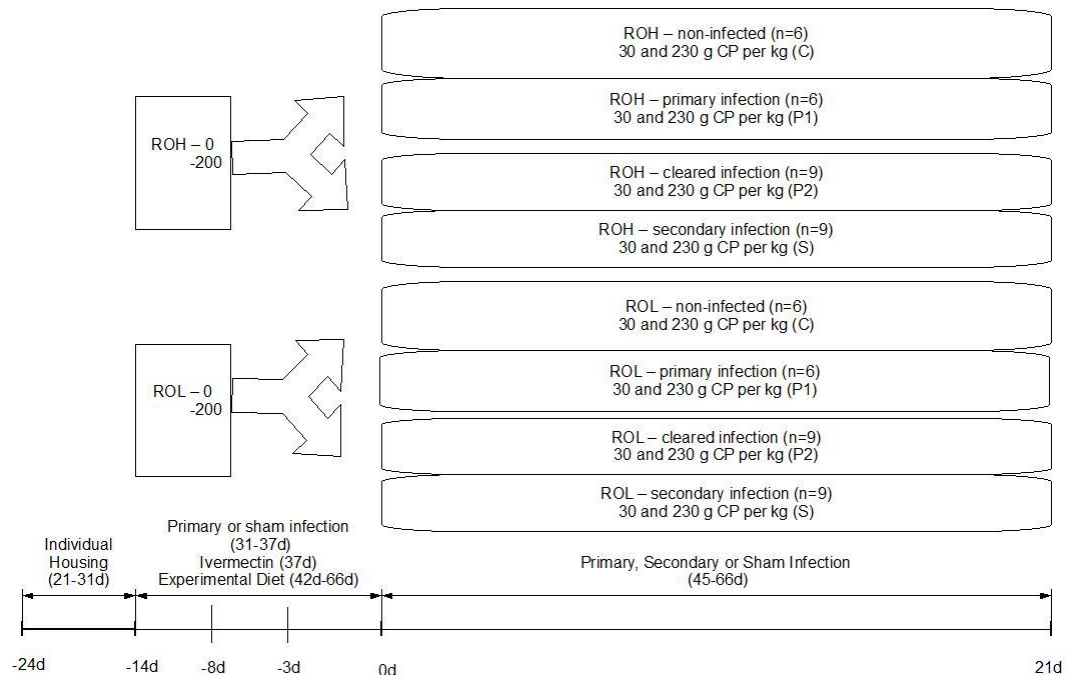


Figure 5.1 - Diagram of experimental design. Timeline in experimental days shown along the top. First *Heligmosomoides bakeri* or sham infection occurred at d-14, all mice were dosed with Ivermectin (20mg/kg) day -8, experimental diet was offered day -3, mice were then infected or sham infected a second time day 0. Dissection occurred day 21. Mice ages are shown in parentheses.

5.3.4 Sample measurements and collection

Body weight and food intake. Between experimental day -14 and 21, mice and food refusals were weighed twice weekly (Tuesday and Friday) resulting in 11 experimental periods for weight gain and food intake. On each of these days food refusals were weighed out and fresh food weighed in. Around 30g was offered to ROH and 15g to ROL mice, which was sufficient to achieve *ad libitum* feeding (Coltherd et al 2009; Chapter 2). Food intake was calculated per day within each experimental period for each mouse.

Colon contents and worm burden. Mice were humanely killed via CO₂ inhalation and dissected to obtain the small intestine and the colon. The small intestine was weighed, divided into quarters, opened up and placed directly into a 5% formaldehyde solution pending assessment of the number of male and female worms in each quarter. The colon contents were weighed and a colon egg count, using a modified flotation technique (Christie and Jackson, 1982) was used to assess faecal egg counts (eggs per g) and performed for every mouse. The resulting colon egg count was then multiplied by the colon contents weight to eliminate any dilution effects and obtain a final number of eggs in colon (EIC) value. The EIC was divided by the number of females counted to estimate *per capita* fecundity (eggs per female).

Fat percentage. To allow the prediction of fat percentage, the mouse carcasses were weighed and bagged upon dissection for subsequent freeze-drying. In preparation for the freeze-drying process, incisions were made, to allow maximal water loss, in the back, tail and head of the animal. When weight loss ceased (approximately 7 days later) the carcasses were removed from the freeze-drier and re-weighed. The following formula was used to calculate fat percentage (Hastings and Hill 1989):

$$\text{Fat percentage} = [(\text{dry weight} \times 1.13) / \text{fresh carcass weight}] - 0.302 \times 100$$

5.3.5 Statistical Analysis

Due to the skewed nature of the data EIC and worm burden data were Log₁₀ (n+1) transformed. To adequately account for the relatively large differences in growth between the mouse lines, body weight gain was expressed on a percentage scale ((Final weight/Initial weight) x 100) – 100) and feed intake data was Log₁₀ (n) transformed before analysis (Falconer & MacKay, 1996). In contrast to Coltherd *et*

al (2009, Chapter 2) and Chapter 2 \log_{10} transformation was not appropriate for this experiment as mice actually lost weight. Therefore, percentage weight gain was deemed a more appropriate transformation as this would also account for the need to scale weight gain effects in heavy and light mice. Repeated measures Analysis of Variance (ANOVA) was used to assess interactive effects of genetic growth potential, dietary protein content, parasite treatment and time on food intake. ANOVA models were also used to assess interactive effects between genetic growth potential, dietary protein content and parasite treatment on average daily weight gain, average food intake and body fat percentage. All statistical analyses were performed using Genstat 11 for Windows release 11.1, 2008 (Lawes Agricultural Trust, Rothamsted, UK).

5.4 Results

5.4.1 Body weight gain and food intake

Figure 5.2 shows the arithmetic mean body weight of all experimental groups across the experimental period. All mice gained weight during the first phase of the experiment, i.e. between day -24 and day -4, with ROH mice gaining more weight on average than ROL mice (0.68 ± 0.050 g/d and 0.23 ± 0.009 g/d respectively). However, after switching to the experimental diets, ROH-P2-LP and ROH-S-LP mice lost weight; their weight gain averaged -0.14 ± 0.071 and -0.21 ± 0.081 g/d, respectively.

Figure 5.3 shows Log_{10} transformed average food intake and percentage weight gain. Genetic growth potential and dietary protein content interacted for food intake over the experiment ($F_{1,102}=38.64$, $p<0.001$; Figure 5.3a). This was due to ROH-HP mice consuming more than ROH-LP mice whilst ROL-LP mice consumed more than ROL-HP mice. As expected ROH mice consumed more food than ROL mice ($F_{1,102}=1605.97$, $p<0.001$). Parasite treatment also affected food consumption with C and P1 mice consuming more food than P2 and S mice ($F_{3,102}=3.12$, $p=0.029$). A trend where mice fed the LP diet ate more than mice fed the HP diet was found during the experimental diet, i.e. day -4 to day 21, phase ($F_{1,102}=3.82$, $p=0.053$), this was due to C and P1 mice consuming significantly more LP diet than C and P1 mice fed the HP diet ($F_{1,40}=4.95$, $p=0.032$). A significant three-way interaction between genetic growth potential, dietary protein content and parasite treatment was observed for mean food intake ($F_{1,62}=5.01$, $p=0.029$), as ROH-S-LP mice ate less than ROH-P2-LP mice, whilst in contrast ROL-S-LP mice ate more than ROL-P2-LP mice.

Time tended to interact with genetic growth potential and parasite treatment for food intake ($F_{33,1122}=1.76$, $p=0.069$; Figure 5.4). This was brought about by anorexia

being shown by ROH-P1, ROL-P1 and ROL-S mice between day -10 and -3; and ROL-P2 mice between day 4 and 11. Whilst ROH-P2 mice reduced their intake between day 4 and 7 and ROH-S mice reduced their intake between day 7 and 10 of the experiment, neither group of mice recovered their intake. There was no interaction with dietary protein contents ($p>0.1$)

Genetic growth potential and dietary protein content interacted for scaled weight gain ($F_{1,102}=82.40$, $p<0.001$; Figure 5.3b). This was due to ROH-HP mice gaining the most weight whilst ROH-LP mice gained the least weight, in fact they gained less weight than ROL mice who, although they lost weight, did not experience as severe a weight loss on the LP diet. Overall, mice fed HP diet had higher weight gain than mice fed LP diet ($F_{1,102}=364.83$, $p<0.001$). There was a tendency for ROH mice to gain more weight than ROL mice ($F_{1,102}=3.5$, $p=0.064$) due to ROH-HP mice gaining significantly more weight than ROL mice ($F_{1,40}=6.38$, $p=0.016$). Parasite treatment also tended to affect weight gain over the entire experiment with C mice gaining more than P1 mice who gained more than P2 mice whilst S mice gained the least weight ($F_{3,102}=2.43$, $p=0.07$), when analysing only the experimental diet phase (d-4 to d21) this pattern of parasite exposure affecting weight gain was significant during this period ($F_{3,102}=4.69$, $p=0.004$).

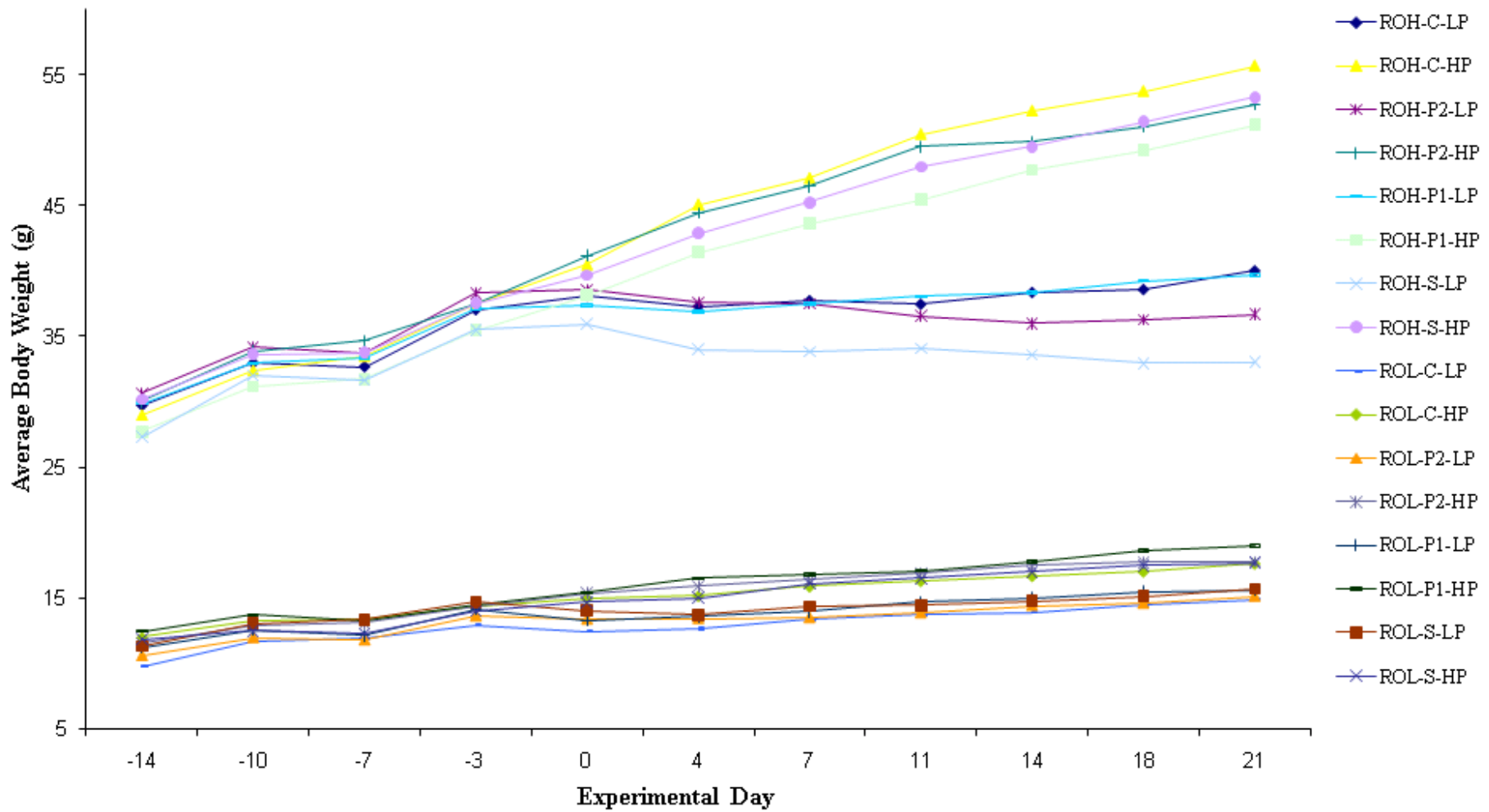


Figure 5.2 Average body weight (in g) over time for all experimental groups.

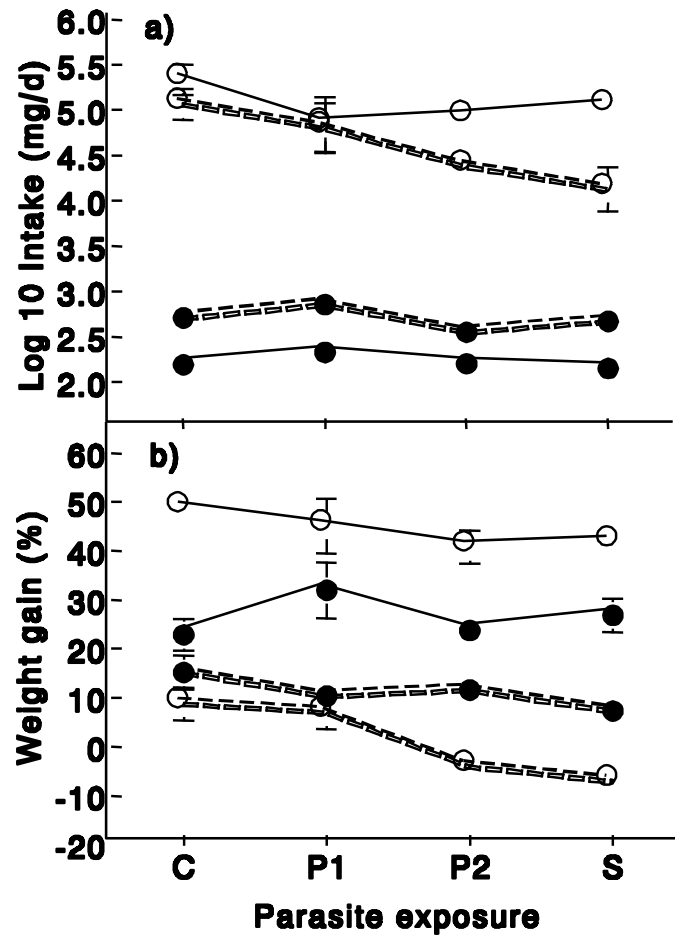


Figure 5.3. a) Log₁₀ transformed daily food intake and b) percentage body weight gain, ((final body weight/initial body weight) x 100) – 100, of high (ROH – open circle) and low (ROL – closed circle) body weight mice averaged across d-3 to d21 of the experiment fed low protein (dashed line) or high protein diets (solid line) whilst experiencing differing parasite exposures.

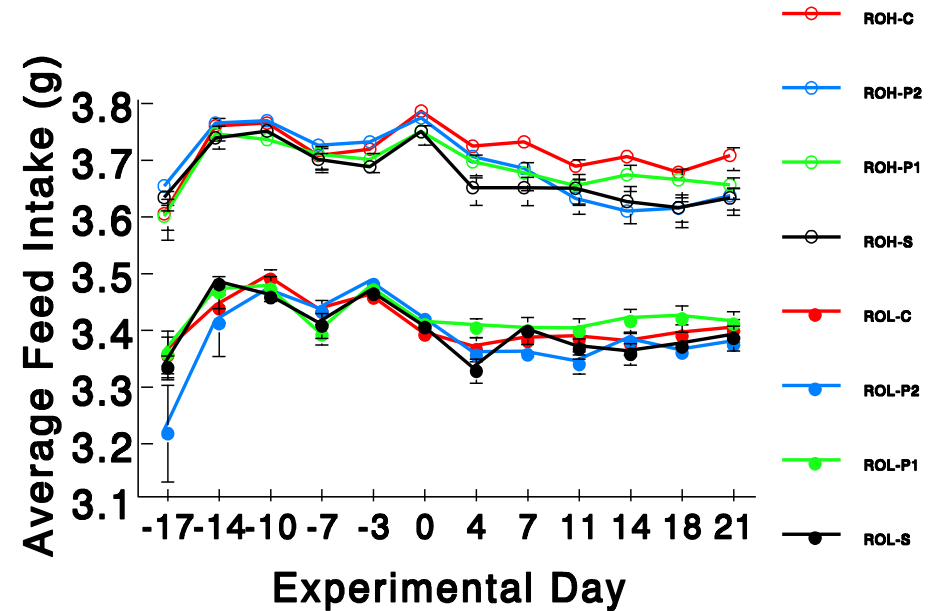


Figure 5.4. Average food intake (g) of ROH (open circles) and ROL (closed circles) mice over the experimental period. Mice are experiencing either a sham (C); early primary (P1, d-14 infection with 200L₃); late primary (P2, d0 infection with 200L₃); or secondary *H. bakeri* infection (S, d-14 and d0 infection with 200L₃).

5.4.2 Body fat percentage -

A significant three-way interaction between genetic growth potential, dietary protein content and parasite treatment was observed ($F_{1,102}=6.11$, $p=0.018$; Figure 5.5). ROH-C-LP mice had a lower body fat percentage than ROH-C-HP, and ROH-P1-LP had a higher body fat percentage than ROL-P1-HP, whilst ROL-C-LP mice had a higher body fat percentage than ROL-C-HP and ROL-P1-LP mice had a lower body fat percentage than ROL-P1-HP. Dietary protein content and genetic growth potential significantly interacted for body fat percentage ($F_{1,102}=4.69$, $p=0.033$). This was observed due to ROH-HP mice having a higher body fat percentage than ROH-LP mice while dietary protein content did not affect body fat percentage in ROL mice. ROH mice had a higher body fat percentage than ROL mice ($F_{1,102}=65.15$, $p<0.001$) and mice fed the HP diet had a higher body fat percentage than mice fed LP ($F_{1,102}=4.06$, $p=0.047$).

5.4.3 Eggs in the colon, worm burden and per capita fecundity

The three-way interaction between genetic growth potential, dietary crude protein and parasite treatment was significant for EIC ($F_{1,61}=4.27$, $p=0.043$; Figure 5.6a); ROH-P2-HP mice had lower EIC than ROH-P2-LP, ROL-S-HP mice had lower EIC than ROL-S-LP whilst dietary protein content did not affect EIC in ROH-S mice or ROL-P2 mice. It was also observed that previous exposure reduced EIC in all mice ($F_{1,61}=65.36$, $p<0.001$) but that this reduction was more marked in ROH mice than in ROL mice ($F_{1,61}=5.62$, $p=0.021$). Consequently, ROL mice had on average a higher EIC than ROH mice ($F_{1,61}=8.78$, $p=0.004$).

Previous exposure reduced total worm burden in all mice ($F_{1,62}=41.47$, $p<0.001$; Figure 5.6b) but, as with EIC, this reduction was more pronounced in ROH mice

than in ROL mice ($F_{1,62}=13.11$, $p<0.001$). Overall, ROL mice had a higher total worm burden than ROH mice ($F_{1,62}=8.79$, $p=0.004$) and mice fed the LP diet tended to have a higher total worm burden than mice fed the HP diet ($F_{1,62}=3.71$, $p=0.059$). Experimental treatment did not affect distribution of worm burden over the small intestine; worms were observed to inhabit the first quarter of the small intestine only. Dietary crude protein and parasite treatment interacted significantly on percentage of female worms ($F_{1,62}=8.60$, $p=0.005$). This was observed due to P2-HP mice having a higher percentage of female worms than P2-LP whilst S-HP mice had a lower percentage of female worms than S-LP. Genetic growth potential also interacted with parasite exposure with ROH-P2 mice having a higher percentage of female worms than ROH-S mice whilst previous exposure did not affect percentage of female worms in ROL mice ($F_{1,62}=20.11$, $p<0.001$). Overall, percentage of female worms was also significantly reduced after previous parasite exposure when compared to the mice experiencing their first infection ($F_{1,62}=14.79$, $p<0.001$).

Genetic growth potential, dietary crude protein and parasite exposure interacted for *per capita* fecundity ($F_{1,61}=4.52$, $p=0.038$; Figure 5.6c). This was due to ROH-HP mice having a lower *per capita* fecundity than ROH-LP mice regardless of parasite exposure whilst the HP diet reduced *per capita* fecundity in ROL-S mice only. ROL mice, overall, had a higher *per capita* fecundity than ROH mice ($F_{1,61}=12.88$, $p<0.001$) whilst previous exposure reduced *per capita* fecundity in all mice ($F_{1,61}=51.29$, $p<0.001$). Mice fed the HP diet also tended to have lower *per capita* fecundity than mice fed the LP diet ($F_{1,61}=3.10$, $p=0.083$).

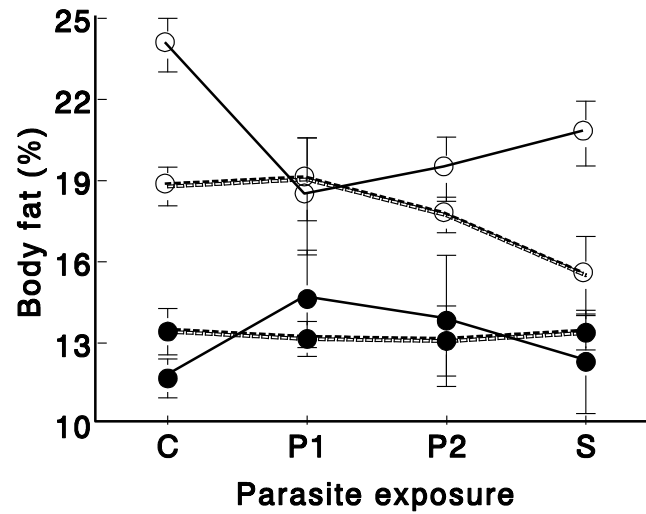


Figure 5.5. Average body fat percentage of high (ROH – open circle) and low (ROL – closed circle) body weight mice either fed low (dashed line) or high (solid line) dietary CP, whilst experiencing differing parasite exposures.

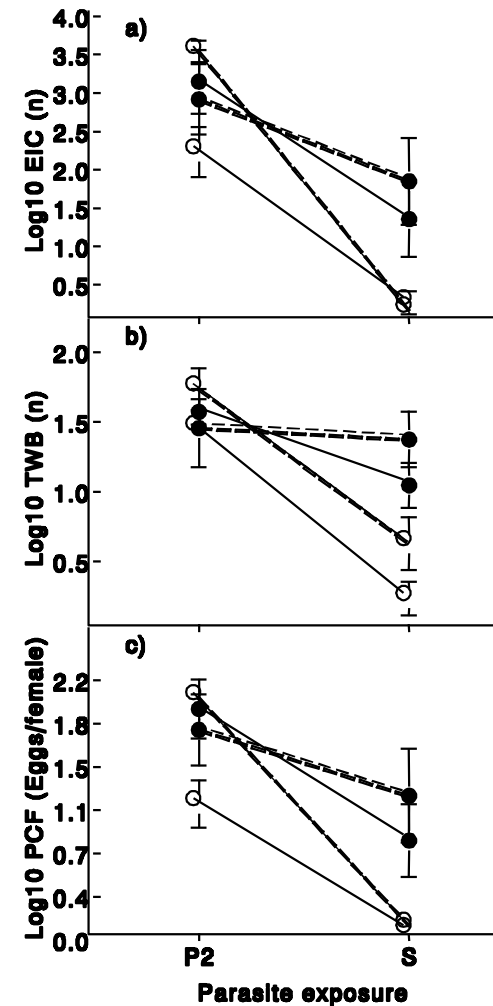


Figure 5.6. Log₁₀ (n+1) transformed: a) Eggs in colon (EIC), b) total worm burden (TWB), and c) *per capita* fecundity (PCF) day 21 post infection for high (ROH – open circle) and low (ROL – closed circle) body weight mice fed a high (solid line) or low (dashed line) crude protein diet, and exposed to a primary (P2) or secondary (S) infection of 200L₃ *Heligmosomoides bakeri*.

5.5 Discussion

The data supports the hypothesis that secondary exposure to parasites leads to an increased penalty on weight gain at times of nutrient scarcity and that these effects are more pronounced in ROH than in ROL mice. This was observed in the expected presence of lower worm burdens during secondary exposure compared to primary infection. The hypothesis that increased protein nutrition would result in the amelioration of the penalty of parasitism on weight gain and lead to reduced worm numbers and egg production was also supported. It should also be noted that this study managed to achieve protein scarcity in both ROH and ROL mice allowing the effect of protein malnutrition to be analysed in both mouse lines in the absence of confounding effects; as discussed, this was not possible in the studies described in Chapters 2 and 3.

5.5.1 Body weight, food intake and fat percentage

Both mouse lines showed a reduction in body weight gain as a result of feeding foods with a low protein content. In the case of ROL mice this reduction in growth was observed despite an increased food intake on the low protein diet compared to the high protein diet. ROH-HP and ROL mice were able to maintain a relatively constant body weight gain over the experimental period regardless of parasite exposure. However, ROH-LP mice lost weight when experiencing a primary (P2) infection or a secondary infection (S). This finding shows that the timing of infection relative to nutrient scarcity is important in animals that are less resilient to infection. Tu *et al* (2007) also showed that, during a challenge infection, feeding a high protein diet to mice that had been fed a protein deficient diet rapidly restored the reduced weight gain seen in these mice to that of mice that had been protein

sufficient throughout the experiment. This further indicates that protein nutrition can be used to ameliorate the loss of production that can occur during parasite exposure.

As seen in the previous experiments (Coltherd *et al.*, 2009, Chapter 2, and Chapter 3) ROL mice were able to increase their food intake on the LP diet compared to the HP diet whilst ROH mice were unable to do this. In fact, ROH mice reduced their food intake when experiencing a late primary (P2) or secondary (S) infection whilst fed the LP diet, compared to their HP fed counterparts. To some extent, this reduction in food intake (averaged over the entire experimental period) may be an outcome of the ROH-P2 and ROH-S mice being smaller (by around 15%) than ROH-C and ROH-P1 mice in addition to dealing with a pathogen whilst experiencing protein scarcity.

All infected groups showed anorexia, a temporary reduction in food intake, after their first exposure to *H. bakeri*, however ROH-S mice also showed anorexia during their challenge infection. In agreement with these observations, Tu *et al* (2007) also found that anorexia was seen during a challenge infection in mice fed both protein sufficient and protein deficient diets. Although there is a distinct lack of evidence in the literature for the presence of anorexia in partially or fully immune individuals (Kyriazakis *et al.*, 1998), the inability to recover intake (or prolonged depression of intake) is frequently reported in ruminant species (Symons *et al.*, 1981; Zaralis *et al.*, 2008). This inability of ROH-P2 and ROH-S mice to recover their intake may be due to an increased immune response, as immune responses *per se* have been implicated in anorexia (Greer *et al.*, 2009). Indeed ROH-S mice and ROH-P2-HP mice whose intake did not recover, did have lower worm burdens than ROL-S and ROL-P2 mice respectively, who recovered their food intake.

Interestingly infection reduced body fat percentage in ROH-HP mice indicating that, although body weight did not reduce, there was an increased energy requirement during parasite exposure that lead to a reduction in fat deposition. This increased energy requirement has been reported in a number of studies, including the experiment detailed in Chapter 2, and can lead to as much as a 20% reduction in body fat when compared to uninfected control animals (Kristan & Hammond, 2006; Coltherd *et al.*, 2009, Chapter 2).

5.5.2 Worm burden and egg output

In contrast to the hypothesis, improved protein nutrition and previous parasite exposure reduced worm burdens and egg counts in ROH mice to a greater extent than in ROL mice. Kloosterman *et al* (1978) also reported that male calves, artificially infected with *Cooperia oncophora* (a gut nematode), with the highest production potential showed the greatest effect of infection on performance whilst also having the highest antibody titres, lowest worm burdens and egg counts and the shortest worms. This may indicate that while animals that have a lower production potential can be seen to be more resilient to infection, it is the animals with a higher production potential that are better able to resist pathogen challenges by directing resources towards immune responses rather than towards obtaining their full production potential. This new hypothesis requires further study, as this contrasts with the current accepted view that selection for productivity is associated with higher parasite loads (Rauw *et al.*, 1998).

Slater and Keymer (1988) and Boulay *et al* (1998) found that improved protein nutrition reduced worm burdens and egg outputs in anthelmintic abbreviated infections. In this study the effect of improved protein nutrition wasn't as clear, as

the HP diet did tend to reduce worm burdens and egg outputs but ROH mice that had been previously exposed to infection only showed a significant effect of HP diet in their total worm burdens. Indeed, the data appears to suggest that protein nutrition has the greatest effect in reducing worm burden and egg counts of ROL mice during a secondary infection. Selection for genetic growth potential may have confounded the effects of protein nutrition, as day 21 may have been too late a time point to observe the effects of increased protein nutrition in ROH mice but may have been too early a time point for ROL mice. Chapter 4 suggests that ROL mice may produce an inflammatory immune response to primary *H. bakeri* infection that is similar to the profile of “slow responder” mice (Menge *et al.*, 2003).

Anthelmintic treatment succeeded in clearing primary infections, as no eggs or worms were found in P1 mice (data not shown), and produced an effective immunity towards a challenge infection as seen by reduced egg counts and worm burdens in S-mice when compared to P2-mice in ROH mice. As egg counts and total worm burdens in P2-mice were comparable to those seen in Chapter 2 (Coltherd *et al.*, 2009) and Chapter 3 it can be presumed that the ivermectin had been metabolised by the mice in the 8 days between drenching and infection, consistent with Wahid *et al* (1989) who estimated that an oral dose of 20mg/kg ivermectin persisted only for between 2 and 4 days.

In conclusion, although selection for high body weight increased the penalty of infection on performance in mice as observed in our previous studies, unexpectedly their overall worm burden and parasitic egg counts were lower than in their low body weight counterparts. Loss of performance in infected ROH mice was restored to that of uninfected ROH mice when fed a high protein diet, whilst the effect of increased

protein nutrition to decrease worm burden and egg counts in ROL mice was more pronounced than in ROH mice. It may be suggested therefore that animals selected for high body weight would benefit from improved protein nutrition to restore production losses during pathogen challenges whilst animals that are resilient to parasitic infection may benefit from improved protein nutrition as part of a non-chemical and sustainable approach to parasite control to reduce pasture contamination arising from such individuals.

Chapter 6. General Discussion

Table 6.1. Summary of experimental chapter hypotheses and results.

Chapter	Hypotheses	Results
Chapter 2	Increasing protein nutrition above a certain level will reduce the penalty of infection, this level will be higher in mice selected for high body weight than in mice selected for low body weight.	Increasing protein nutrition above 130 g/kg crude protein (CP) ameliorated the penalty of infection on weight gain in ROH mice. ROL mice did not show any effect of infection on weight gain. Body fat was reduced due to infection in both lines. Both mice lines also showed clear anorexia. ROH mice had higher parasite worm burdens and egg counts than ROL mice, increasing protein nutrition did not affect on worm burdens and egg counts in either mice line.
Chapter 3	Increasing larval dose above a certain level will increase the penalty of infection, this level will be lower in mice selected for high body weight and than in mice selected for low body weight. Increased protein nutrition is expected to increase the larval dose required to produce this effect in both lines of mice.	During protein scarcity larval doses of 150L ₃ and above caused a reduction in weight gain for ROH mice. Infection dose did not affect ROL mice consistently. Feeding a high CP diet ameliorated the effect of infection on ROH mice weight gain. No anorexia was observed. ROL mice had slightly higher parasite worm burdens and egg counts, feeding a high CP diet did not affect parasite outcomes. Both mice lines produced IgE and IgG1 antibodies in response to infection with ROH mice producing a consistently higher IgG1 response.
Chapter 4	Immune responses to GI nematodes are expected to be impaired (reduced) in mice selected for high body weight compared to mice selected for low body weight. Increased protein nutrition is expected to enhance immune responses towards GI nematodes.	Cytokine data were not polarised towards a Th2 phenotype. Low CP diets and ROL mice produce a more inflammatory immune response upon infection.
Chapter 5	Secondary infections with GI nematodes are expected to increase the penalty of infection on mice selected for high body weight compared to their low body weight counterparts. Increased protein nutrition is expected to ameliorate this penalty.	During protein scarcity, infection further reduced weight gain in ROH mice but not in ROL mice. Feeding a high CP diet ameliorated this penalty of infection. Anorexia was observed in both lines. ROL mice had the highest parasite worm burdens and egg counts. Feeding a high CP diet had a greater effect in reducing parasite worm burdens and egg counts in ROH mice than in ROL mice.

Table 6.2. Summary of parasite outcomes and animal performance for each chapter

Chapter	Protein Content	Results		
		Parasite measures	Food intake/anorexia	Animal performance
Chapter 2	LP-30-80 g/kg Crude Protein (CP)	ROH had higher worm burden and egg counts than ROL	Infection reduced food intake in ROH mice. Anorexia was observed in all infected mice	Infection reduced weight gain in ROH mice and body fat percentage in both mice.
	HP-130-280 g/kg CP	No significant effect of HP	No significant effect of HP	Infection does not reduce weight gain
Chapter 3	LP-40 g/kg CP	ROL mice had higher worm burden and egg counts than ROH mice	No effect of infection on food intake. No anorexia observed.	Infection over 150L ₃ larvae decreased body weight in ROH mice. No consistent effect of infection on body fat in either line
	HP-230 g/kg CP	No significant effect of HP	LP increased food intake in ROL mice at low larval doses (<100L ₃)	HP increased weight gain in ROH mice and produced no effect of increasing larval dose
Chapter 5	LP-30 g/kg CP	ROH and ROL had a similar worm burden and egg count in a primary infection. ROL mice had a higher worm burden and egg count during a secondary infection	Late primary and secondary infection decreased intake in ROH mice. Anorexia was observed in all infected mice	Late primary infection or secondary infection reduced weight gain and body fat percentage in ROH mice. No effect of infection status in ROL mice.
	HP-230 g/kg CP	HP reduced worm burden and egg counts in ROH mice during a primary infection and ROH worm burden during a secondary infection. HP tended to reduce worm burdens and egg counts in ROL mice	HP increased food intake in ROH mice and decreased intake in ROL mice. HP had no effect on anorexia	HP increased weight gain in both mouse lines and reduced the effect of infection status in ROH mice. Body fat percentage was increased in late primary and secondarily infected ROH mice

The overall aim of this thesis was to investigate the potential effects of extreme phenotypic selection of a production trait (growth in this case) on an animals' ability to cope with a pathogen challenge. A mouse model was used in order to reduce genetic variation that is present in livestock species due to a background of a mixture of different breeding goals. Using a mouse model also enabled a controlled environment to be maintained and the diet to be manipulated easily. The overarching hypothesis of this thesis was that selection for high body weight would decrease resilience and resistance to a parasite infection when compared to low body weight counterparts. It was also expected that improved protein nutrition would be able to ameliorate this penalty of infection in mice selected for high body weight. As each experimental chapter (2, 3, 4 and 5) contains a comprehensive discussion of the results, the purpose of this general discussion will be to briefly summarise the results across the chapters and then to draw together the experimental results and discussions and relate them to their implications for other species and to future work that should be considered. Table 6.1. provides a summary of the hypotheses and results for each experimental chapter.

6.1. Selection for body weight affects resilience to pathogen challenge during protein scarcity

Table 6.2. summarises the food intake and performance data gathered in this thesis which has been used to assess resilience traits. The data shows that selection for high body weight has impaired resilience to infection during protein scarcity. It also shows that feeding a diet with a higher protein content can ameliorate this penalty on weight gain and, to an extent, body fat percentage (seen in Chapter 5; Table 6.2).

Mice selected for low body weight were extremely resilient to infection and a high protein diet did not affect the outcome of performance traits. The presence of anorexia in all infected ROH and ROL mice in Chapters 2 and 5 (Table 6.2) was also unaffected by protein nutrition.

This thesis provides evidence that selection for high body weight in mice results in a decreased resilience (growth despite infection) during protein scarcity. This is in agreement with Zaralis *et al* (2008) who also found that infection reduced weight gain in growing lambs with a high growth potential but not in lambs with a lower growth potential. These lambs also showed anorexia that lasted the entire infection period which presumably caused nutritional restriction in these animals, where this was not seen in the lambs with low growth potential (Zaralis *et al.*, 2008). Selection for low body weight resulted in no weight loss due to infection in all experiments detailed in this thesis. This lack of any loss of resilience to infection during protein scarce conditions may be due to the mice selected for low body weight showing hyperphagia on the low protein diet. This was able to prevent protein scarcity in mice described in Chapter 3 due to the protein content of the diet being 40 g/kg CP instead of the 30 g/kg diets from Chapters 2 and 5. In Chapter 5 ROL mice were unable to compensate for the low protein diet despite again showing hyperphagia but all weight loss was due to the low protein diet and infection did not incur any additional penalty on weight gain. The inability of ROH mice to similarly increase their food intake in response to low dietary protein contents is an interesting one. Perhaps this was due to differences in circulating leptin levels, a hormone responsible for signalling satiety in mammals (Schwartz *et al.*, 2000), or due to an

inability of ROH mice to deal with the excess energy intake that would result from increasing their food intake on this diet (Emmans and Kyriazakis, 2000). From personal observation, the ROL mice showed increased activity (i.e. bar climbing) and perhaps expended some of the excess energy in this way.

Chapter 2 also provided the first characterisation of the time course of anorexia during an *H. bakeri* infection, which was unchanged by selection for body weight. Chapter 5 showed that anorexia was again present in both Roslin lines but that ROH mice that were infected during the experimental diet phase (d-3 to d21) were unable to recover their intake before the end of the experiment. This inability to recover intake during a prolonged parasite challenge has also been seen in livestock studies (Symons *et al.*, 1981; Zaralis *et al.*, 2008). The lack of a significant interaction between time and infection for food intake in Chapter 3 is thought to be due to anorexia only being present at the highest infection doses, and that the presence of infection doses below this threshold for anorexia diluted the effects that were able to be picked up during the analysis of this experiment.

The results of Chapter 3 suggest that during protein scarcity there is a minimum level of incoming larvae above which a mouse selected for high body weight changes its resource allocation rules, resulting in increased control of the parasite burden at the expense of weight gain. It makes evolutionary sense that an animal would have to make trade-off decisions between costly traits (Lochmiller and Deerenberg, 2000). Although there are indications that, above certain population densities, parasite numbers are controlled by host immune responses (Paterson and Viney, 2002; Bleay

et al., 2007) these studies do not report on host growth and therefore cannot assist in developing this argument further.

During the third experiment (Chapter 5) an interesting reduction in body fat percentage was observed in the ROH mice fed the high protein content diet. This was namely, that despite not showing any reduction in weight gain, their body fat percentage was decreased in response to infection. This decrease by around 20% (of total body fat percentage comparative to an uninfected mouse) is in keeping with the reduction seen during the first experiment (Chapter 2) and indicates that energy may play a role during parasitic infection in agreement with other studies (Kristan and Hammond, 2001; Kristan, 2008). It is unclear, however, whether this decrease in body fat was an inability for the animal to gain the body fat or a catabolism of existing body fat reserves.

6.2. Selection for body weight affects resistance to pathogen challenge

The data in this thesis indicates that selection for high body weight does not impair resistance traits (excluding Chapter 2 results; Table 6.2). ROL mice showed higher egg counts and worm burdens and whilst experiencing a secondary infection (Chapter 5; Table 6.2) showed a greater ability of high protein nutrition to reduce these measures. High protein nutrition also reduced primary infection measures in ROH mice that were experiencing a late infection (Chapter 5; Table 6.2) whilst protein nutrition did not affect parasite worm burdens or egg counts previously. The results of Chapter 2 are discussed below.

Unexpectedly, during the course of this thesis, resistance seemed to be impaired in ROH mice in the first experiment (Chapter 2), ROL resistance was slightly impaired in the second experiment (Chapter 3) whilst finally the third experiment (Chapter 5) showed that ROL mice definitely had impaired resistance to infection. This impaired resistance of ROH mice seen in Chapter 2 may have been due to a low-level pinworm contamination of the experiment, one or two pinworm eggs were observed in twenty individuals' faecal egg counts (FEC), only four of whom were undergoing *H. bakeri* infection. This pinworm outbreak may have primed the immune system to a helminth infection *in utero* or during post natal development (Cai *et al.*, 2009) and thus may have confounded the worm burden and parasite egg count results in Chapter 2. However, it remains unclear as to why the ROL mice may have been affected more than the ROH mice, and hence doubtful that this was the only cause of this result. Since both the second and third experiment (Chapters 3 and 5) indicated that ROL mice show higher worm burdens and egg counts, it is assumed that this is the true outcome of the model. Indeed the tendency for ROH mice to have lower worm burdens and egg counts was accompanied by increased IgG1 titres, and some indication of Th2 cytokines (IL-5 and IL-13) being up-regulated (Chapters 3 and 4). This leads to the assumption that the immune responses of ROL and ROH mice are inherently different and warrant further investigation of how different and why this is the case.

Chapter 4 indicated that although Th2-type antibodies were produced by both lines (Chapter 3) *H.bakeri* antigen re-stimulated mesenteric lymph nodes (MLN) did not produce a Th2 skewed cytokine profile. As this *H. bakeri* antigen was a simple

preparation of adult worms there is a chance that immunomodulatory proteins (Rzepecka *et al.*, 2006) could be present and be able to inhibit normal cytokine production. As such a comprehensive analysis of the MLN stimulated by a mitogen (e.g. anti-CD3) would enable any confounding effects of the *H. bakeri* antigen preparation to be noted. The presence of any immunomodulatory proteins *in vitro* would mimic the conditions *in vivo* and enable the recording of the cytokines that would be up and down regulated as a result of the presence of adult worm within the host. For the purposes of this thesis, the differences between the mouse lines under these immunomodulatory conditions are of the most importance. It makes sense that an animal's ability to cope with a pathogen challenge, where that pathogen can regulate the host immune response, is only as good as the host's ability to resist this regulation.

6.3. The mouse model

The mouse model used in this thesis appears to resemble the research performed on growing lambs, where a high growth potential caused a loss of resilience (anorexia presumably causing some nutritional restriction) compared to lambs with a low growth potential (Zaralis *et al.*, 2008). This indicates that a mouse model may prove useful in dissecting out the underlying causes, both genetic and nutritional, of loss of resilience during pathogen challenges in animals with differing production potentials.

With regards to the effect of selection for high body weight on resistance to a nematode infection, the genetics of the resistance would need to be further elucidated. If genetic linkage of two or more genes is the cause of high body weight

mice being more resistant then it may be that genetic linkage causes any co-selection of growth and immunity traits, whether detrimental or not, in livestock species. If this is the case then selective breeding may be able to separate these traits, where the outcome is detrimental to one or the other, to enable improvement. If the cause turns out to be due to pleiotropy then there is little scope for separating the traits.

6.4. Future direction

The literature for divergently selected mouse lines being infected with parasites and studied is limited to this thesis, however other studies have used *H. bakeri* to assess the effect of protein malnutrition in mice (Boulay *et al.*, 1998; Ing *et al.*, 2000; Tu *et al.*, 2007). There is also some ability to use livestock studies (usually sheep experiments) to provide some comparisons between the interactive effects of differing growth potentials, dietary protein content and infection. This thesis did allow some theories to be formed with regard to extrapolation of results to livestock studies, however, in order to get the most out of this model a few limitations should be addressed in the future.

Firstly, an unselected line made of the same background (C57BL/6J x DBA/2J) should be used in conjunction with the ROH and ROL lines. This would enable a comparison of each selected line with an unselected “baseline” line, enabling the assessment of how selection for high or low body weight actually changed other traits. ROH mice are observed to have the C57BL/6J coat colour whilst ROL mice have the DBA/2J coat colour and as such it may be that alleles linked to coat colour in mice have been segregated as such too. Keightley and Bulfield (1993) found that

quantitative trait loci (QTL) associated with growth traits were also associated, through genetic linkage rather than directly, with coat colour in mice. This leads to questions as to which other traits may be linked.

Secondly, in order to satisfy that the effects seen in the Roslin line mice are not due to genetic drift, random mutations within the ROH and ROL populations, other divergently selected lines (and their unselected counterpart if possible) should be used in this model. Bünger *et al* (2001a) detail several different lines that have been divergently selected for different body weight and growth traits. Using a selection of different lines would also allow for further extrapolation of results and an enhanced ability to draw conclusions for livestock species. Prior to the start of this thesis, a pilot study was performed on four divergently selected mouse lines (for high or low body weight). These mouse lines were infected with 250L₃ *H. bakeri* or sham infected with water, where it was found that high growth potential reduced their weight gain in response to infection more than their low growth counterparts (Houdijk & Bünger, 2006). Faecal egg counts were also found to be higher in the high growth potential counterparts (Houdijk & Bünger, 2006). Whilst the Roslin line was chosen for further work because they showed the greatest magnitude of effects during the pilot studies, the pilot data suggests that the Roslin line may not be unique in the depression of growth due to infection in the high body weight mice. However, further investigations are required to solidify the model.

Thirdly, the major-histocompatibility complex (MHC) haplotype should be classified in ROH and ROL mice. For instance, if the MHC haplotype has segregated with

coat colour and body weight then this may explain the higher resistance observed in the ROH line during the final experiment (Chapter 5). If this is the case, then it should be investigated as to whether this segregation is true for all divergently selected mouse lines (referred to by Bünger & Hill, 1999; and Bünger *et al.*, 2001a) and whether this segregation is due to genetic linkage. Differing MHC haplotypes have been shown to confer large variation in resistance to *H. bakeri* infection in mice (Behnke & Wahid, 1991) and to infection in cattle and sheep (Glass *et al.*, 2000; Hickford *et al.*, 2004). In addition, some evidence for growth traits being influenced by MHC haplotype has been shown in mice and pigs (Simpson *et al.*, 1982; Jung *et al.*, 1989).

Finally, cytokine and antibody measurements from the third experiment (Chapter 5) will enable further characterisation of the Roslin lines, providing a more detailed insight into the underlying immune responses and effects of intensive selection on the expression of immunity. Tied into these measurements it would have been useful to be able to characterise the commensal gut bacteria of the Roslin lines, and the possible effects of infection and dietary protein contents to the gut flora. Commensal bacteria that colonise the gut have been found to provide critical roles in the development of gut-associated lymphoid tissues (GALT) and the physiology of the gut through experimental manipulation studies (Hooper, 2004; and reviewed in Artis, 2008). Creamer *et al* (1967) also observed that germ-free animals had a reduced rate of epithelial cell turnover in the gut, while a high rate of epithelial cell turnover has been implicated in the expulsion of intestinal parasite (Cliffe *et al.*, 2005). Commensal gut flora, therefore, appears to be a key piece of the immunity puzzle

and warranting of further investigation during any gut associated infection and/or dietary manipulation experiments.

6.5. Conclusions

This thesis provides evidence that improved protein nutrition can be used to ameliorate the penalty of infection on resilience in hosts selected for high body weight. It also provides evidence that the allocation of scarce resources in hosts selected for high body weight is dependent on the intensity of the infection pressure. At low infection intensities weight gain is still the priority during a primary infection whilst controlling the parasite becomes the priority at higher infection intensities. This thesis also lead to the unexpected conclusion that hosts showing increased resilience to pathogen challenge may have a reduced resistance and that this reduced resilience is sensitive to protein nutrition. These conclusions are in keeping with research using improved protein nutrition in livestock breeds to reduce weight loss and parasite egg excretion. Thus, 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 even in intensively selected animals. 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.

The exact genetic effect of selection for high and low body weight in the Roslin lines remains to be established and may enable further conclusions to be drawn to livestock breeds. Furthermore, using other divergently selected mouse lines (and

non-selected counterparts) in this model will also require further work to rule out genetic drift in these lines.

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