Energetic, morphologic and physiologic responses during *Heligmosomoides bakeri* (Nematoda) infection and protein deficiency in pregnant and lactating mice

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ABSTRACT

This research investigated the concurrent effects of a gastrointestinal nematode infection *Heligmosomoides bakeri,* pregnancy, and protein deficiency (PD) during late pregnancy throughout lactation on energetic, morphologic and immunological responses in CD1 mice and their offspring. Our novel findings can be summarized broadly into three key themes: (i) energetics and resting metabolic rate, (ii) bone metabolism and (iii) immune development.

This work highlights the largely independent ways in which the mouse responds to competing demands of pregnancy, infection, and PD. Pregnancy increased RMR while PD lowered RMR; a trickle infection was not associated with any change. During pregnancy, these additional energetic demands were met by increased food intake and fat utilization. During infection mice lowered their body temperature. Finally, the reduction in RMR during PD was associated with higher serum corticosterone and leptin concentrations.

A second novel finding was that both infection and PD impacted on maternal and neonatal bone development. Infection lowered maternal femur bone area which was associated with elevated serum IFN-γ in heavily infected pregnant mice and reduced foetal crown-rump length consistent with higher amniotic fluid IL-1β. Lower bone mineralization in PD dams was associated with elevated serum corticosterone and leptin whereas it was associated with elevated serum IL-1β and IL-6 during infection. The elevated serum IL-1β, lower leptin and IGF-1 in pups of PD and infected dams were consistent with the shorter crown-rump length.

Finally, we explored for the first time the impact of maternal PD and infection on neonatal immune development. Both maternal infection and PD reduced lymphoid organ mass in pups whereas the percentage of T cells and T:B cell ratio in the spleen was increased only by maternal PD. These changes

were associated with elevated corticosterone and IL-6 concentration in milk, and lower pup serum leptin and IGF-1 in pups from PD infected dams.

Together, these studies show that pro-inflammatory cytokines, corticosterone and leptin up-regulated during nematode infection and PD modify bone metabolism, and impair neonatal linear growth and immune development. This mouse model provides a novel framework to study the impact of diet and nematode infection on growth and development.

ABRÉGÉ

L'impact sur les réactions immunologiques, énergétiques et morphologiques a été étudié suite à une combinaison de facteurs tels une infection par *Heligmosomoides bakeri*, un nématode du système gastro-intestinal, la grossesse et la déficience protéique (DP) durant les derniers mois de grossesse et pendant la lactation chez les souris CD1 et leur descendance. Nos découvertes peuvent se résumer selon trois thèmes clés: (i) énergétique et métabolisme de base (MB), (ii) métabolisme osseux et (iii) développement immunitaire.

Ce travail met en évidence les diverses voies indépendantes utilisées par la souris pour répondre à trois évènements: la grossesse, l'infection et la DP. La grossesse a augmenté le MB tandis que la DP l'a diminué. Une infection peu sévère n'a été associée à aucun changement. Durant la grossesse, les besoins énergétiques supplémentaires ont été comblés en augmentant l'apport nutritionnel et l'utilisation des graisses. Pendant la période d'infection, la température corporelle des souris a diminué. Enfin, la réduction du MB lors de la DP a corrélé avec une plus grande concentration de corticostérone et de leptine.

Notre seconde découverte montre que l'infection et la DP ont un impact sur le développement osseux maternel et néonatal. L'infection a diminué l'os du fémur et a entraîné une forte concentration d'IFN-γ dans le sérum des souris gestantes fortement infectées. Elle a aussi diminué la distance vertex-coccyx tout en augmentant IL-1β dans le fluide amniotique. Une plus faible minéralisation osseuse chez les souris souffrant de DP coïncidait avec une forte concentration de corticostérone et de leptine dans le sérum bien qu'elle

coïncidait avec une forte concentration d'IL-1β et d'IL-6 lors de l'infection. Une concentration élevée de IL-1β mais plus faible de leptine et de IGF-1 chez les souriceaux de mères infectées et souffrant de DP était compatible avec des distances vertex-coccyx plus basses.

Enfin, pour la première fois, on s'est intéressé à l'impact de la DP maternelle et de l'infection sur le développement du système immunitaire néonatal. Chez les souriceaux, l'infection maternelle et la DP ont diminué la masse des organes lymphoïdes. Cependant, le pourcentage de lymphocytes T et le ratio LT:LB dans la rate ont seulement pu être augmentés par la DP maternelle. Ces changements ont été accompagnés par une forte concentration en corticostérone et en IL-6 dans le lait, mais par une plus faible concentration de leptine et d'IGF-1 dans le sérum des souriceaux de mères infectées et souffrant de DP.

Ces études montrent : que les cytokines pro-inflammatoires, la corticostérone et la leptine surrégulent lors d'une infection par un nématode, que la DP modifie le métabolisme osseux et qu'elle altère la croissance néonatale et le développement immunitaire. Ce modèle animal procure un nouveau cadre de recherche pour étudier l'impact de la diète et de l'infection par les nématodes sur la croissance et le développement.

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CONTRIBUTIONS OF AUTHORS

This thesis is written in the form of manuscripts according to the "Guidelines Concerning Thesis Preparation". This thesis was written by Maurice R. Odiere. The thesis consists of three manuscripts, all of which were co-authored with my academic advisors, Dr. Marilyn E. Scott and Dr. Kristine G. Koski as the research was developed in collaboration with them. The first manuscript is published, whereas the second and third manuscripts have been submitted. I was responsible for all the experimental work (experimental design, experimental manipulation of mice, sample collection and analyses), statistical analysis of the data and data interpretation within the context of available literature. In Chapter III and IV, Dr. Hope A. Weiler was involved in the training and design of body and bone composition measurements using the Dual-energy X-ray absorptiometry (DXA) technique. In Chapter V, Dr. Florence Dzierszinski was involved in training on cell harvesting and flow cytometry procedures. In Chapter V, Louis-Philippe Leroux assisted in flow data acquisition and FACS analysis. Dr. M. E. Scott and Dr. K. G. Koski served as research/thesis supervisors, provided financial resources for the laboratory work, were involved in the design of experiments, critiqued data presentation and analyses, and pre-edited all written manuscripts and the thesis.

STATEMENT OF ORIGINALITY

1. Physiological responses during trickle *H. bakeri* infection and pregnancy reduced maternal femur bone area and impaired normal foetal linear growth. This is the first study to report this negative effect of maternal intestinal nematode infection on foetal skeletal linear growth. The reduction in maternal femur bone area and the shorter foetal crown-rump lengths induced by *H. bakeri* infection were associated with elevated serum IFN-γ and amniotic fluid IL-1β respectively.

2. In protein deficient mice, bone area and bone mineral content were reduced compared to protein sufficient mice, and this was accompanied by higher serum osteoprotegerin (OPG) and lower receptor activator of nuclear factor-k B ligand (RANKL) concentration. The *H. bakeri* infection-induced reduction in maternal bone mineral density was accompanied by higher serum OPG and pro-inflammatory cytokines IL-1β and IL-6, but with no effect on RANKL concentration. The markers of bone remodeling (OPG and RANKL) exhibited non-classical patterns during these pathological conditions of PD and infection.

3. The elevated serum IL-1β and lower IGF-1 in pups of protein deficient dams, and lower serum leptin and IGF-1 in pups of *H. bakeri*-infected dams were consistent with the lower pup body mass and the shorter crown-rump length of pups. We succeeded in creating a novel animal model for studying stunting effects.

4. In protein deficient mice, worm burden was elevated. Leptin and corticosterone were elevated suggesting that these two hormones were associated with the delayed worm expulsion.

5. Both maternal infection and PD reduced thymus and spleen organ mass in pups whereas the percentage of T cells and T:B cell ratio in the spleen was increased only by maternal PD. These changes in neonatal immune system were associated with elevated corticosterone and IL-6 concentration in milk, and lower serum leptin and IGF-1 in pups from PD infected dams.

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LIST OF ABBREVIATIONS

AF	Amniotic fluid
ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
ATP	Adenosine triphosphate
BA	Bone area
BMC	Bone mineral content
BMD	Bone mineral density
BMR	Basal metabolic rate
BSA	Bovine serum albumin
CD3, 4 & 8	Cluster of differentiation 3, 4 & 8
DXA	Dual-energy X-ray absorptiometry
EPG	Eggs per gram
FACS	Fluorescent activated cell sorting
GC	Glucocorticoid
GH	Growth hormone
GI	Gastrointestinal
GR	Glucocorticoid receptor
HSD	Hydroxysteroid dehydrogenase
IFN-γ	Interferon-gamma
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IL-1β	Interleukin 1 beta
IP	Intraperitoneal
L_3	Third-stage larvae
L_4	Fourth-stage larvae
M-CSF	Macrophage-colony-stimulating factor
MCP-1	Monocyte chemotactic protein-1
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex

MMC	Mucosal mast cell
mIgM & D	Membrane-bound Immunoglobulin M & D
NRC	National Research Council
OPG	Osteoprotegerin
PBS	Phosphate buffered saline
PCD	Programmed cell death
PD	Protein deficient
PS	Protein sufficient
PP	Post-partum
RAG	Recombination activating gene
RBC	Red blood cell
RANKL	Receptor activator for nuclear factor kappa B ligand
RMR	Resting metabolic rate
RQ	Respiratory quotient
STAT	Signal transducer and activator of transcription
Th 1	T helper type 1
Th 2	T helper type 2
TCR	T cell receptor
TGF-β	Transforming growth factor - beta
TNF-α	Tumour necrosis factor - alpha
VEGF	Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

Gastrointestinal (GI) nematodes are chronic pervasive infections that contribute to worldwide morbidity and mortality in humans (WHO 1992; Albonico *et al.* 1999) and livestock (van Houtert and Sykes 1996). Such morbidity often involves malnutrition (Crompton 1992). Unfortunately, intestinal parasitism and malnutrition share a similar geographical distribution, with the same individuals experiencing both disease states simultaneously (Pelletier 1994). Interactions between malnutrition and infection have been envisaged as a negative spiral whereby malnutrition promotes infection, and infection leads to malnutrition (Koski and Scott 2001).

GI nematodes may reduce the hosts' energy intake through induction of anorexia (Roberts *et al.* 1999; Mercer *et al.* 2000), impairment of assimilation efficiency through malabsorption, maldigestion and gastrointestinal losses (Koski and Scott 2001), or may increase hosts' energy expenditure via up-regulation of immune defense mechanisms and/or tissue repair (Lochmiller and Deerenberg 2000). As a corollary, one might predict an intimate and complex association between the host and the parasite with regards to energy partitioning by the host. This complex relationship hinges upon factors that affect parasite populations such as host's nutritional status and physiological conditions such immunity. For instance protein energy malnutrition (PEM) often coexists with GI nematode infections (Koski and Scott 2001). Since host's nutritional requirements for metabolizable protein (MP) increase substantially in late gestation and lactation (Adams and Liu 2003), co-occurrence of nematode infection and protein deficiency (PD) during this reproductive period imposes considerable physiological demands due to energy and protein required for immunity, foetal growth, lactation and maintenance of maternal tissue (Adams and Liu 2003). In addition, many components of the immune effector responses

are highly proteinaceous in nature (Coop and Holmes 1996), and thus are influenced by dietary PD (Boulay *et al.* 1998; Ing *et al.* 2000).

When animals are well-nourished, they presumably have more than sufficient resources to meet competing demands associated with infection and other important energy demanding activities. However, during periods of increased energy demands and/or reduced nutrient supply, hosts prioritize energy towards maintenance functions e.g. tissue repair (Coop and Kyriazakis 1999), possibly at the expense of functional immunity and/or short-term reproduction (Kristan 2002a). Competition among different organismal functions for limited internal resources often results in trade-offs (Zera and Harshman 2001). In line with this, because immune responses are energetically costly (Buttgereit *et al.* 2000), the adaptive suppression of such responses reallocates the released resources to other important functions (Sheldon and Verhulst 1996). The nutrient-partitioning framework suggests that allocation of scarce nutrients to functions associated with parasite control is less prioritized relative to reproductive effort (Coop and Kyriazakis 1999), which may explain the breakdown of immunity against GI nematodes in pregnant (Houdijk *et al.* 2003a; Ng'ang'a *et al.* 2006) and lactating animals (Houdijk *et al.* 2003b; Ng'ang'a *et al.* 2004; Normanton *et al.* 2007).

Effects of GI nematode infection are not confined to the immune system, but may be manifest in other organs such as bone. Skeletal tissue is an integral part of the endocrine energy regulation mechanism (Wolf 2008), indicating that increased energy demand associated with up-regulation of immunity may necessitate plasticity in bone. Although growth stunting in nematode-infected children has commonly been reported (Casapía *et al.* 2007; Payne *et al.* 2007; Jardim-Botelho *et al.* 2008), there are surprisingly few studies on the effect of GI nematode infections on bone and on mechanisms through which such infections may modulate bone tissue. Mechanisms through which GI nematodes may affect bone composition include impairment in

intestinal calcium absorption and/or up-regulation of mediators such as pro-inflammatory markers and hormones e.g. leptin (Tu *et al.* 2008) that inhibit bone formation and/or induce resorption (Nguyen *et al.* 1991; Kotake *et al.* 1996; Ducy *et al.* 2000). Up-regulation of energetically costly immune responses (Buttgereit *et al.* 2000) as may occur during GI nematode infection may also reduce energy allocation to other tissues such as bone, consequently affecting bone remodeling processes in the infected host. Furthermore, changes in bone remodeling may be directly related to immunopathology induced during GI nematode infection. For instance, chronicity to helminth infections that leads to malnutrition is propagated by Th1 pro-inflammatory cytokines including IFN-γ, IL-6, IL-12 and IL-18 (Else *et al.* 1994; Hayes *et al.* 2004) that also modify bone metabolism (Nguyen *et al.* 1991; Kotake *et al.* 1996; Ducy *et al.* 2000). On the other hand, bone is one of the most protein dense tissues in the body (Heaney 1998), and undergoes constant turnover and remodeling to repair micro-damaged areas (Raisz 1999). Therefore, besides the effect of infection, bone remodeling is influenced by dietary PD (Delmi *et al.* 1990; Ammann *et al.* 2000; Bourrin *et al.* 2000).

It is expected that the effects of GI nematode infection and PD should be more dramatic in pregnant and lactating hosts. There is evidence that adverse maternal conditions such as infection or nutrient deficiency during reproductive period can alter transfer of immunity to the pup and maternal effort (e.g. nursing) subsequently affecting offspring growth, immunocompetence and survival (Saino *et al.* 1997; Kristan 2002b). However, although the prenatal and antenatal period is thought to be one of high susceptibility to infection and diet-induced physiological changes, there have been few studies on the effects of GI nematode infection and PD on foetal and neonatal skeletal linear growth and immune development. Sub-optimal maternal protein nutrition during the reproductive phase may lead to elevated glucocorticoids (GCs) (Hill *et al.* 1995) which impair glucose transport resulting in foetal growth retardation (Hahn *et al.* 1999). In addition, elevated GCs and pro-inflammatory cytokines

associated with maternal stress during infection or PD can be transferred from maternal system to neonates via milk (Catalani *et al.* 2000; Nguyen *et al.* 2007). GCs and pro-inflammatory cytokines are known to inhibit the growth hormone-insulin-like growth factor (IGF)-1 axis (Beauloye *et al.* 1999; MacRae *et al.* 2006), which may consequently not only inhibit foetal and neonatal axial skeletal growth, but also impair development of the immune system in the neonate. For instance, elevated GCs are known to cause thymic atrophy (Cunningham-Rundles *et al.* 2005), programmed cell death (PCD) of immature thymocytes during intra-thymic T cell selection (Ashwell *et al.* 1996), and apoptosis of thymic T cells (Gruber *et al.* 1994) and B cells (Lill-Elghanian *et al.* 2002).

Research on immunological-based costs and trade-offs to date has involved non-natural conditions involving the use of non-replicating or non-living antigens (Demas *et al.* 1997; Smits *et al.* 1999; Martin *et al.* 2003). However, we know that this is far from what happens in natural populations since the immune response invoked is limited compared with parasite-induced immune responses, which involve tissue damage and repair as an additional cost (Lochmiller and Deerenberg 2000). Factoring these additional costs into the energy expenditure equation provides a more realistic perception of costs of immunity in terms of trade-off decisions. In this regard, my studies selected to use the common intestinal parasite of murine rodents, *Heligmosomoides bakeri* (a living organism that releases a variety of antigens during its life), which would induce a more ecologically relevant antigenic challenge. *H. bakeri* (*Nematospiroides dubius*; *Heligmosomoides polygyrus*) is used commonly in the laboratory mouse-nematode model (Monroy and Enriquez 1992), but most experimental studies have been conducted using single-pulse primary infections (Behnke *et al.* 1993; Ben-Smith *et al.* 1999; Kristan and Hammond 2006) that are chronic (Gause *et al.* 2003), or challenge infections (Shi *et al.* 1998; Ing *et al.* 2000; Tu *et al.* 2008) that induce protective immunity (Gause *et al.* 2003). Studies in the present research employed the trickle (repeated) infection

protocol with *H. bakeri* that induces a much more vigorous immune response (Behnke and Robinson 1985; Enriquez *et al.* 1988a; Enriquez *et al.* 1988b; Robinson *et al.* 1988; Scott 1991), is presumably more energetically costly, and perhaps better reflects conditions in the field, where animals are exposed to frequent but generally low-intensity challenge (Berding *et al.* 1986).

Another important measure in the present research was body and bone composition. Analyses of body composition are important as they provide insights into the impact of experimentation (e.g. parasitism, dietary nutrient deficiency). Unfortunately, such analyses have been limited in the past, because the traditional approach for body composition measurements in animals (for instance determination of fat mass) has been through killing of animals before, during, or after treatment and chemically extracting total lipid from the dried carcass. This requirement for destructive sampling limits the number of animals that can be used and prevents repeated measurements on the same animal. I was fortunate to have access to the Dual-energy X-ray absorptiometry (DXA) equipment to measure fat, lean mass and bone composition. This methodology is quick and can be used on live animals.

Therefore, in summary, the overall objective of this study was to provide an insight into the impact of GI nematode infection and PD during pregnancy and lactation, by looking at the energetic, morphologic and physiologic responses involved during these interactions, using the *H. bakeri* - mouse model. My work subjected laboratory mice to simultaneous demands that are within the ranges that are likely to occur in nature but remain relatively unstudied: (i) repeated nematode infection during pregnancy and (ii) repeated nematode infection and PD throughout late pregnancy and lactation. This is important because in nature, animals likely face a multitude of simultaneous demands, not just an individual demand. Of particular interest in the current research was the differential allocation of resources and physiological responses that occur not only within the mother, but also at the maternal-foetal interface

and within the neonate post-partum. In Chapter III, I investigated the energetic, immunological, physiological and skeletal responses to infection and pregnancy in the maternal and foetal compartments - specifically whether a stronger immune response during higher dose infection would increase RMR, and whether shifts in cytokine profiles in infected pregnant mice would promote nematode survival, induce maternal bone remodeling, and impair skeletal development in the foetus. In Chapter IV, I investigated whether PD and GI nematode infection during late pregnancy and lactation would modify RMR, maternal body composition and bone mineralization, and neonatal growth, and the hormone and cytokine regulatory mechanisms related to these responses. In Chapter V, I sought to confirm that PD during late pregnancy and lactation impairs maternal immune response to trickle *H. bakeri* infection, and further explored whether PD and *H. bakeri* infection during pregnancy and lactation would impair development of immune system in the neonate. Details of these studies are provided in the three papers that follow. The first paper has been published in Parasitology journal, the second paper has been accepted in The Journal of Nutrition and the third paper has been submitted to Infection and Immunity journal.

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CHAPTER II

LITERATURE REVIEW

A. *HELIGMOSOMOIDES BAKERI* - MOUSE MODEL

H. bakeri is a trichostrongyloid intestinal parasite of murine rodents. It was introduced as an experimental infection in laboratory mice by Spurlock in 1943 (Behnke *et al.* 1991), and has been proposed as an experimental model for human hookworm infection (Bartlett and Ball 1972) and other nematode infections of veterinary importance e.g. *Ostertagia ostertagi* (Monroy and Enriquez 1992). The parasite has had a series of name changes from *Nematospiroides dubius* (Spurlock 1943) to *Heligmosomoides polygyrus* (Behnke *et al.* 1991) and to *Heligmosomoides bakeri* (Cable *et al.* 2006). Parasite molecular sequence divergence between laboratory and wild mice led to the recommendation by Cable *et al.* (2006) that *H. polygyrus* be confined to wild mice whereas *H. bakeri* be used for the laboratory mice. Therefore, I have used the taxonomic designation *H. bakeri* throughout this thesis.

1. Lifecycle of *H. bakeri*

The lifecycle of *H. bakeri* has been described by Bryant (1973). It involves both free-living and parasitic stages and occurs predominantly in the small intestine of its murine host. Parasites eggs are passed in the faeces of the infected host, hatch within approximately 36 hours to become the first larval stage (L_1), which then undergo two moults to form the ensheathed non-feeding infective third larval stage (L_3). Ingested L_3 exsheath and migrate through the lumen of the stomach (during the first 24 hours after infection) (Sukhdeo *et al.* 1984), and migrate to the small intestine where they penetrate the serosal musculature and mature to form the fourth larval stage (L_4). Within 7-8 days post-infection (PI), the pre-adults migrate out of the intestinal mucosa and emerge into the intestinal lumen. Adult worms migrate preferentially to the anterior duodenum where they mature, mate and produce eggs. The anterior duodenum contains longer villi, and *H. bakeri* rely on host intestinal epithelial

tissue for their food resource (Bansemir and Sukhdeo 1994). The preferential location for adult worms therefore is linked to the sufficient food resource and enhanced support/attachment by coiling around the longer villi (Bansemir and Sukhdeo 1996). Upon mating, significant levels of egg output are observed within 12-14 days PI (Bryant 1973). An average of 1200-1500 eggs per worm per day are released in the feces of mice infected once with 100 infective L_3 (Kerboeuf 1985).

2. Host Immune Response to *H. bakeri*

Mice produce both a cell- and antibody-mediated immune response to *H. bakeri* that involves both the spleen and small intestine (Dehlawi and Wakelin 1988; Scott and Koski 2000). There is a consensus that immunity to intestinal-dwelling nematode worms is critically dependent on a type 2 cytokine response (controlled by CD4+ T helper type 2 cells that secrete the cytokines IL-4, IL-5, IL-9 and IL-13), while susceptibility to chronic infection is propagated by type 1 cytokine responses (characterized by production of IL-12, IL-18 and interferon-gamma (IFN) -γ) (Maizels and Yazdanbakhsh 2003; Cliffe and Grencis 2004; Hayes *et al.* 2004). There is also evidence for the role of IL-25 (IL-17E) in the development of Th2-cell responses and worm expulsion (Fallon *et al.* 2006; Owyang *et al.* 2006). While IL-4 stimulates fluid secretion in the gut, IL-13 promotes goblet cell responses and both cytokines induce smooth muscle contraction (Urban *et al.* 1998; Akiho *et al.* 2002; Finkelman *et al.* 2004; Madden *et al.* 2004), which together are thought to induce expulsion of adults.

Whereas L_4 stages provide the most antigenic stimulus, functional immunity to *H. bakeri* is achieved through expulsion of adult worms (Behnke *et al.* 1983). The Th2-dependent immunity (antibody-dependent) also involves effectors such as IgE-dependent eosinophils and mucosal mast cells (MMC) (Wakelin and Donachie 1983). Systemic responses reported during *H. bakeri* infections include splenomegaly, mesenteric lymphadenopathy, leukocytosis,

eosinophilia, neutrophilia, activation of macrophages and activation of complement, particularly during challenge infections (Wakelin and Donachie 1983; Pentilla *et al.* 1985). The primary infection of *H. bakeri* is usually chronic (Gause *et al.* 2003), whereas both challenge infection (after worm clearance with anti-helminthics) or repeated 'trickle' re-infection elicit a vigorous Th2 response (Pritchard *et al.* 1984; Brailsford and Behnke 1992) and induce protective immunity (Behnke *et al.* 2003; Gause *et al.* 2003).

3. Genetics of Resistance/Susceptibility of Mice to *H. bakeri*

Resistance can be assessed by the host's ability to limit the initial establishment, growth, development and reproduction of the worms, to expel primary infections from the intestines and to interfere with the establishment of secondary infections (Rothwell 1989). Additionally, hosts may show resilience by limiting the pathological consequences of infection (Bisset and Morris 1996). This implies that variation in overall resistance status reflects variation in immune and inflammatory responsiveness.

Quantitative trait loci (QTL) analysis have enabled designation of mice strains relative to others as strong ("resistant") or weak ("susceptible") responders to *H. bakeri* (Iraqi *et al.* 2003; Menge *et al.* 2003). The rapidity and levels of resistance achieved upon challenge infection are dependent upon the dose and frequency of the immunizing inoculum (Menge *et al.* 2003), host genetics, the infectivity of larvae and the parasitic stages to which mice are exposed during the primary infection (Jacobson *et al.* 1982; Behnke and Robinson 1985), the parasite culture (Kristan and Hammond 2006), infection protocol (Behnke and Robinson 1985) and sex of mice (Zuk and McKean 1996). The immune response phenotype in *H. bakeri* infection is influenced by both background and major histocompatibility complex (MHC) genes (Behnke and Robinson 1985).

Mouse strains differ not only in their response to primary and challenge infections to *H. bakeri*, but also in their ability to control repeated infections (Behnke *et al.* 2003). In most mouse strains, the immune response is much more vigorous during challenge or trickle infection (Behnke and Robinson 1985; Enriquez *et al.* 1988; Scott 1991). CD1 are outbred mice, their gene locus is H-2q and their background genes are CD-1 (Arcellana-Panlilio and Schultz 1994), and are presumed to be stronger responders to *H. bakeri* since they share similar genetic traits to SWR (H-2q haplotype) (Iraqi *et al.* 2003).

4. Pathology of *H. bakeri* Infection

H. bakeri undergoes a short histotropic larval phase in the intestinal mucosa, which is characterized by a strong inflammatory response in the gastric and gut walls, followed by a long adult luminal phase in the duodenum. The first phase provokes the most dramatic pathologic change that results in the formation of granulomata at the site of encystment, particularly in resistant mice (Monroy and Enriquez 1992). As with other intestinal parasites, *H. bakeri* induces large increases in intraepithelial lymphocytes, globular leukocytes, and goblet and paneth cells, particularly in resistant strains (Monroy and Enriquez 1992). Infection with *H. bakeri* has also been shown to cause hyperplasia of the epithelium, elongation of intestinal glands, distortion and hypertrophy of villi and increase in numbers of goblet cells compared with uninfected mice (Fakae *et al.* 2000). Morphological changes as reflected by the increase in small intestine mass both in regions occupied by *H. bakeri* (proximal) and unoccupied (mid and distal), increases in spleen and liver sizes have also been documented (Kristan and Hammond 2006). It is possible that *H. bakeri* also elicits complement responses in mice which may account for an increased liver size due to increased function during complement production (Kristan 2002a).

Challenge and trickle infections induce effective protective immune responses which expels adult worms (Behnke *et al.* 2003; Gause *et al.* 2003), and data on immunopathology of *H. bakeri* are often based on secondary

responses. Challenge infection causes patchy lifting of epithelial surface cells, an increase in goblet cell numbers and mucous production (Morimoto *et al.* 2004), increase in paneth cells (Shea-Donohue *et al.* 2001) and eosinophilia (Fakae *et al.* 2000). Sites of larval development become the foci of intense reactions resulting in prominent granulomatous lesions (Jones and Rubin 1974). The severe intestinal pathology that results may be due to the direct damage from the attachment, migration and feeding activities of worms and/or the immune responses (Gause *et al.* 2003). Evidence also suggests that inflammatory and/or p athophysiological effectors secondary to the reduced Th2 response may also contribute to worm expulsion (Shea-Donohue *et al.* 2001; Artis *et al.* 2004; Cliffe *et al.* 2005).

B. THE ENERGY BUDGET AND ENERGETIC TRADE-OFFS

Animals have evolved adaptations to maintain a balanced energy budget (Bronson and Heideman 1994). Balancing an energy budget becomes challenging during times such as winter or drought when food supplies dwindle and thermoregulatory demands increase. These adaptations allow animals to partition available energy to bodily functions where it is most needed (Wade and Schneider 1992). Such challenges in balancing energy budgets may also occur during parasitism necessitating energy trade-offs. Definitions of key trade-off terms such as costs, constraints, and trade-off often vary among studies (Antonovics and Tienderen 1991; Zera and Harshman 2001). For example, in some cases trade-off is defined as the result of physiological or fitness costs (Leroi *et al.* 1994), while in other cases the term cost is used to define a trade-off (Reznick 1985; Reznick 1992). In the current study, the term trade-off will be restricted to cases where data will indicate a negative functional interaction between traits.

If internal resources are limited and insufficient to provide construction and maintenance costs for two traits that share a common resource pool, then a trade-off results: an increment of resources allocated to one trait necessitates a decrement of resources to another trait [the traditional "Y" model of resource allocation (Fig. 2.1), (Noordwijk and Jong 1986)]. Trade-offs can occur between physiological traits expressed during the same or different times in the life cycle (Chippindale *et al.* 1996; Stevens *et al.* 1999; Zera and Cisper 2001) and they can result from variation in genetic factors (e.g., pleiotropy), environmental factors, or combinations of these two types of factors that give rise to negative interactions between traits (Zera and Harshman 2001).

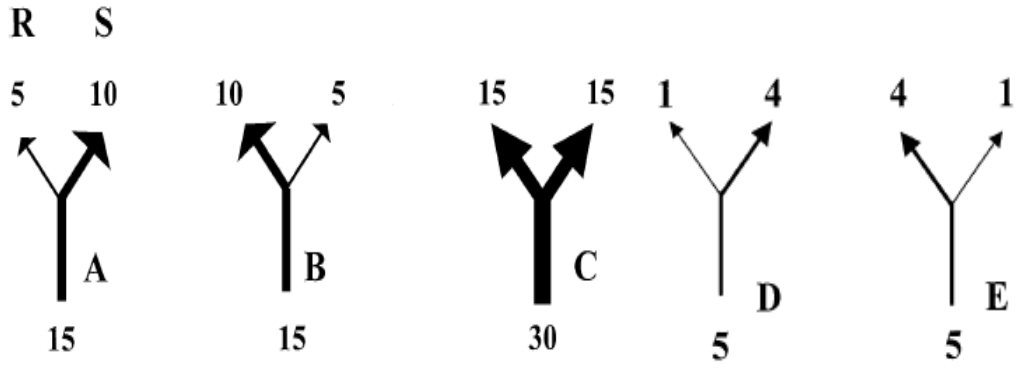

Fig. 2.1 Diagrammatic representation of trade-offs using the **"Y"** model of resource allocation (Reprinted with permission from Zera and Harshman 2001)[†].

Each **"Y allocation tree"** illustrates the amount of resource input (acquisition; number at the base) and the pattern of resource allocation (numbers at the tips of the branches) for a particular phenotype. **R** denotes allocation to reproduction, while **S** denotes allocation to soma. ***A*** and ***B*** illustrate a standard trade-off (differential allocation of a limiting internal resource). Relative to trees ***A*** and ***B***, tree ***C*** illustrates the effect of increased resource input on a trade-off (resource input matches physiological costs of both traits), while trees ***D*** and ***E*** illustrate the exacerbating effect of decreased nutrient input on a trade-off. Trees ***A*** and ***B***, relative to ***D*** and ***E***, illustrate plasticity of a resource-based trade-off (Zera and Harshman 2001).

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1. Phenotypic Plasticity in Response to Environmental Demands

One common way that animals respond to environmental demands is by changing their morphology or physiology [i.e. phenotypic plasticity or flexibility, (Piersma and Lindstrom 1997)]. Benefits of phenotypic plasticity are immediately apparent when we consider that demands will fluctuate, sometimes rather quickly, and a capacity for relatively quick response to these demands is necessary for survival (Kristan and Hammond 2006).

Studies on the effects of acute and sustained environmental demands such as cold exposure, calorie restriction and parasite infection on morphological and physiological plasticity have found evidence that (1) multiple demands elicit responses that either are independent of each other or interact with each other depending on the type of demands that are combined (Kristan and Hammond 2006); (2) the intensity of a demand affects phenotypic plasticity (Kristan 2002b); and (3) combinations of certain demands (e.g. lactation and cold exposure) bring animals close to their limit (Hammond *et al.* 1994) or exceed their capacity to respond leading to mortality (Kristan and Hammond 2006). Not only is the number of demands important, but also the type and the duration of the demands (Kristan and Hammond 2004), and their combined effects (Kristan and Hammond 2000; Kristan and Hammond 2001).

Energy repartitioning in response to concurrent energy demands involves changes in body composition, visceral organ size and energy metabolism (Kristan and Hammond 2006). For example, visceral organs make up approximately 10% of the whole body mass but are responsible for 70% of resting energy expenditure (Ramsey and Hagopian 2006). Therefore any change in organ mass is likely associated with changes in energy expenditure as measured using resting metabolic rate (RMR) as is the case during *H. bakeri* infection in mice (Kristan and Hammond 2006).

2. Resting Metabolic Rate (RMR)

Resting metabolic rate (RMR) represents the 'minimum cost of life', or the metabolic rate of an organism during its inactive phase while at its thermoneutral temperature (Ricklefs *et al.* 1996). The release of energy in this state is sufficient only for the functioning of the vital organs for survival. RMR accounts for about 50-70% of total daily energy expenditure and represents the cost of processes such as the maintenance of transmembrane ion gradients and resting cardiopulmonary activity (Ravussin and Bogardus 1992). As a consequence, fat-free mass together with total body weight, age and gender account for about 80% of the variance in RMR (Ravussin and Bogardus 1992). Other factors that affect RMR include genetics, body surface area, diet, body temperature, external temperature and infection. Men have a higher RMR than women of the same body weight because they have more muscle mass, while decreases in RMR with aging are mainly due to a decrease in muscle mass (Bogardus *et al.* 1986; Bouchard *et al.* 1989).

RMR is measured by gas analysis through either direct or indirect calorimetry (Krol *et al.* 2003; Kristan and Hammond 2006) or from equations employing the energy balance method (Gurr *et al.* 1980; Rothwell and Stock 1987). Changes in RMR can be attributed to an induced physiological condition, and previous studies have used this technique to assess the costs of many physiological activities including moult, organ growth, egg production and antibody response (Lindstrom *et al.* 1993; Klaasen 1995; Svensson *et al.* 1998). Illness, previously consumed food and beverages and stress levels can affect one's overall energy expenditure, and can affect one's RMR (Ruggiero and Ferrucci 2006). The thermic effect of feeding, which is the energy required for the digestion, transport and deposition of nutrients, accounts for about 10% of total 24 h energy expenditure; while non-resting energy expenditure, i.e. all the remaining energy consumption, which mainly occurs in the form of physical activity, represents about 30% of daily energy expenditure (Menozzi *et al.* 2000).

A closely related term is basal metabolic rate (BMR) which is the minimal rate of energy expenditure to maintain life's essential processes (Henry 2005). BMR is measured under stricter conditions than RMR, for instance the subject must be completely rested, the subject must be fasted for at least 10-12 hours prior to measurements, measurements must be at thermoneutral temperature and the subject should be free from emotional stress and familiar with the apparatus used (Henry 2005).

3. Impact of Parasitism on Host's Energy Budget

Energy budgets have provided physiological ecologists with a vital link between environmental variables and individual performance in the field (Voltura and French 2000). Animals strive to maintain a relatively constant flow of energy (i.e., energy intake = energy expended) to the body, despite potentially large fluctuations in energetic availability in their environment (Wade and Schneider 1992). Helminth parasitism can affect energy budgets by imposing an additional metabolic cost (immune defense) (Holmes 1987) or by reducing the overall amount of energy taken in as a result of anorexia, malabsorption or competition for nutrients (Mercer *et al.* 2000) or by decreasing the assimilation efficiency (Crompton 1984). Either process will reduce the energy available for growth or reproduction.

a. Energy Repartitioning involving Body composition During *H. bakeri* Infection

Based on studies involving *H. bakeri* in non-pregnant mice (Kristan 2002a; Wong *et al.* 2007), the impact of infection on whole body mass appears to be directly related to shifts in lean body mass. In experiments involving obese resistant SWR/J mice, where *H. bakeri* infection caused a decrease of whole body mass during calorie restriction, both lean mass and fat mass decreased (Wong *et al.* 2007). In contrast, in studies involving Swiss-Webster mice, when whole body mass increased during *H. bakeri*, only lean mass and visceral organ mass increased (Kristan 2002a).

b. The Immune System and Energetic cost of Immunity

Metabolic requirements are increased by infectious diseases both because of the need for increased tissue repair and because of up-regulation of the immune system (Demas *et al.* 1997; Colditz 2002). Mounting an immune response requires resources that could otherwise be allocated to other biological functions such as growth and reproduction (Sheldon and Verhulst 1996). Therefore, the immune function should be "optimized" so that individuals can tolerate small infections if the energetic costs of mounting an immune response outweigh the benefits (Behnke *et al.* 1992). A general energy deficit can increase the risk of infection and death because insufficient energy reserves may be available to mount and sustain immunity (Demas *et al.* 1997).

Several studies have found that mounting and maintaining an immune response is energetically costly. Demas *et al*. (1997) found that the antibody response of laboratory mice (*Mus musculus*) to keyhole limpet haemocyanin (KLH) challenge elevated RMR by 27%. Similarly, Ots *et al.* (2001) found a 9% increase in BMR of wild great tits (*Parus major*) after challenge with sheep red blood cells (SRBCs). Using a more ecologically relevant parasite, several studies have reported elevated RMR in response to *H. bakeri* primary infection, that was also associated with elevated visceral organ mass (Kristan and Hammond 2000; Kristan and Hammond 2001). However, there are also examples where visceral organ mass increases in response to nematode infection but RMR does not (Kristan 2002a; Kristan and Hammond 2004; Kristan and Hammond 2006). Recently, our laboratory reported that *H. bakeri* challenge infection reduced RMR (Tu *et al.* 2008b). Collectively, these studies suggest that up-regulation and/or maintenance of a competent immune response affects the host's energy budget and may necessitate trade-offs with other organ systems or functions.

c. Trade-offs During Immune Response

Few studies have experimentally addressed the question about the existence of a resource-based trade-off with respect to immune response, though several workers have more or less implicitly assumed this when interpreting observed cases of immunosuppression in experimental studies (Gustafsson *et al.* 1994; Deerenberg *et al.* 1997; Saino *et al.* 1997). Trade-offs presumably exist between immune function and other energetically demanding biological functions (e.g., growth, thermoregulation and reproduction) (Demas and Nelson 1996; Sheldon and Verhulst 1996). Costly immune responses are known to be suppressed during stressful activities such as parental care, strenuous exercise, and the released resources from this immunosuppression are then adaptively reallocated to cover other costly activities (Gustafsson *et al.* 1994; Hoffman-Goetz and Pedersen 1994; Nierman and Nehlsen-Cannarella 1994; Sheldon and Verhulst 1996).

4. Effect of Nematode Parasitism During Pregnancy and Lactation

Maternal nematode infection during the reproductive period has adverse consequences on maternal performance and offspring growth. Relatively few studies have examined the impact of GI nematode infection on reproductive outcomes (Scott 1990; Willis and Poulin 1999; Kristan 2002a; Kristan 2004). Scott (1990) reported that a single maternal infection with *H. bakeri* had no detectable effect on litter size or pup growth. Similarly, neither litter size nor mass of the laboratory rat (*Rattus rattus*) was affected by the intestinal tapeworm, *Hymenolepis diminuta* either at birth or at weaning (Willis and Poulin 1999). Evidence to suggest that infection reduces investment in reproduction is seen in the work of Kristan (2002a) who reported that mice infected with *H. bakeri* were unable to sustain large litter sizes over several pregnancies. After adjusting for parity and litter size, parasitized mothers produced female pups that were 6% smaller at weaning than female pups from unparasitized mothers. In contrast, another study showed that reproductive investment increased in infected females (Kristan 2004). Parasitized females

had larger litter size both at birth and at weaning, pups at birth from parasitized mothers were 1.2% larger than those from unparasitized mothers, but pup size at weaning was not affected by maternal parasite infection (Kristan 2004). This pattern was observed in four consecutive litters. Although some studies have shown that litter size and pup weight are unaffected by *H. bakeri* infection (Scott 1990; Kristan 2002b), recent literature in developmental programming considers that birth weight is a very poor measure of changes (Bautista *et al.* 2008), and important modifications may occur in the pups without a change in the overall weight. Such modifications may alter offspring morphology and growth trajectories.

The effect of GI nematodes on reproductive outcomes is not confined to rodents, but is also evident in the veterinary field. In sheep and goats, GI nematode infection reduces birth weights and growth rates of offspring, with lower mortality in lambs and kids with higher birth weights (Gatongi *et al.* 1997). GI nematode infection during reproduction also affects productivity, for example periparturient rise in parasite egg counts results in decreased milk production in the ewe (Connan 1973) which consequently impairs weight gain in lambs (Darvil *et al.* 1978). GI nematode infection also delays lambing and kidding (Gatongi *et al.* 1997), thereby reducing reproductive performance.

In addition to the above effects on offspring, a considerable demand for protein by activation of immunity-associated response to nematode infection during pregnancy presumably diminishes the host's nutrient status (Liu *et al.* 2003). This total demand for protein is often not met by increase in food intake by the host and results in immunosuppression to cover for the deficit, with the consequent increase in faecal egg count (Adams and Liu 2003; Liu *et al.* 2003). The observed immunosuppression is therefore attributable to the optimal resource allocation between reproductive and immune function depending on competing energetic demands and their associated costs and benefits (Sheldon and Verhulst 1996).

Two hypotheses, one based on resource limitation and the other on avoidance of immunopathology have been put forward to explain the purpose of adaptive immunosuppression during pregnancy (Raberg *et al.* 1998). The resource allocation hypothesis is based on the principle that reproductive functions are important for species propagation and therefore immunosuppression of the energetically costly immune defenses occurs to reduce competition with reproduction for scarce resources (Raberg *et al.* 1998; Coop and Kyriazakis 1999). There is an increasing body of evidence that at least some aspects of immunity are sensitive to changes in nutrient supply (Coop and Holmes 1996; van Houtert and Sykes 1996; Coop and Kyriazakis 1999; Coop and Kyriazakis 2001). The immunopathology hypothesis states that adaptive immunosuppression during reproduction serves to avoid inappropriate immune responses (Wegmann *et al.* 1993; Raberg *et al.* 1998). The above hypotheses may not be mutually exclusive and it is possible that both are functional and important during reproduction.

Adaptive responses in resource allocation and energy repartitioning may not be confined to the immune system, but may involve changes in other tissues such as bone. Indeed, recent research shows that the skeleton exerts an endocrine regulation of energy metabolism (Karsenty 2006; Lee *et al.* 2007), indicating that bone remodeling is an important component of energy repartitioning. This is especially so, considering that bone tissue undergoes constant turnover and remodeling (Raisz 1999).

5. Bone Remodeling and Energy Repartitioning

The skeleton is able to adapt to local biomechanical changes and to repair microdamaged regions by balancing rates of bone formation and resorption through coordination between osteoclasts which resorb bone, and osteoblasts which deposit mineral matrix in previously resorbed areas (Raisz 1999). These processes are under complex regulation involving hormones, soluble local growth factors and cytokines (Baron *et al.* 1983; Mann *et al.*

1994). Some of the mediators that govern the cross-talk between osteoblasts/stromal cells and osteoclast hemopoietic precursor cells are osteoprotegerin (OPG) and the receptor activator of nuclear factor-k B ligand (RANKL) (Silvestrini *et al.* 2007). Both OPG and RANKL are expressed by osteoblasts (Thomas and Martin 2005), and also act as regulators of vascular cell metabolism and calcification (Hofbauer and Schoppet 2004). Other factors that appear to have a prominent role in bone remodeling are glucocorticoids (GCs), pro-inflammatory cytokines (IFN-γ, IL-1β, IL-6, TNF-α) and leptin.

a. Osteoprotegerin (OPG)

OPG is a cytokine expressed by stromal cells and osteoblasts (Lacey *et al.* 1998) and is a soluble member of the Tumor Necrosis Factor receptor family (Simonet *et al.* 1997). OPG is a RANK homolog, and works by binding to RANKL on osteoblast/stromal cells. It inhibits osteoclast differentiation by acting as a decoy receptor for the receptor activator of nuclear factor-k B ligand (RANKL). It prevents RANKL from binding to, or activating, the receptor activator of nuclear factor-k B (RANK) located on the pre-osteoclastic cell surface (Hofbauer and Schoppet 2004), and which is the downstream signaling receptor for RANKL (Yeung 2004). This consequently inhibits the differentiation of the osteoclast precursor into a mature osteoclast.

b. Receptor activator of nuclear factor-k B ligand (RANKL)

RANKL was discovered by several groups (Anderson *et al.* 1997; Wong *et al.* 1997; Lacey *et al.* 1998; Yasuda *et al.* 1998), and was given different names depending on the function being studied. RANKL is essential in the development and activation of osteoclasts (Takayanagi *et al.* 2002). RANKL is produced by activated T cells (Yeung 2004) and its expression is up-regulated by the pro-inflammatory cytokines, including IL-1β and TNF-α (Hofbauer *et al.* 1999). In addition to its essential role in the development and activation of osteoclasts, RANKL has been identified as an important co-stimulation

molecule involved in T cell-dendritic cell communication and in dendritic cell survival (Anderson *et al.* 1997; Bachmann *et al.* 1999).

c. Glucocortiocoids (GCs)

GCs induce osteoclast differentiation, proliferation and activity (Defranco *et al.* 1992; Manolagas 2000), which decreases bone mineralization (Karabelyos *et al.* 1998). GCs also inhibit osteoblast function and proliferation (Lukert and Raisz 1990; Canalis and Giustina 2001) and stimulates apoptosis of osteoblasts (Manolagas 2000) and reduction of osteoblast replication (Canalis 2003) leading to bone loss (Canalis 2005). GC-induced osteoblast apoptosis and increased osteoclast survival and activity appear to be mediated by the OPG/RANKL system (Khosla 2001). The OPG/RANKL ratio is a crucial determinant of osteoclast differentiation and activation (Takayanagi *et al.* 2000). A decrease in OPG/RANKL ratio favours a pro-inflammatory and pro-resorptive response (Silvestrini *et al.* 2007). For instance, the catabolic effects of corticosterone on bone occur together with an OPG fall and a RANKL rise (Silvestrini *et al.* 2007), and OPG falls in GC-induced osteoporosis (Sasaki *et al.* 2001).

The exact mechanism of how GCs affect bone remodeling is still a controversial issue. It has been suggested that GCs stimulate bone resorption directly via activation of mature osteoclasts (Defranco *et al.* 1992). On the other hand, it has been suggested that GCs inhibit the recruitment and/or differentiation of bone-resorbing cells, as they were found to decrease osteoclastogenesis in mice, and administration of hydrocortisone to cat bone marrow cultures led to complete inhibition of osteoclast generation (Suda *et al.* 1983).

d. Pro-inflammatory cytokines

Pro-inflammatory cytokines such as IFN-γ, IL-1β and IL-6 inhibit bone formation and/or induce bone resorption (Nguyen *et al.* 1991; Rodriguiz *et al.* 1993; Mann *et al.* 1994; Manolagas 1998). Effects of IFN-γ on bone remodeling appear to vary depending on the "health status". In healthy animals, IFN-γ is a bone-sparing cytokine that decreases serum calcium and osteoclastic bone resorption in mice (Tohkin *et al.* 1994; Vermeire *et al.* 1997). However, under pathological conditions both in rats and in humans, IFN-γ indirectly promotes bone resorption (Mann *et al.* 1994; Key *et al.* 1995) and a reduction of the trabecular soft inner bone matrix of the long bones such as tibia and femur (Nanes *et al.* 1990; Mann *et al.* 1994), by stimulating antigen-dependent T cell activation and T cell secretion of the osteoclastogenic factor RANKL (Gao *et al.* 2007).

The cellular mechanisms through which pro-inflammatory cytokines such as IFN-γ and IL-1β modulate bone growth have yet to be elucidated, however a common mechanism, such as the impairment of the IGF-1 axis, may be involved (MacRae *et al.* 2007) with cascading effects through IL-6, TNF-α, and macrophage-colony-stimulating factor (M-CSF) leading to increased on osteoclastogenesis (Srivastava *et al.* 1994), and decreased production of OPG (Hofbauer *et al.* 2000).

e. Leptin

Leptin, the product of *ob* gene is a 16 kDA protein derived from adipose tissue (Faggioni *et al.* 2001). Leptin is a pleiotropic molecule; beyond its initial function in regulating appetite and energy expenditure, this hormone plays important roles in regulation of endocrine and immune functions, and is also considered a pro-inflammatory cytokine (Faggioni *et al.* 2001).

Leptin mediates the skeleton's regulation of energy metabolism through its interaction with osteocalcin and adiponectin (Karsenty 2006; Lee *et al.*

2007). Binding of leptin to receptors in the brain causes stimulation of the sympathetic nervous system and activation of the b2-adrenergic receptor gene (Adrb2) in bone, which decreases osteoblast proliferation and bone formation. The osteoblast, in turn, influences energy metabolism by expressing osteotesticular protein tyrosine phosphatase (OT-PTP) which in turn influences the vitamin K dependent γ-carboxylation of osteocalcin (Wolf 2008). Uncarboxylated osteocalcin increases β cell proliferation and insulin secretion in the pancreas, and further influences energy metabolism by affecting adipocyte secretion of adiponectin, an insulin-sensitizing adipokine (Wolf 2008).

Effects of leptin on bone are complex; it can promote bone formation (Goulding and Taylor 1998; Steppan *et al.* 2000), or inhibit bone formation (Ducy *et al.* 2000; Pogoda *et al.* 2006) depending upon bone location, skeletal maturity and whether leptin is functioning directly on osteoblasts through receptors (Cornish *et al.* 2002) or indirectly through the hypothalamus (Hamrick *et al.* 2007). Osteoblasts, osteoclasts and bone marrow stromal (stem) cells all express leptin receptors (Thomas 2004); thus leptin can regulate bone metabolism directly. In early life, leptin can stimulate bone growth and bone size through direct angiogenic and chondro-osteogenic activity. In the mature skeleton it may decrease bone remodeling when trabecular bone turnover is high, by decreasing the OPG/RANKL ratio (Thomas 2004). Interestingly, leptin is up-regulated by GCs both *in vivo* (Miell *et al.* 1996; Zakrzewska *et al.* 1999) and *in vitro* (Russell *et al.* 1998).

6. Impact of Nematode Infection on Bone

There are limited studies on the effect of GI nematode infections on bone. An earlier study reported that hookworm infection caused lesions on bone (O'Connor *et al.* 1971). More recent studies in sheep have shown that GI nematode infection leads to thinning of the trabecular structure and reduced bone formation (Thamsborg and Hauge 2001) and is associated with reduced

deposition of phosphorus and calcium in bone (Sykes *et al.* 1979; Coop and Field 1983). Other tissue-dwelling nematodes such as *Trichinella spiralis*, *Wuchereria bancrofti*, *Brugia malayi* and *Dracunculus medinensis* have been reported to affect the musculoskeletal system (Chhem *et al.* 2001). Mechanisms through which GI nematodes may affect bone composition include impairment in intestinal calcium absorption and/or up-regulation of mediators such as pro-inflammatory markers and hormones e.g. leptin (Tu *et al.* 2008a) that modify bone remodeling processes (Nguyen *et al.* 1991; Kotake *et al.* 1996; Ducy *et al.* 2000).

C. PROTEIN DEFICIENCY AND THE HOST

1. Protein Deficiency and the Host's Energy expenditure

Studies on the effect of PD on energy expenditure as measured by RMR are inconsistent. Some studies using the energy balance method reported increased RMR during PD in pigs (Gurr *et al.* 1980), whereas other studies of RMR during PD in rats reported decreased RMR (Hartsook *et al.* 1973) or no effect (Rothwell and Stock 1987). On the other hand, studies using open flow respirometry technique to measure RMR reported no effect of PD on RMR in rats (Specter *et al.* 1995) and mice (Tu *et al.* 2008b). Given that PD decreases lean mass (Chauveau *et al.* 1999), a strong predictor of RMR (Odiere *et al.* 2010), it is more plausible that PD should decrease RMR.

2. Protein Deficiency and Immunity to *H. bakeri* Infection

Effects of PD on host immune system are generally inhibitory (Cunningham-Rundles *et al.* 2005), and include: reduced homing of mucosal lymphocytes to the gut (McDermott *et al.* 1982), reduced cytokine production by both Th1 (Doherty *et al.* 1994) and Th2 cells (Ing *et al.* 2000), reduced numbers of memory cells (Najera *et al.* 2001), depressed blood eosinophilia and IgG1 (Slater and Keymer 1988; Boulay *et al.* 1998), and delayed worm expulsion (Tu *et al.* 2008a). Consequently, PD prolongs survival of *H. bakeri*

(Slater and Keymer 1988; Boulay *et al.* 1998; Ing *et al.* 2000), increases its fecundity (Keymer and Tarlton 1991) and may increase susceptibility to incoming larva. Furthermore, re-feeding rapidly restores protection against *H. bakeri* in protein-deficient mice (Tu *et al.* 2007). It is possible that elevated GCs during PD (Weber *et al.* 1990; Hill *et al.* 1995) are associated with the impaired immune function during concurrent nematode and PD, considering the inhibitory and harmful effects of GCs on the immune system (Stevenson *et al.* 1989; Gruber *et al.* 1994; Cunningham-Rundles *et al.* 2005).

Ironically, *in vitro* studies show that GCs induce a shift from Th1 to Th2 cytokine secretion by promoting a Th2 cytokine response by CD4+ T cells (Ramirez *et al.* 1996), and enhancing the capacity of macrophages (DeKruyff *et al.* 1998) and monocytes (Blotta *et al.* 1997) to induce Th2 cytokine synthesis in CD4+ T cells. Blocking IL-12-induced STAT4 phosphorylation, without altering IL-4-induced STAT6 phosphorylation (Franchimont *et al.* 2000) and down-regulation of the IL-12R $ß_1$- and $ß_2$-chain expression (Wu *et al.* 1998) mediate suppressive actions of GCs on Th1 cellular immune response, and may help explain the GC-induced shift towards the Th2 humoral immune response. This may suggest that GCs should enhance resistance against nematode infections. However, this is certainly not the case since functional immunity against nematode infections is impaired during PD (Slater and Keymer 1988; Boulay *et al.* 1998; Ing *et al.* 2000), implying that a decrease in protein nutrient plays a more significant role than the GC-induced physiological shift from Th1 to Th2 immunity. The GC-induced shift from Th1 to Th2 has not been demonstrated *in vivo*, indicating that there are differences between synthetic and endogenous glucocorticoids produced by the adrenal glands in terms of their biological functions. Such differences in biological function between synthetic and endogenous GCs emanate from differences in their regulatory mechanisms, for example, differences in binding to the corticosteroid binding globulin (CBG), tissue-specific metabolism, the affinity for the diverse GC receptors and in the interaction with transcription factors (Wilckens 1995).

GCs affect the immune system by regulating gene expression either through transactivation or through transrepression (Newton 2000). During transactivation, GCs bind to the cytosolic glucocorticoid receptor (GR) forming a receptor-ligand complex that translocates itself into the cell nucleus, where it binds to glucocorticoid response elements (GRE) in the promoter region of the target genes resulting in the regulation of gene expression (Newton 2000). During transrepression, the activated hormone receptor interacts with specific transcription factors such as the activator protein 1 (AP-1) and nuclear factor kappa B (NF-κB) and prevents the transcription of targeted genes (Newton 2000). GCs are able to prevent the transcription of cytokine genes, including interleukins (IL-1β, IL-4, IL-5, and IL-8), chemokines, granulocyte macrophage colony stimulating factor (GM-CSF), and TNF-α genes (Newton 2000). GCs suppress the cell-mediated immunity by inhibiting genes that code for the cytokines IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8 and IFN-γ (Leung and Bloom 2003). In addition, GCs not only reduce T cell proliferation, but also induce apoptosis in immature T cells in the thymus and peripheral T cells (Gruber *et al.* 1994; Ashwell *et al.* 1996). GCs also suppress the humoral immunity, by reducing B cell numbers (Stevenson *et al.* 1989) which reduces antibody synthesis.

Elevated GCs during PD may also impair immune responses against GI nematodes via indirect mechanisms. For instance, GCs up-regulate leptin concentration (Miell *et al.* 1996) that is known to impair functional immunity to *H. bakeri* (Tu *et al.* 2008a).

3. Protein Deficiency and Body Mass

Body weight and lean mass are decreased during PD (Chauveau *et al.* 1999) but not fat mass (Chauveau *et al.* 2003), indicating that decrease in lean mass accounts for the observed loss in body weight (Quevedo *et al.* 1994). Elevated plasma GC contributes, with other hormones, to the induction of a hypermetabolic state characterized by increased energy expenditure, accelerated

net protein breakdown and negative nitrogen balance, increased gluconeogenesis and hyperglycemia, insulin resistance, and hyperinsulinemia (Goldstein and Elwyn 1989) and protein wasting (Quan and Walser 1992). Pro-inflammatory cytokines such as IL-l and TNF are known to increase protein catabolism (Flores *et al.* 1989; Zamir *et al.* 1993) via GC-dependent (TNF) (Hall-Angeras *et al.* 1990) or GC-independent (IL-1α) (Zamir *et al.* 1993) mechanisms.

4. Protein Deficiency and Bone Composition

Approximately one third of bone mass is protein, making bone one of the most protein-dense tissues of the body (Heaney 1998). Synthesis of new bone matrix requires essential amino acids (Heaney 1998), therefore PD may affect bone directly. For instance, bone growth is stunted in protein-energy malnutrition, and outcomes of hip fracture are dramatically improved with protein supplements in the typical elderly victim of osteoporotic fractures (Bastow *et al.* 1983; Delmi *et al.* 1990). PD decreases total bone mass and lumbar or hip site bone mass (Chauveau *et al.* 2003), decreases bone mineral mass leading to decreased bone strength (Ammann *et al.* 2000; Bourrin *et al.* 2000b) and reduces plasma osteocalcin and insulin-like growth factor (IGF)-1 (Bourrin *et al.* 2000a). In addition, PD may affect bone composition through effects of other mediators such as GCs and leptin that are known to be up-regulated during PD (Hill *et al.* 1995; Tu *et al.* 2008a).

D. NEONATAL IMMUNE DEVELOPMENT

In species that give birth to non-precocious offspring such as mice, a large proportion of the immune development occurs during late gestation and the postnatal period (Merlot *et al.* 2008). Therefore, stressful insults such as PD and infection on the maternal system during late gestation and during the neonatal phase may affect immune development in the neonate.

1. T cell Development in the Mouse

Progenitor T cells begin to migrate to the thymus from the early sites of hematopoiesis at about day 11 of gestation in mice (Kindt *et al.* 2007). Most early thymic T cell progenitors are cortical thymocytes that do not express the TCR-CD3 complex or the co-receptors CD4 and CD8 (Galy *et al.* 1993). These progenitor cells have not yet rearranged their TCR genes and do not express the proteins such as recombination activating gene (RAG)-1 and RAG-2, that are required for rearrangement (Kindt *et al.* 2007). At approximately day 17 of foetal mouse development, the TCR αβ-heterodimer is expressed on the majority of thymocytes (Tentori *et al.* 1988).

During approximately three weeks of development in the thymus, the differentiating T cells pass through a series of stages involving rearrangement of the germ-line TCR genes and marked by characteristic changes in their cell surface phenotype (Kindt *et al.* 2007) (Fig. 2.2). Key surface markers during the double negative (DN) phase of early thymocytes include c-kit, the receptor for stem cell growth factor; CD44, an adhesion molecule; and CD25, the α chain of the IL-2 receptor (Kindt *et al.* 2007). The ultimate T cell repertoire is shaped by two selection processes: positive selection permits the survival of only those T cells whose TCRs are capable of recognizing self-MHC molecules, whereas negative selection eliminates T cells that react too strongly with self-MHC or with self MHC plus self peptides (Kindt *et al.* 2007).

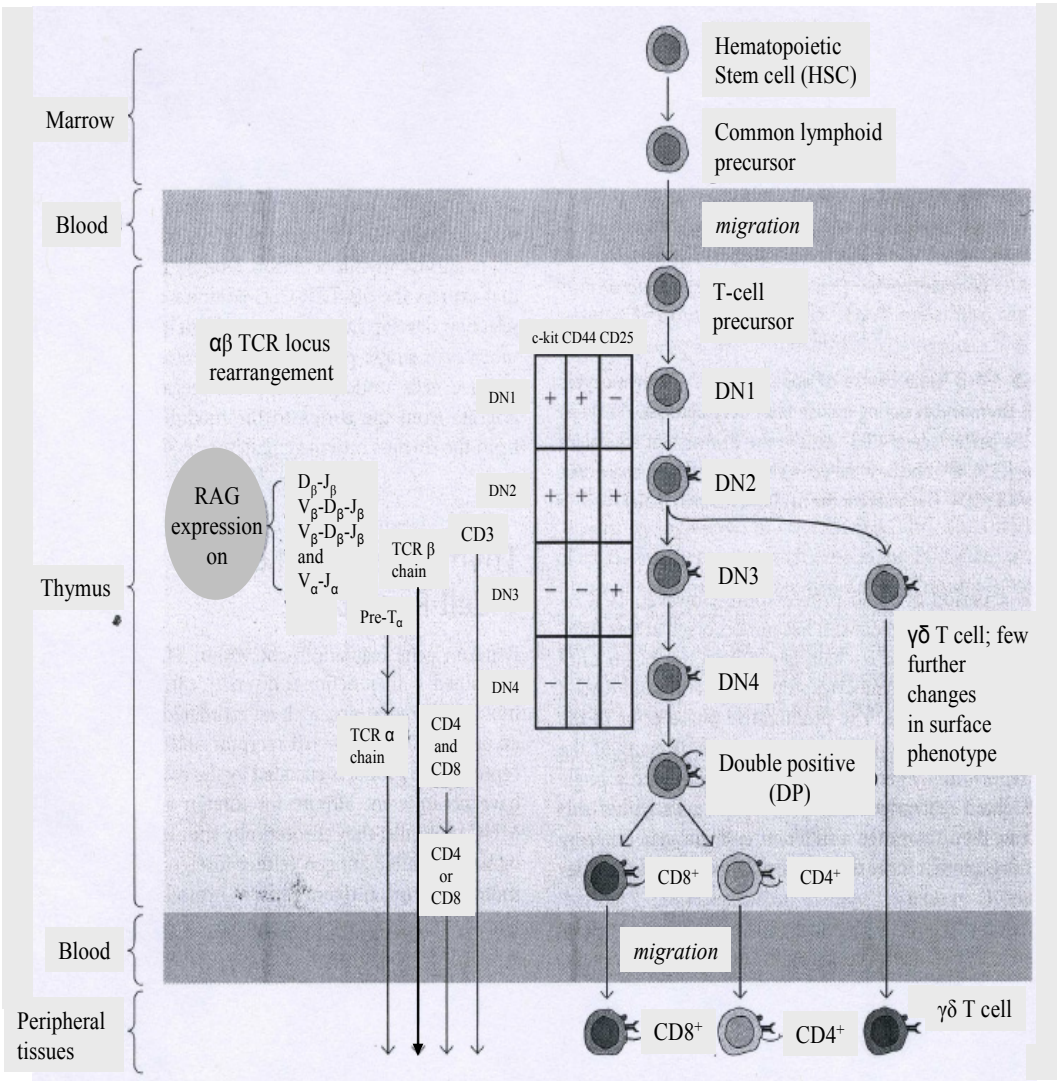

Fig. 2.2 Development of T cells in the mouse

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2. B cell Development in the Mouse

Generation of mature B cells first occurs during embryonic stages, involving the yolk sac, foetal liver and foetal bone marrow; but after birth, the bone marrow is the main site for generation of mature B cells (Kindt *et al.* 2007). B lymphopoiesis can be identified in mouse foetal liver by days 12-13 of gestation (Hayakawa *et al.* 1994). B cell development begins as lymphoid precursor cells differentiate into the earliest B-lineage cell - the progenitor B cell (pro-B cell), which then differentiate into precursor B cells (pre-B cells) (Kindt *et al.* 2007). Pre-B cells can be found in the foetal liver of mice by day 14, and B lymphocytes, which are for the most part restricted to IgM expression, can be found by days 16-17 (Holladay and Smialowicz 2000). B cell development involves three stages, first the antigen-independent maturation phase which leads to generation of mature, immunocompetetent B cells – here, immature B cells expressing membrane IgM are generated in the bone marrow, they then enter the bloodstream and develop into mature naïve B cells that express both mIgM and mIgD. Second, the antigen-dependent activation of mature B cells when they react with antigen in the peripheral lymphoid organs such as spleen and lymph nodes. Third, the antigen-dependent differentiation of activated B cells (those bearing high affinity mIgM) into plasma and memory B cells (Kindt *et al.* 2007), some of which will also undergo class switching, changing the isotypes of the antibodies they produce from μ to γ, α or ε. About 2×10^9 cells constitute the recirculating pool of B cells in mice (Kindt *et al.* 2007).

Developmental progression of B cells, like for T cells, involves changes in surface markers. Among the unique markers are CD45R (sometimes called B220 in mice), a protein tyrosine phosphatase found on leukocytes; Ig-α/Ig-β, which are found in association with the membrane forms of antibody; CD19, part of the B-cell co-receptor), CD43, leukosialin; CD24, heat stable antigen (HAS); c-kit, a receptor for a growth-promoting ligand on stromal cells, and

CD25, the α chain of the IL-2 receptor (Kindt *et al.* 2007). IL-7, secreted by stromal cells, drives the B cell maturation process (Parrish *et al.* 2009).

E. IMPACT OF NEMATODE INFECTION AND PROTEIN DEFICIENCY ON FOETAL AND NEONATAL DEVELOPMENT

Sub-optimal environments in the womb and during early neonatal life alter growth and predispose individuals to lifelong health problems in both humans (Strauss 1997) and animal models (Nathanielsz 2006). Such environments can result from increased energy demands associated with infection or decreased nutrients such as protein (Bautista *et al.* 2008).

Maternal condition during reproductive period and maternal effort (e.g. nursing, brooding) can affect offspring growth, immunocompetence and survival (Sorci and Clobert 1995; Merino *et al.* 1996; Saino *et al.* 1997; Hõrak *et al.* 1999; Kristan 2002b). For instance, maternal *H. bakeri* infection in pregnant mice results in offspring that express one of two resistance phenotypes upon infection with the same parasite at weaning (Kristan 2002b). Kristan (2002b) reported some protection to offspring (35 days old) because only offspring from parasitized mothers were able to clear their infection. On the other hand, the same study found that if offspring born to parasitized mothers did not clear their infection, they had a greater infection intensity compared with pups born to unparasitized mothers. These offspring phenotypes show that maternal nematode infection can induce physiological changes in growing mice sufficient to alter susceptibility/resistance to parasite infection.

The hypothesis of foetal programming of Barker (1998) proposes that adverse intrauterine environments can alter foetal growth, leading to malfunction of organ systems later in life. 'Programming' describes the process whereby a stimulus or insult at a critical period of development has lasting or lifelong effects (Lucas 1994), e.g. PD during foetal life and development up to

weaning accelerates reproductive ageing in female progeny (Guzman *et al.* 2006). Among the important mediators of foetal and neonatal programming are GCs from maternal stress (Fowden and Forhead 2004), which are able to breach the 11β-hydroxysteroid dehydrogenase-2 (11β-HSD2) placental barrier (Benediktsson *et al.* 1997) or can reach the neonate via breast milk (Catalani *et al.* 2000).

Maternal PD during the reproductive phase is also associated with deleterious effects in the offspring. For instance, feeding of low protein diets during pregnancy over-exposes the foetus to imprinting effects of maternal corticosteroids and results in increased systolic blood pressure (17 mmHg) in the resulting offspring in early adulthood (Langley-Evans *et al.* 1996). In rats, maternal protein restriction (10% protein) results in delayed sexual maturation (delayed vaginal opening and timing of the first oestrus) and premature ageing of reproductive function (reduced oestradiol, progesterone at day 21 PP, luteinizing hormone at day 70 PP) in female progeny (Guzman *et al.* 2006). In addition, maternal protein restriction (10% protein) during either pregnancy and/or lactation alters postnatal growth, appetitive behaviour, leptin physiology, triglycerides and cholesterol concentrations and modifies glucose metabolism and insulin resistance in a gender and time window of exposure-specific manner (Zambrano *et al.* 2006). The normal postnatal leptin rise in pup serum is delayed by prenatal protein undernutrition (Bautista *et al.* 2008), suggesting that foetal nutrition modifies timing of neonatal leptin surge and may contribute to the development of altered appetite and metabolic disorders in later life (Bautista *et al.* 2008).

In mice, impaired pup immune development during maternal PD and maternal infection may be associated with atrophy of the lymphoid tissues (Deitch *et al.* 1992), reduction of granulocyte-macrophage colony-forming units (Borelli *et al.* 2007) and interference with germinal centre development (Bell *et al.* 1976). In both human and animal models, immunodepression

associated with PEM is related to lymphoid involution, the thymus being the organ most affected (Woodward 1992).

1. Mediators of Maternal Stress on Foetal and Neonatal Development

Maternal PD and nematode infection during reproduction induce significant stress both to the host and offspring, the physiological response largely mediated by GCs (Weber *et al.* 1990; Munck and Náray-Fejes-Tóth 1995). Normal maternal levels of GCs, cytokines and other hormones are important as they act as nutritional and maturational signals for foetus (Fowden and Forhead 2004). However, elevated levels of GCs from maternal stress can reach the foetus and cause tissue programming in utero (Fowden and Forhead 2004), or can exert their effects on the neonate via breast milk (Catalani *et al.* 2000). Furthermore, the activity of placental 11β-HSD2, which protects the foetus from maternal GCs, is attenuated by 33% in activity by mild protein restriction (9% protein) (Langley-Evans *et al.* 1996).

a. Hormones in Breast Milk

One of the most important neonatal factors involved in developmental programming is the adequacy of nutrition during the lactation period (Bautista *et al.* 2008). Breast milk contains a wide variety of biologically active hormones - including GCs (Hamosh 2001), leptin (Houseknecht *et al.* 1997), insulin and IGF (Fowden and Forhead 2004) and cytokines (Kverka *et al.* 2007). These hormones pass from maternal circulation to neonates during lactation and induce effects in neonates such as alteration of stress response and protection against neonatal necrotizing enterocolitis (Catalani *et al.* 2000; Minekawa *et al.* 2004). Therefore, understanding milk composition is important because it reflects not only neonatal nutriture but also neonatal exposure to other biological agents.

There is evidence that GCs readily cross the gut in rodents. GC permeability of the gut of the rat is very high in early postnatal life until 17-18 days of age, after which it declines markedly (Henning and Sims 1979). Furthermore, the number of GC receptors in the rat pup's brain is highest in the first two postnatal weeks (Olpe and McEwen 1976; Pavlik and Buresova 1984). Oral administration of corticosterone to rat pups from the 2nd to 15th day post-partum (simulating elevated stress during suckling) delays their locomotor development (Pavlovska-Teglia *et al.* 1995). Administration of exogenous corticosterone to dams reaches the pups via milk as demonstrated by its higher levels in the milk and by the presence of labelled corticosterone in the plasma and brain of pups when the labelled hormone was added to the drinking water of the dams (Angelucci *et al.* 1985). Collectively, these studies suggest that the suckling pup is most susceptible to the harmful effects of high GC concentration in the mother's milk during the first 2-3 weeks post-partum.

b. Glucocorticoids, Neonatal Growth and Immune Development

Elevated maternal GCs and cytokines have harmful effects on foetal and neonatal development. GCs inhibit glucose transport in the placenta (Hahn *et al.* 1999) and skeletal muscle (Weinstein *et al.* 1998), and inhibit the GH/IGF-1 axis (MacRae *et al.* 2007) which is critical in embryonic and neonatal bone development (Wang *et al.* 2006). GC-mediated inhibition of the function of mature osteoblasts and suppression of IGF-1 synthesis (Canalis 2005), and inhibition of placental glucose transport (Hahn *et al.* 1999) consequently suppresses linear growth that is evident during protein restriction (Allen 1995).

Undernutrition in critical periods of gestation and neonatal maturation and during weaning impairs the development and differentiation of a normal immune system, effects that may be mediated through GCs (Cunningham-Rundles *et al.* 2005). Increased circulating levels of GCs cause thymic atrophy and affect hematopoiesis (Cunningham-Rundles *et al.* 2005). GCs are involved in programmed cell death (PCD) of immature thymocytes during intra-thymic T

cell selection (Ashwell *et al.* 1996), and apoptosis of thymic T cells (Gruber *et al.* 1994). Antenatal corticosteroid treatment transiently suppresses several foetal monocyte functions, decreases lymphocyte proliferation, induces thymic involution, increases the CD4/CD8 T-cell ratio in the blood and decreases T-cell numbers in the spleen (Murthy and Moya 1994; Bakker *et al.* 1995; Bakker *et al.* 1998; Kramer *et al.* 2004). In addition, GCs reduce splenic B cell numbers (Stevenson *et al.* 1989), selectively inhibit early cell cycle events in tonsillar B cells (Bowen and Fauci 1984), induce loss of precursor B cells (Garvy *et al.* 1993) and apoptosis in early B cells (Lill-Elghanian *et al.* 2002).

Pro-apoptotic effects of GCs on thymocytes involve the activation of the glucocorticoid receptor (GR) (Radu *et al.* 2006), a member of the nuclear receptor family of ligand-dependent transcription factors (Beato *et al.* 1995). Gene transactivation function of GR is required in GC-induced thymocyte apoptosis (Reichardt *et al.* 1998), a process that is ATP dependent (Stefanelli *et al.* 1997) involving activation of the "mitochondrial" apoptotic pathway in an apoptotic protease activating factor (Apaf-1)-dependent (Yoshida *et al.* 1998) and caspase-9-dependent (Hakem *et al.* 1998) manner.

c. Leptin in Neonatal Growth and Immune Development

Leptin stimulates release of growth hormone (Dixit *et al.* 2003) and is important not only during neonatal development, but also in reversing adverse pre-natal developmental metabolic programming events (Vickers *et al.* 2005). In rodents, serum leptin concentrations are higher in neonates than in adults (Devaskar *et al.* 1997; Mistry *et al.* 1999). In rats, leptin levels are higher during the first days of postnatal life than those observed later, with a surge in leptin around postnatal days 10-14 (Rayner *et al.* 1997), and are lower in 21-day old pups from undernourished than from well-nourished mice (Chisari *et al.* 2001), supporting a role for leptin in neonatal growth.

Leptin is also important in neonatal immune development. Leptin has been shown to prevent starvation-induced thymic atrophy in mice (Howard *et al.* 1999; Mito *et al.* 2004), through either increased thymopoiesis (Hick *et al.* 2006) or inhibition of apoptosis (Mansour *et al.* 2006). The inhibition of thymocyte apoptosis by leptin occurs through a mechanism that is independent of the activation of Janus kinase (JAK)-2 but depends on the engagement of the insulin receptor substrate (IRS)-1/phosphatidylinositol 3-kinase pathway (Mansour *et al.* 2006).

d. Pro-inflammatory Cytokines in Neonatal Growth and Immune Development

Increase in maternal stress and the associated elevation in GCs causes priming of maternal immune system towards a pro-inflammatory, Th1-cytokine response (IL-1, IL-6, TNF-α, IFN-γ) in the mammary tissue (Wockel *et al.* 2007). In addition, GCs such as corticosterone elevate pro-inflammatory cytokines such as TNF and IL-6 (Liao *et al.* 1995). These pro-inflammatory cytokines have deleterious effects on neonatal growth including inhibition of bone development or induction of bone resorption (Nguyen *et al.* 1991; Rodriguiz *et al.* 1993; Mann *et al.* 1994; Manolagas 1998). For instance, IL-1β directly inhibits growth plate chondrocyte dynamics leading to growth disorders (MacRae *et al.* 2006).

Maternal transfer of cytokines via milk changes over time (Kverka *et al.* 2007), suggesting that there are regulatory mechanisms in the mammary glands that concentrate or filter different cytokines. Maternal cytokines (TGF-β, IL-6 and IL-10) in milk could contribute to the development and differentiation of IgA-producing cells (Bottcher *et al.* 2000) and maturation of the naive intestinal immune system (Donnet-Hughes *et al.* 2000). It has been suggested that low cytokine production in neonates may in part be compensated by cytokines in breast milk (Ehlers and Smith 1991), and that higher concentrations of pro-

inflammatory Th1-cytokines in breast milk may lead to local and systemic immunological effects of the newborn (Knackstedt *et al.* 2005).

Can cytokines withstand the digestive effects and acidic environment in the gut? There is evidence that cytokines in breast milk can withstand digestion in the gut and get transferred to the neonate during lactation (Letterio *et al.* 1994; Goldman *et al.* 1996; Nguyen *et al.* 2007). Some of the cytokines are bound to other proteins, for example to their receptors (Rudloff *et al.* 1993; Garofalo *et al.* 1995) or are compartmentalized (Goldman *et al.* 1996), and are thereby protected from digestion (Bottcher *et al.* 2000). Furthermore, it has been shown in humans that milk also contains anti-proteases such as α-1-antichymotrypsin and α-1-antitrypsin that interfere with proteolysis (Lindberg *et al.* 1982). Some cytokines in milk are also resistant to proteolysis, for example IL-1β in human milk (Kobayashi *et al.* 1991). Cytokines may also readily cross the intestinal tract since the digestive capacity is not fully developed in the neonate (Goldman *et al.* 1996). Susceptibility of the neonate to the actions of cytokines in milk may be more pronounced in the mucosal immune system of the proximal parts of the alimentary tract/respiratory system where there is little proteolytic activity (Goldman *et al.* 1996). In addition, developmental delays in the production of gastric acid by the neonate may also favour the survival of these acid-sensitive cytokines in the gut (Goldman *et al.* 1996). For instance, epidermal growth factor (EGF) and transforming growth factor (TGF)-α in human milk may enhance the developmental delay in the production of gastric HCl (Goldenring *et al.* 1993; Guglietta and Lesch 1993).

In summary, the energetic, morphologic and physiologic responses induced during concurrent *H. bakeri* infection and PD in pregnant and lactating mice are not well studied. Most research on energetics of infection has employed non-living antigens. The present research employed not only a living parasite, but also the trickle (repeated) infection protocol that induces a much more vigorous immune response (Behnke and Robinson 1985; Scott 1991) that

is presumably more energetically costly, and that perhaps better reflects conditions in the field, where animals are exposed to frequent but generally low-intensity challenge (Berding *et al.* 1986). Analyses of body composition and morphology are important as they provide not only an insight into the impact of experimentation, but also the phenotypic plasticity associated with organs. Few studies have examined the impact of GI nematode infection at the maternal-foetal interface and on foetal development. Several studies have looked at the effect of PD during pregnancy (Langley-Evans *et al.* 1996; Guzman *et al.* 2006; Zambrano *et al.* 2006; Bautista *et al.* 2008), combined PD and GI nematode infection (Boulay *et al.* 1998; Ing *et al.* 2000; Tu *et al.* 2008a) and GI nematode infection during pregnancy (Scott 1990; Kristan 2002a; Kristan 2004). Both PD and GI nematode infection likely alter the energy repartitioning framework, thereby modulating physiological responses such as immune function. For instance, PD has been demonstrated to delay worm expulsion during GI nematode infection through suppressed IL-4, IgE, eosinophilia, mast cell proliferation, but with elevated IFN-γ (Ing *et al.* 2000).

The impact of combined PD and GI nematode infection during late pregnancy and lactation on maternal and offspring outcomes remains relatively unstudied. Both PD and infection induce significant stress to an animal (Hill *et al.* 1995; Munck and Náray-Fejes-Tóth 1995), and it has been suggested that elevated levels of GCs, pro-inflammatory cytokines and other hormones from maternal system can have deleterious effects on foetal and neonatal development (Fowden and Forhead 2004). Little work has been done on regulatory mechanisms that underlie impaired foetal and neonatal development as a result of maternal PD and GI nematode infection, despite the fact that the prenatal and antenatal period is thought to be one of high susceptibility to diet and infection-induced physiological changes. For instance, stunting is common in GI nematode infected children and is largely attributed to malnutrition (Payne *et al.* 2007; Jardim-Botelho *et al.* 2008). In addition, lactation is energetically demanding (Kristan 2002a) and a superimposition of GI nematode

infection is often accompanied with elevated worm burden (Normanton *et al.* 2007).

The studies in this thesis were performed to better understand the combined effect of *H. bakeri* infection and protein deficiency in pregnant and lactating mice through evaluation of the energetic, morphologic and physiologic responses. The first experiment involved concurrent *H. bakeri* infection and pregnancy, whereas the second experiment involved combined *H. bakeri* infection and PD during late pregnancy throughout lactation. 24% and 6% dietary protein were used as protein sufficient and protein deficient diets, respectively. In Chapter III, I investigated whether a stronger immune response during higher dose infection would increase RMR, and whether shifts in cytokine profiles in infected pregnant mice would promote nematode survival, induce maternal bone remodeling, and impair skeletal development in the foetus. I then explored whether PD and GI nematode infection during late pregnancy and lactation would modify RMR, maternal body composition and bone mineralization, and neonatal growth, and the hormone and cytokine regulatory mechanisms related to these responses (Chapter IV). In Chapter V, I further examined the effect of maternal PD and nematode infection on functional immune response and the development of immune system in the neonate.

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CHAPTER III

CONCURRENT NEMATODE INFECTION AND PREGNANCY INDUCE PHYSIOLOGICAL RESPONSES THAT IMPAIR LINEAR GROWTH IN THE MURINE FOETUS

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SUMMARY

This study examined concurrent stresses of nematode infection and pregnancy using pregnant and non-pregnant CD1 mice infected three times with 0, 50 or 100 *Heligmosomoides bakeri* larvae. Physiological, energetic, immunological and skeletal responses were measured in maternal and foetal compartments. Resting metabolic rate (RMR) was elevated by pregnancy, but not by the trickle infection. Energy demands during pregnancy were met through increased food intake and fat utilization whereas mice lowered their body temperature during infection. Both infection and pregnancy increased visceral o rgan mass and both altered regional bone area and mineralization. During pregnancy, lumbar mineralization was lower but femur area and mineralization were higher. On the other hand, infection lowered maternal femur bone area and this was associated with higher IFN-γ in maternal serum of heavily infected pregnant mice. Infection also reduced foetal crown-rump length which was associated with higher amniotic fluid IL-1β.

Key words: *Heligmosomoides bakeri*, nematode, costs of trickle infection, costs of pregnancy, resting metabolic rate, bone development, periparturient immunosuppression, IFN-γ, IL-1β, body temperature.

INTRODUCTION

During concurrent pregnancy and nematode infection, physiological demands for energy and protein required for foetal growth and maternal immunity may be met either by increased food intake (Bronson and Heideman, 1994), or by mobilizing stored triglycerides and adipose tissue (Spiegelman and Flier, 2001) or lean body mass (Wong *et al.* 2007). If either food intake or energy reserves are insufficient, animals re-allocate resources to maintenance functions (Coop and Kyriazakis, 1999), at the expense of functional immunity, current reproduction and lifetime reproductive success (Forbes, 1993). For example, pregnancy induces immunosuppression in livestock and rodents infected with nematodes (Houdijk *et al.* 2003; Ng'ang'a *et al.* 2006; Normanton *et al.* 2007), and nematode infections reduce reproductive outcomes including litter size and pup mass (Kristan, 2002). Energy demands can also be accommodated through phenotypic plasticity in size and function of internal organ systems (Piersma and Lindstrom, 1997), perhaps including bone.

Bone formation and resorption are regulated by hormones, soluble local growth factors as well as cytokines including interferon (IFN)-γ (Baron *et al.* 1983; Mann *et al.* 1994). Shifts in IFN-γ concentrations disrupt the tight cytokine balance involved in bone remodeling and lead to net bone loss as is observed during generalized inflammation (Lerner, 2006). IFN-γ is also an important regulatory cytokine in chronic nematode inflammation (Else *et al.* 1994; Tu *et al.* 2008). Thus, up-regulation of the maternal immune system during nematode infection may induce bone remodelling in the mother as an adaptive response (Lochmiller and Deerenberg, 2000) and reduce the maternal investment in foetal skeletal growth.

This study investigated energetic, immunological, physiological and skeletal responses to infection and pregnancy in the maternal and foetal compartments. We hypothesized that a stronger immune response during higher dose infection would increase resting metabolic rate (RMR). We further

hypothesized that the shift in cytokine profiles in infected pregnant mice would promote nematode survival, induce maternal bone remodeling, and impair skeletal development in the foetus.

MATERIALS AND METHODS

Experimental Design, Mice and Parasite

A 2 x 3 factorial design was employed that combined two conditions (pregnant and non-pregnant mice) with 3 doses of trickle infection (Sham: 3 x 0 L_3; Low: 3 x 50 ± 3 L_3; High: 3 x 100 ± 3 L_3) with the murine gastrointestinal nematode (*Heligmosomoides bakeri*, previously named *Heligmosomoides polygyrus*; see Cable *et al.* (2006). The trickle infection protocol and the selected doses are typical of real world infection dynamics (Monroy and Enriquez, 1992) and induce a vigorous and persistent immune response (Behnke and Robinson, 1985; Scott, 1991). A total of 70 female, 8 week-old outbred CD1 mice (Charles River Breeding Laboratories, Quebec, Canada) were housed individually in Nalgene cages (Fisher Scientific, Montreal, Canada) with stainless steel covers in a temperature controlled mouse room (22-25 ^{0}C), with a 14:10 h light:dark cycle. Normal pelleted rodent diet [8640 Teklad 22/5 mouse chow; 22.6% protein, 5.2% fat, 3.9% fiber, 3.1 kcal/g metabolizable energy] (Harlan Teklad, Madison, WI) and water were provided to the mice *ad libitum*.

As it was impossible to process all mice at the same time, the experiment was staggered into five sets initiated over a period of 15 weeks. Previously uninfected, non-pregnant mice and primiparous pregnant mice (day 4 of pregnancy, defined as experimental day 0) were received from Charles River (Quebec, Canada). Mice were randomized into 3 infection doses, and infected on experimental days 2, 7 and 12 (days 6, 11 and 16 of pregnancy) to allow three infections prior to parturition. L_3 were obtained by faecal culture of stock parasites (originally provided by Dr. M. V. K. Sukhdeo, Rutgers University, New Brunswick, New Jersey) maintained in outbred CD1 mice

(Charles River) in our laboratory for over 15 years. Larvae were counted in 10 sham doses for accuracy before infecting the mice. The same larval culture was used for all infections within each set of mice.

Food intake and body temperature and weight were recorded between 0900-1000 hours every second day beginning on experimental day 1 (day 5 of pregnancy). At selected times during the experiment, resting metabolic rate (RMR) and maternal body composition were measured. At necropsy (experimental day 15, day 19 of pregnancy), mice were anaesthetized using Ketamine/Xylazine (100/10 mg/kg body weight) intraperitoneally (IP), then exsanguinated by cardiac puncture. Maternal serum samples were collected and frozen at -20^0C until later analysis for cytokines and chemokines. Immediately after cardiac puncture, a cesarean section (C-section) was performed and intact uteri were removed. Amniotic fluid (AF), collected from each individual foetal sac using a 3 ml syringe fitted with a 22-gauge needle, was pooled for each dam, then immediately snap-frozen in liquid nitrogen. The pooled AF was stored at -80°C for later analysis of cytokines and chemokines. Foetuses were removed and then killed by exsanguination. Reproductive outcomes (number of foetuses, foetal mass and crown-rump length) were determined immediately thereafter. Foetal crown-rump length was measured from the top of the head to the base of the tail. Maternal organ masses for heart, spleen, liver, lungs, kidneys, pancreas, small intestine and small intestine length were also determined. All procedures were approved by the McGill Animal Care Committee according to guidelines of the Canadian Council on Animal Care (CCAC, 1993).

Energy expenditure

RMR of each mouse was measured on four separate days chosen to correspond with key phases of infection. Baseline RMR was obtained on the day prior to first infection (experimental day 1). RMR was also measured on experimental day 4 when the first dose of L_4 would be within the serosal

mucosa, on experimental day 9 when pre-adults would first be emerging into the intestinal lumen, and on experimental day 13 when mature adults would be present in the intestinal lumen. RMR was assessed at room temperature (24 ± 1°C) during the light phase (between 0800h and 1700h) when mice were in their inactive phase by an open-flow respirometry system (Multi-Channel Respirometry Gas Exchange System) connected to a paramagnetic oxygen analyser (Qubit Systems Research, Kingston, Ontario). Immediately prior to placing them in the respirometry chamber, non-fasted mice were weighed and their body temperature measured (to within 0.1^0C) using a thermometer (Microtherma 2T, model TW2-193; Braintree Scientific, Braintree, MA), by inserting a prelubricated rectal probe for mice (RET-3, shaft ¾" long, 0.028" dia. Tip dia. 0.065"; Physitemp Instruments, Clifton, NJ) two cm into the rectum. RMR was measured in groups of 4 mice at a time. Each mouse was placed in one of four cylindrical Perspex respirometry chambers with rubber stoppers (volume = 625 ml). The flow of tank air (dried with self indicating, 4-8 mesh soda lime; Spectrum Chemical Mfg, Gardena, CA) was maintained by a pump and measured by a gas flow meter upstream of the chamber. Flow rate was 650–700 ml min^{-1}. Gases leaving the mouse chambers were dried (magnesium perchlorate; Fisher Scientific, Montreal, Canada) and passed through the O_2 and CO_2 analysers (Qubit Systems Research) at approximately 150 mL min^{-1}. We did not absorb CO_2 before determination of O_2 content of the excurrent gases, in order to maximize accuracy in the derived estimate of energy expenditure (Koteja, 1996). O_2 consumption and CO_2 production of each of the 4 mice were recorded sequentially for 10 min with a flush out with reference air for a period of 2.5 min between mice. This cycle was repeated 3 times over a period of 2.5 h for each group of 4 mice.

Data were automatically recorded by the customized software (Qubit Systems Research) as average O_2 consumption and CO_2 production for every 10 second interval. For each mouse, values of O_2 consumption ($\dot{V}O_2$, ml / min) and CO_2 production ($\dot{V}CO_2$; ml / min) were calculated from the mean of the

lowest stable 30 sec interval from each of the three 10 min runs, and these were used to determine the respiratory quotient (RQ). The RMR (Watts = Joules/sec) for each animal was then calculated from the derived RQ using the Weir equation (Weir, 1949) and converted into KJ/day (Bartholomew, 1977). We have used the term RMR, although we note that room temperature (22-25 ^{0}C) was below the thermoneutral temperature for mice (30 ± 1^{0}C) (Hart, 1971).

Parasitological indicators

At necropsy the small intestine was excised, and the length and mass determined. The duodenum (10 cm immediately distal to the pyloric sphincter) was divided into three equal length portions (proximal, mid and distal) and the remaining small intestine was divided equally into the jejunum and ileum in order to assess worm distribution. The sex and number of adult *H. bakeri* in each section were determined by examining intestines that were slit open onto a glass plate and scanned using a dissection microscope. The number of 4th-stage larvae (L_4) of *H. bakeri* remaining in the serosal musculature was also recorded. Percentage of worms and percentage of L_4 recovered were computed based on the cumulative infection dose (150 or 300).

Body composition and bone measurements

We used the dual-energy X-ray absorptiometry (DXA) technique (Model # QDR 4500A, version 12.5; Hologic Inc., Waltham, MA) to measure maternal body composition (Nagy and Clair, 2000) and bone mineralization (Amman *et al.* 1992). On each day, two manufacturer-specific aluminum/lucite bone phantoms were scanned for quality control prior to scanning animals. On experimental day 14 (day 18 of pregnancy), adult mice were weighed, immobilized by anaesthesia (Ketamine/Xylazine, 100/10mg/kg body weight) IP, followed by whole body and high resolution regional scans. Mice were placed in the anterior-posterior position with limbs extended, spine straight, and tail placed around and not under the animal to permit regional analysis. Whole body composition was measured in triplicate without repositioning using the

small animal software. Scans were conducted for approximately 8 minutes per mouse by one investigator (M.R.O.). The scan analysis recorded grams of body mass, lean and fat mass, percent fat, bone mineral content (BMC), bone mineral density (BMD), and bone area (BA). The average intra-individual coefficients of variation for each mouse using the triplicate scans were 2.7% for whole body BMC, 2.2% for BMD, 1.4% for lean mass, and 9.2% for fat. The data were examined for the whole body and for specific skeletal regions (lumbar vertebrae 1-5, femur and tibia using high resolution scanning) and values for triplicate scans were averaged.

Cytokine and chemokine measurements

Multiplex suspension bead array immunoassay was performed on serum and AF using a Luminex 200^{TM} xMAP analyzer (Luminex Corp., Austin, TX) to quantify cytokines and chemokines known to play an important role in pregnancy (Orsi *et al.* 2006) and immunity to *H. bakeri* (Urban *et al.* 1998; Gause *et al.* 2003). The Mouse Cytokine 10-Plex kit (LINCO Research, St. Charles, Missouri, USA, # MCYTO-70K) was used to measure IL-1ß, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IFN-γ, tumor necrosis factor (TNF)-α, and monocyte chemotactic protein (MCP)-1, according to manufacturer specifications. Assays were performed in duplicate and assay sensitivity ranged between 0.3 and 10.3 pg/ml, depending on analyte (manufacturer's details). Serial dilutions prepared from a lyophilized standard were transferred to appropriate microtiter wells (Microtiter 96-well filter plate, Millipore, Bedford, MA, # MSBVN12), and 25µl of assay buffer and 25µl of serum matrix or serum or AF samples were added to the appropriate microtiter wells. After sonication (Branson Sonicator, Branson Co., Shelton, CT, model # 3200), 25µl of diluted antibody-coated bead complexes mixed with assay buffer were then added to each well. Samples were sealed, covered with aluminum foil and incubated with agitation on a plate shaker (Sanofi Diagnostics Pasteur, Chaska, MN, Model # 2202) overnight (16 h) at 4^0C. Fluid was gently removed by vacuum filtration. After washing twice with wash buffer (200µl/well), 25µl

cocktail biotinylated detection antibody was added and sealed, covered plates were incubated for 1 h at room temperature with vigorous agitation, but without vacuuming after incubation. Thereafter, 25µl of Streptavidine-Phycoerythrin was added and plates were incubated with agitation for 30 min at room temperature. All microtiter wells received a final wash (3 times with 200µl/well), removing wash buffer by vacuum filtration between each wash. Sheath fluid (100µl) was added to all wells, and the beads were resuspended by shaking sealed, covered plates on a plate shaker for 5 minutes. Samples were then analyzed on the Luminex using MasterPlex® CT software version 1.2 (MiraiBio, Inc., Alameda, CA). A minimum of 50 events (beads) was collected for each of the 10 cytokines and median fluorescence intensities (MFI) were obtained. Cytokine concentrations were automatically calculated based on standard curve data using MasterPlex® QT analysis software version 3.0 (MiraiBio, Inc., Alameda, CA), and were expressed as pg/ml±S.E.M.

Statistical analysis

All analyses were performed using Proc Mixed model in SAS statistical software (v. 9.1; SAS Institute Inc., Cary, NC, USA) and P values < 0.05 were considered statistically significant. Unless otherwise indicated, values are presented as least square means ± S.E.M. All parameters were tested for normality prior to analysis. Post-hoc Bonferroni adjustment (where appropriate) was used to account for multiple comparisons.

For variables measured once, we used either a 2 X 2 or 2 X 3 design to study the main effects of pregnancy (non-pregnant vs pregnant) and either infection (uninfected vs infected) or dose of infection (sham, low and high), depending on the variable. When there were no significant interactions among main effects, the data were pooled according to main effects, and re-analysed.

RMR, body temperature and food intake were analysed using repeated-measures analysis of variance (ANOVA) with main effects of pregnancy, dose of infection, and time (experimental days 4, 9 and 13 for RMR, 7, 9, 11, 13, 15 and 17 for body temperature, and 3, 5, 7, 9, 11 and 13 for food intake) modelled

as fixed effects and individual mice (dams) as random effects, after correcting for baseline values using a spatial power covariance structure for RMR or auto-regressive covariance structure for other variables. For foetal crown-rump lengths and foetal weight, infection status was modelled as a fixed effect and individual dams as random effects nested within infection status. Linear regression was used to determine whether RMR was predicted by whole body mass, lean mass, food intake or litter size.

The cytokine data sets were not normally distributed. Therefore serum cytokines were compared between pregnant and non-pregnant mice using a one-way non-parametric Kruskal-Wallis ANOVA. The subset of data used to examine infection effects within pregnancy states was normally distributed; hence the effect of infection dose on serum and AF cytokine concentration was examined using one-way ANOVA.

RESULTS

Parasite outcomes

All mice infected with *H. bakeri* developed mature infections, and no sham mice became infected. Significant main effects of pregnancy and dose were found for percent worm recovery and no significant interaction was detected. The percentage of worms recovered was higher in pregnant mice and in high dose infected mice compared to non-pregnant mice and low dose infected mice respectively (Fig. 1A). The percentage of L_4 was also higher in pregnant compared to non-pregnant mice (Fig. 1B), but no main effect of dose or interaction between dose and pregnancy state were detected. In all infected mice, the highest percentage of worms was in the proximal duodenum (Fig. 2A, B) and few were found in the jejunum or the ileum. Within the duodenum, worms in pregnant mice were more anteriorad, as shown by the lower percentage of worms in the distal duodenum, compared with non-pregnant mice (Fig. 2A).

Food intake

Parasite infection had no effect on food intake regardless of the dose, but the pattern of food intake over time differed between non-pregnant and pregnant mice (Fig. 3). Whereas average daily food intake gradually decreased in non-pregnant mice, food intake initially declined but then increased steadily during the final week of pregnancy (Fig. 3).

Visceral organ responses

Both pregnancy and infection increased the length of the small intestine and the mass of most visceral organs (Table 1), with no statistical interaction. Whereas the effect of infection was only evident at the high dose for liver and lung mass, small intestine mass increased in a dose-dependent manner. In the case of spleen (Fig. 4A) and heart mass (Fig. 4B), there was a significant interaction between pregnancy and infection dose. Spleen mass increased in a dose-dependent manner in non-pregnant mice, and was elevated in infected pregnant mice, but not in a dose-dependent manner (Fig. 4A). Post-hoc multiple comparisons of heart mass showed that all pregnancy groups had higher heart mass than all non-pregnant mice (Fig. 4B).

Body composition and bone measurements

Pregnancy had a stronger impact on many dimensions of maternal body composition than infection (Table 2). On experimental day 14, pregnant mice had higher whole body mass and lean mass, whole body bone area (BA), bone mineral content (BMC), but lower fat mass, percent fat and bone mineral density (BMD), compared with non-pregnant mice. Infection dose had no effect on whole body fat mass or percent fat, BMC or BMD (Table 2) and no interaction was detected. However, lean mass was borderline greater ($F_{2,62.5}$ = 3.01, P = 0.0566) in high dose than low dose infections.

As a further assessment of bone composition, regional analyses were performed for BA, BMC and BMD using lumbar, femur and tibia regions

(Table 2). In no case was a significant interaction detected. Pregnant mice had higher femur BA, BMC and BMC but lower lumbar BMC and BMD, compared with non-pregnant mice. In contrast, femur BA was reduced in high dose compared with uninfected mice, but neither femur BMC or BMD were affected by infection dose. Post-hoc multiple comparisons demonstrated that pregnant mice had greater lumbar BA in all infection groups. Neither infection dose nor pregnancy had any effect on tibia BA, BMC and BMD (data not shown).

Reproductive outcomes

High dose infection decreased crown-rump length compared to sham infection ($F_{2,17}$ = 10.03, P = 0.001). Furthermore, crown-rump length declined significantly with increasing adult worm burden (Fig. 5). However, infection dose had no impact on litter size (average litter size 12 ± 1) or foetal weight (average weight of foetus 1.21 ± 0.07g).

Energy expenditure and body temperature

Pregnancy increased maternal RMR across all infection doses on experimental days 9 and 13 but infection had no detectable impact on RMR (Fig. 6). Linear regression showed a positive relationship between RMR and average food intake on experimental days 4, 9 and 13 (Table 3). RMR also increased linearly with whole body mass, lean mass and litter size measured at the end of the experiment (Table 3).

Body temperature was higher in pregnant (37.60 ± 0.08 ^{0}C) compared with non-pregnant (37.32 ± 0.07 ^{0}C) mice ($F_{1,62.9}$ = 9.75, P = 0.0027). Body temperature was lower in mice with high dose infection (37.36 ± 0.08 ^{0}C) compared with uninfected mice (37.63 ± 0.09 ^{0}C) ($F_{2,62.8}$ = 3.71, P = 0.0301). There was no interaction between pregnancy state and infection. However, there was a significant interaction between pregnancy state (pregnant vs non-pregnant) and time ($F_{2,258}$ = 2.94, P = 0.0135) whereby body temperature declined during pregnancy but remained constant in non-pregnant mice.

Cytokines in serum and amniotic fluid

The effect of pregnancy on serum cytokines was evident through lower serum concentrations of IL-4, IL-5, IL-13 and MCP-1 (Table 4). Infection also influenced serum cytokines. Among the non-pregnant mice, serum IL-4 and IL-5 increased with dose (Fig. 7A) and serum IL-13 was higher in infected compared with uninfected mice (Fig. 7A). Among pregnant mice, IFN-γ was elevated in high dose compared with uninfected mice (Fig. 7B).

In amniotic fluid, infected dams had elevated IL-1β ($F_{2,14} = 3.64$, $P = 0.0535$), but decreased IL-4 ($F_{2,15} = 36.59$, $P < 0.0001$), relative to uninfected dams. Both IL-5 ($F_{2,15} = 17.66$, $P = 0.0001$) and IFN-γ ($F_{2,14} = 3.41$, $P = 0.05$) were reduced in amniotic fluid in a dose-dependent manner (Fig. 7C).

DISCUSSION

This work highlights the largely independent ways in which the mouse responds to competing demands of infection and pregnancy. Whereas the energetic costs of infection were met through reduced body temperature, the costs of pregnancy and associated increase in RMR were met through increased food intake and a reduction of fat reserves. Skeletal responses to infection and pregnancy also differed. Pregnancy increased the area of weight-supporting bones such as the femur at the expense of the lumbar region, whereas infection reduced maternal femur area and stunted foetal growth. These important shifts in bone both in the maternal and foetal compartments are consistent with known mechanisms of bone remodeling involving elevated IFN-γ (Nanes *et al.* 1990; Mann *et al.* 1994) and IL-1β (Nguyen *et al.* 1991) concentrations in maternal serum and amniotic fluid, respectively.

During pregnancy, the increased visceral organ lean mass, foetal growth, RMR and body temperature were accompanied by increased food intake and reduced fat mass. Given that increased food intake provided energy, but also led to an increase in the size of the digestive tract, as reported

previously (Hammond and Diamond, 1997; Piersma and Lindstrom, 1997), its net energy benefit may have been minimal (Piersma and Drent, 2003). Instead, the RQ of 0.73 at experimental day 13 (data not shown) indicates that energy was generated through fat mobilization (McClave *et al.* 2003).

Infection, in contrast, increased mass of spleen, liver and small intestine, and also induced cytokine production, but was not accompanied by increased RMR or food intake, or reduced fat reserves. Only rarely has increased food intake been reported during *H. bakeri* infection (Wong *et al.* 2007); in fact, Tu *et al.* (2007) observed a transient decrease in food intake immediately following a challenge infection. Instead, our *H. bakeri*-infected mice reduced their body temperature by 0.27^0C. A 0.5^0C decrease in body temperature decreases RMR by approximately 4% if conductance and evaporative heat loss remain the same (Kristan and Hammond, 2006). This has been proposed as a mechanism to decrease energy expenditure during *H. bakeri* (Wong *et al.* 2007). To date, literature has been inconsistent with regard to the effect of *H. bakeri* on RMR (Kristan and Hammond, 2000; Kristan and Hammond, 2001; Kristan, 2002; Kristan and Hammond, 2004; Kristan and Hammond, 2006). We suggest that the decreased body temperature without increased food intake served to reduce the energy costs of infection-induced immune responses and the increase in visceral tissue mass, resulting in no increase in RMR.

When infection was superimposed on pregnancy, RMR and food intake remained elevated and fat mass remained low. In addition, the Th2 immune response that is necessary for intestinal epithelial cell turnover (Cliffe *et al.* 2005) leading to worm expulsion (Urban *et al.* 1993) was suppressed and worm recovery was elevated compared with infected, non-pregnant mice. These results are consistent with other reports of Th2 suppression during reproduction of GI nematode-infected birds and mammals as evidenced by depressed antibody production (Sulila and Mattsson, 1990; Deerenberg *et al.* 1997), down-regulated cell-mediated immunity (Medina *et al.* 1993; Gustafsson *et al.*

1994; Saino *et al.* 1997), and elevated fecal egg output (Houdijk *et al.* 2003; Ng'ang'a *et al.* 2006; Normanton *et al.* 2007). It is likely that suppression of the Th2 response may have resulted from up-regulation of IFN-γ evident at the high dose infection in pregnant mice. Furthermore, pregnancy lowered MCP-1 concentrations, and this cytokine has been associated with infiltration of macrophages around the larval parasite (Sarafi *et al.* 1997) and expulsion of adult *H. bakeri* (Tu *et al.* 2008). Thus, both our elevated IFN-γ and reduced MCP-1 would have contributed to the higher worm recovery in pregnant mice.

Our results on maternal bone remodeling during pregnancy are consistent with previously reported increases in whole body and femur BA and BMC (Miller *et al.* 1986; Zeni *et al.* 1999) attributed to increased mechanical loading (De Boer and Wood, 1989; Glauber *et al.* 1995). Furthermore, foetal demands for calcium are first met from those maternal bone regions with a high metabolic turnover such as trabecular-rich areas (Zeni *et al.* 2003), including the lumbar region. This would explain the lower lumbar BMC and BMD in our pregnant mice. In contrast to pregnancy, high dose infection reduced maternal femur BA. Studies in sheep have shown that GI nematode infection leads to thinning of the trabecular structure and reduced bone formation (Thamsborg and Hauge, 2001) and is associated with reduced deposition of phosphorus and calcium in bone (Sykes *et al.* 1979; Coop and Field, 1983). The reduced maternal femur BA in our infected mice may be an indirect consequence of elevated maternal serum IFN-γ concentrations, either through its promotion of bone resorption as occurs in long bones such as the femur under pathological conditions (Nanes *et al.* 1990; Mann *et al.* 1994), or through its inhibition of bone formation (Mann *et al.* 1994) in mice that have not yet reached their peak femur bone mass (Ward *et al.* 2007).

In the foetal compartment, linear growth of the foetus was reduced in response to *H. bakeri* infection of the dams, an effect that increased in severity with increasing numbers of intestinal worms. To our knowledge this has not

been observed with other GI nematode infections, although growth stunting in nematode-infected children is common (Casapía *et al.* 2007; Payne *et al.* 2007; Jardim-Botelho *et al.* 2008). We also observed a high concentration of IL-1β in amniotic fluid, over and above that required as a preparatory step in the initiation of labour (Romero *et al.* 1989). IL-1β has been reported to inhibit bone formation *in vivo* (Nguyen *et al.* 1991) which could explain our observed decrease in foetal crown-rump length in response to infection.

In summary, GI nematode infection induced region-specific shifts in bone area and mineralization in the maternal compartment and reduced foetal crown-rump length. Evidence is provided that pro-inflammatory cytokines in maternal serum and amniotic fluid may be involved. We propose that the *H. bakeri* – pregnancy model may provide a useful animal system for studying bone remodeling during GI nematode infection.

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Table 1. Main effects of pregnancy (pooled across dose) and *Heligmosomoides bakeri* infection dose (pooled across pregnancy state) on organ masses (g) and small intestine length (cm)[1].

Organ	Pregnancy state		Infection dose		
	Non-pregnant (n = 46)	Pregnant (n = 24)	Sham (n = 20)	Low (n = 24)	High (n = 26)
Liver[2]	1.19 ± 0.03	2.02 ± 0.04*	1.53 ± 0.04[a]	1.58 ± 0.04[a]	1.71 ± 0.04[b]
Lungs[3]	0.179 ± 0.003	0.212 ± 0.004*	0.188 ± 0.004[a]	0.193 ± 0.004[ab]	0.206 ± 0.004[b]
Kidneys[4]	0.351 ± 0.005	0.431 ± 0.007*	0.381 ± 0.007	0.389 ± 0.007	0.402 ± 0.007
Pancreas[5]	0.101 ± 0.004	0.174 ± 0.006*	0.126 ± 0.006	0.139 ± 0.006	0.146 ± 0.006
Small Intestine					
Mass[6]	1.75 ± 0.04	2.37 ± 0.06*	1.59 ± 0.07[a]	2.16 ± 0.06[b]	2.44 ± 0.06[c]
Length[7]	46.6 ± 0.6	52.4 ± 0.8*	46.1 ± 0.9[a]	50.2 ± 0.8[b]	52.1 ± 0.8[b]

[1] A significant effect of pregnancy is represented by * whereas different superscripts represent differences among infection doses.

[2] Pregnancy state: $F_{1,62.6} = 420.50$, $P < 0.0001$; Infection dose: $F_{2,62.7} = 7.81$, $P = 0.0009$; Pregnancy state x dose: NS

[3] Pregnancy state: $F_{1,63.3} = 47.30$, $P < 0.0001$; Infection dose: $F_{2,63.4} = 6.03$, $P = 0.004$; Pregnancy x dose: NS

[4] Pregnancy state: $F_{1,62.9} = 104.29$, $P < 0.0001$; Infection dose: $F_{2,63} = 2.80$, $P = 0.0683$; Pregnancy x dose: NS

[5] Pregnancy state: $F_{1,66} = 106.06$, $P < 0.0001$; Infection dose: $F_{2,66} = 3.08$, $P = 0.0529$; Pregnancy x dose: NS

[6] Pregnancy state: $F_{1,66} = 68.72$, $P < 0.0001$; Infection dose: $F_{2,66} = 45.72$, $P < 0.0001$; Pregnancy x dose: NS

[7] Pregnancy state: $F_{1,63.3} = 34.88$, $P < 0.0001$; Infection dose: $F_{2,63.4} = 13.37$, $P < 0.0001$; Pregnancy x dose: NS

Table 2. Main effects of pregnancy (pooled across dose) and infection dose (pooled across pregnancy state) on maternal whole body mass measured on experimental day 14 (day 18 of pregnancy), and on lean and fat mass, and femur and lumbar bone area (BA), bone mineral content (BMC) and bone mineral density (BMD), as measured using a QDR 4500 Hologic densitometer[1].

	Pregnancy state		Infection dose		
	Non-pregnant (n = 46)	Pregnant (n = 24)	Sham (n = 20)	Low (n = 24)	High (n = 26)
Whole body					
Mass (g)[2]	26.8 ± 0.8	51.5 ± 0.9*	38.9 ± 1.0	38.3 ± 0.9	40.2 ± 0.9
Lean mass (g)[3]	20.6 ± 0.7	47.0 ± 0.8*	34.3 ± 0.9[ab]	32.3 ± 0.8[a]	34.9 ± 0.8[b]
Fat mass (g)[4]	5.4 ± 0.3	3.8 ± 0.4*	4.1 ± 0.4	4.9 ± 0.4	4.7 ± 0.4
Percent fat[5]	19.7 ± 0.9	7.4 ± 1.2*	12.3 ± 1.3	14.8 ± 1.2	13.5 ± 1.2
BA (cm^2)[6]	8.7 ± 0.1	10.7 ± 0.2*	9.6 ± 0.2	9.8 ± 0.2	9.7 ± 0.2
BMC (g)[7]	0.73 ± 0.01	0.81 ± 0.01*	0.76 ± 0.02	0.77 ± 0.02	0.77 ± 0.02
BMD (g/cm^2)[8]	0.084 ± 0.001	0.076 ± 0.001*	0.080 ± 0.001	0.079 ± 0.001	0.080 ± 0.001

Lumbar					
BA (cm^2)[9]	0.56 ± 0.01	0.72 ± 0.01*	0.63 ± 0.01	0.63 ± 0.01	0.65 ± 0.01
BMC (g)[10]	0.079 ± 0.003	0.067 ± 0.003*	0.073 ± 0.003	0.072 ± 0.003	0.074 ± 0.003
BMD (g/cm^2)[11]	0.139 ± 0.005	0.095 ± 0.005*	0.121 ± 0.005	0.115 ± 0.005	0.115 ± 0.005
Femur					
BA (cm^2)[12]	0.333 ± 0.005	0.382 ± 0.007*	0.367 ± 0.008[a]	0.365 ± 0.007[ab]	0.341 ± 0.007[b]
BMC (g)[13]	0.031 ± 0.001	0.045 ± 0.001*	0.039 ± 0.001	0.038 ± 0.001	0.037 ± 0.001
BMD (g/cm^2)[14]	0.095 ± 0.003	0.119 ± 0.003*	0.106 ± 0.003	0.104 ± 0.003	0.110 ± 0.003

[1] A significant effect of pregnancy is represented by * whereas different lowercase letters represent significant differences among infection doses.

[2] Pregnancy state: $F_{1,62.5} = 803.32$, $P < 0.0001$; Infection dose: NS; Pregnancy x dose: NS

[3] Pregnancy state: $F_{1,62.5} = 1089.69$, $P < 0.0001$; Infection dose: $F_{2,62.5} = 3.01$, $P = 0.0566$; Pregnancy x dose: NS

[4] Pregnancy state: $F_{1,63.2} = 10.58$, $P = 0.0018$; Infection dose: NS; Pregnancy x dose: NS

[5] Pregnancy state: $F_{1,63.2} = 70.17$, $P < 0.0001$; Infection dose: NS; Pregnancy x dose: NS

[6] Pregnancy state: $F_{1,62.8} = 125.52$, $P < 0.0001$; Infection dose: NS; Pregnancy x dose: NS

[7] Pregnancy state: $F_{1,62.7} = 22.06$, $P < 0.0001$; Infection dose: NS; Pregnancy x dose: NS

[8] Pregnancy state: $F_{1,62.3} = 83.85$, $P < 0.0001$; Infection dose: NS; Pregnancy x dose: NS

[9] Pregnancy state: $F_{1,66} = 95.22$, $P < 0.0001$; Infection dose: NS; Pregnancy x dose: NS

[10] Pregnancy state: $F_{1,62.2} = 24.18$, $P < 0.0001$; Infection dose: NS; Pregnancy x dose: NS

[11] Pregnancy state: $F_{1,62.1} = 236.72$, $P < 0.0001$; Infection dose: NS; Pregnancy x dose: NS

[12] Pregnancy state: $F_{1,66} = 30.20$, $P < 0.0001$; Infection dose: $F_{2,66} = 4.13$, $P = 0.0204$; Pregnancy x dose: NS

[13] Pregnancy state: $F_{1,62.6} = 127.87$, $P < 0.0001$; Infection dose: NS; Pregnancy x dose: NS

[14] Pregnancy state: $F_{1,62.6} = 57.13$, $P < 0.0001$; Infection dose: NS; Pregnancy x dose: NS

Table 3. Relationship between maternal resting metabolic rate (RMR) and average daily food intake, whole body mass, lean mass and litter size.

Independent Variable	Experimental Day	Slope ± SE	Intercept ± SE	r^2	Prob > *F*
Food intake (g)	3	1.68 ± 0.32	20.98 ± 1.65	0.28	$P < 0.0001$
	9	2.86 ± 0.72	18.29 ± 3.12	0.19	$P = 0.0002$
	13	4.77 ± 0.53	13.60 ± 2.39	0.54	$P < 0.0001$
Whole body mass (g)	14	0.82 ± 0.03	4.35 ± 1.27	0.90	$P < 0.0001$
Lean mass (g)	14	0.79 ± 0.03	9.99 ± 0.97	0.91	$P < 0.0001$
Litter size	15	1.28 ± 0.46	31.89 ± 5.50	0.27	$P = 0.01$

Table 4. Main effect of pregnancy (pooled across dose) on serum cytokine profiles (concentration in pg/ml) at day 19 of pregnancy[1].

Cytokine / chemokine	Serum		*P > H value*
	Non-pregnant (n = 46)	Pregnant (n = 24)	
IL-1β	13.4 (2.0)	3.9 (2.5)	NS
IL-4	4.0 (3.7)	3.0 (2.8)	0.0085
IL-5	77 (58)	27 (15)	0.0139
IL-6	49 (25)	88 (39)	NS
IL-10	30.9 (27.9)	45.7 (31.5)	NS
IL-12(p70)	134 (85)	219 (57)	NS
IL-13	34.7 (17.5)	17.2 (8.9)	0.0761
IFN-γ	16.7 (11.0)	10.8 (7.6)	NS
MCP-1	89 (64)	42 (40)	0.0222
TNF-α	6.5 (6.3)	5.5 (5.4)	NS

[1] Values are arithmetic means obtained from Kruskal-Wallis analysis, and values in parentheses are medians.

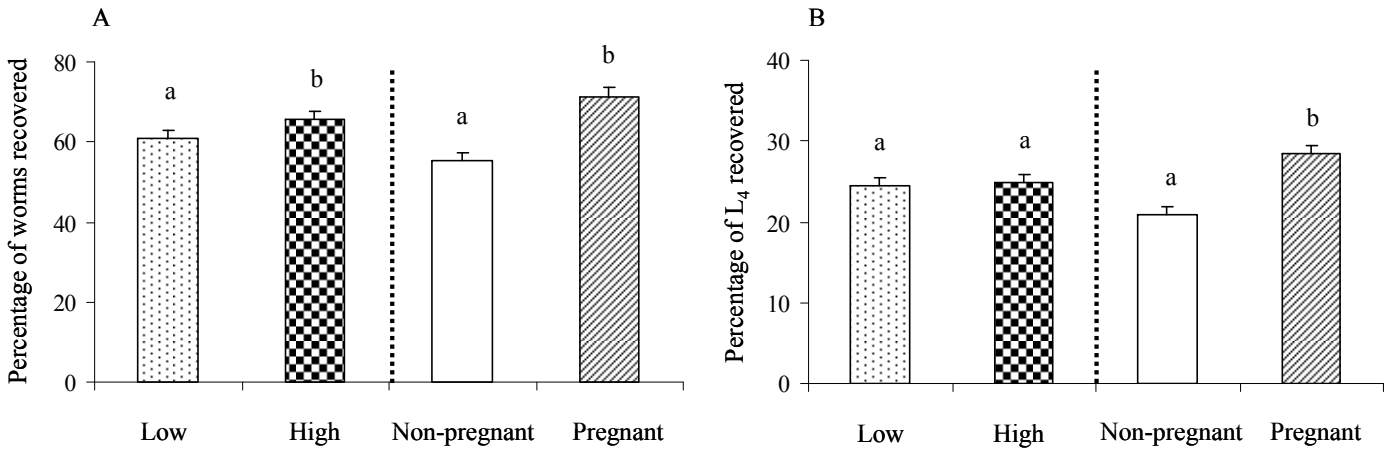

Fig. 1. Effect of infection dose (low, high) and pregnancy on recovery of *Heligmosomoides bakeri.* (A) combined adult worms and L_4 recovery as a percentage of dose (main effect of dose: $F_{1,43.7} = 7.34$, $P = 0.0096$; main effect of pregnancy state: $F_{1,43.5} = 77.7$, $P < 0.0001$)；interaction of pregnancy state * dose: $F_{1,43.1} = 0.56$, $P = 0.4597$); (B) L_4 recovery as a percentage of dose (main effect of dose: $F_{1,43.6} = 0.34$, $P = 0.5646$; main effect of pregnancy state: $F_{1,43.4} = 98.48$, $P < 0.0001$)； interaction of pregnancy state * dose: $F_{1,42.8} = 4.07$, $P = 0.0598$). Different lowercase letters represent significant differences between pregnant and non-pregnant mice, or among infection doses ($P < 0.05$).

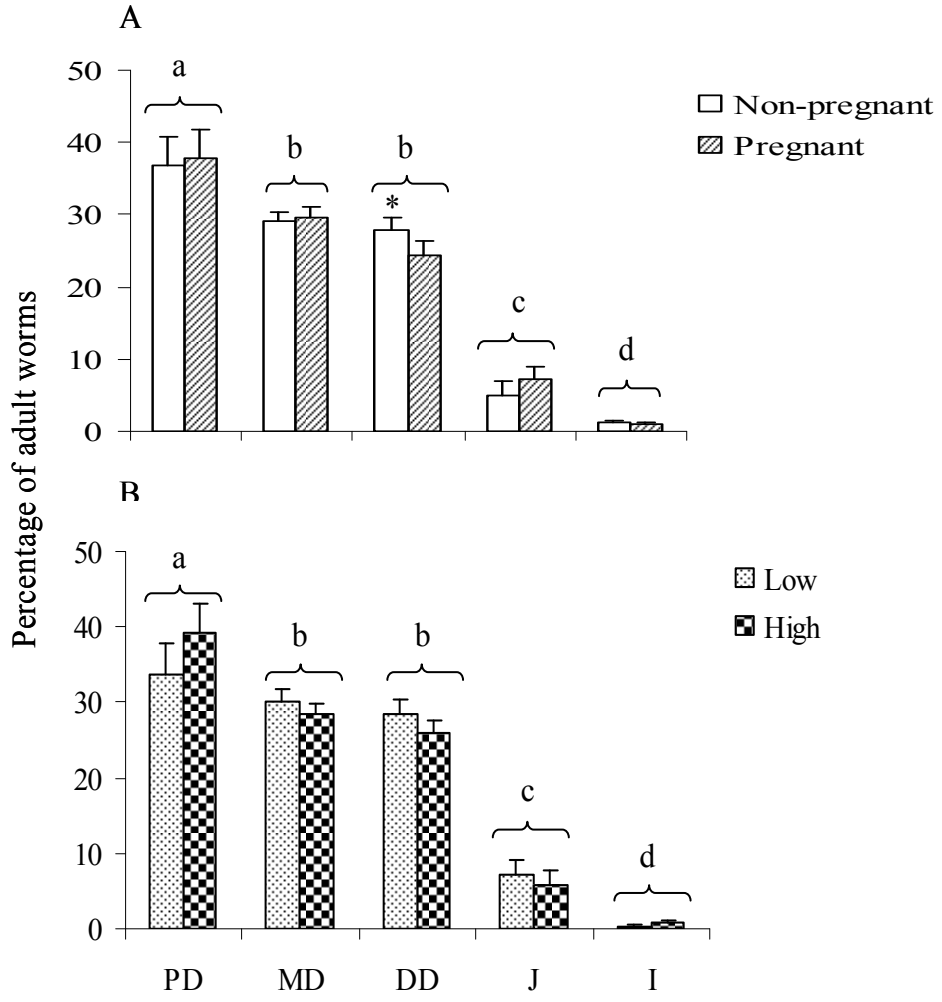

Fig. 2. Percentage distribution of *Heligmosomoides bakeri* along the small intestine in non-pregnant vs pregnant mice (A) and between low and high dose infections (B). PD, MD, DD, J and I refer to proximal duodenum, mid duodenum, distal duodenum, jejunum and ileum respectively. Different lowercase letters represent significant differences among regions (pooled data for pregnant and non-pregnant mice); * indicates higher recovery in non-pregnant compared with pregnant mice within a region ($P < 0.05$).

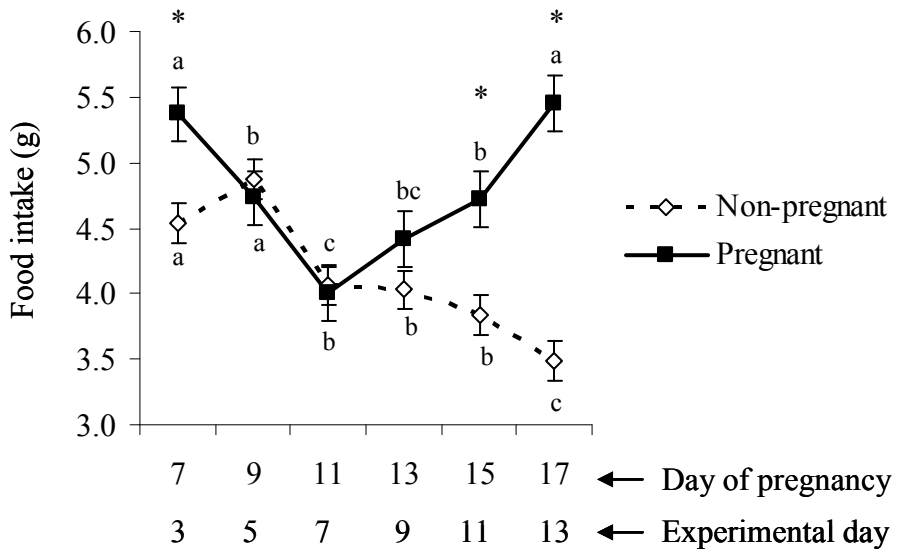

Fig. 3. Effect pregnancy on average food intake per day over time (data pooled across infection groups). Main effect of pregnancy state: $F_{1,66} = 14.38$, $P = 0.0003$; main effect of dose: $F_{2,64.2} = 0.87$, $P = 0.4237$; main effect of time: $F_{5,265} = 9.19$, $P < 0.0001$; interaction of pregnancy state * time: $F_{5,265} = 10.12$, $P < 0.0001$. Differences between pregnancy states at specific time-points are represented by *; different lowercase letters represent significant differences across time within each group ($P < 0.05$).

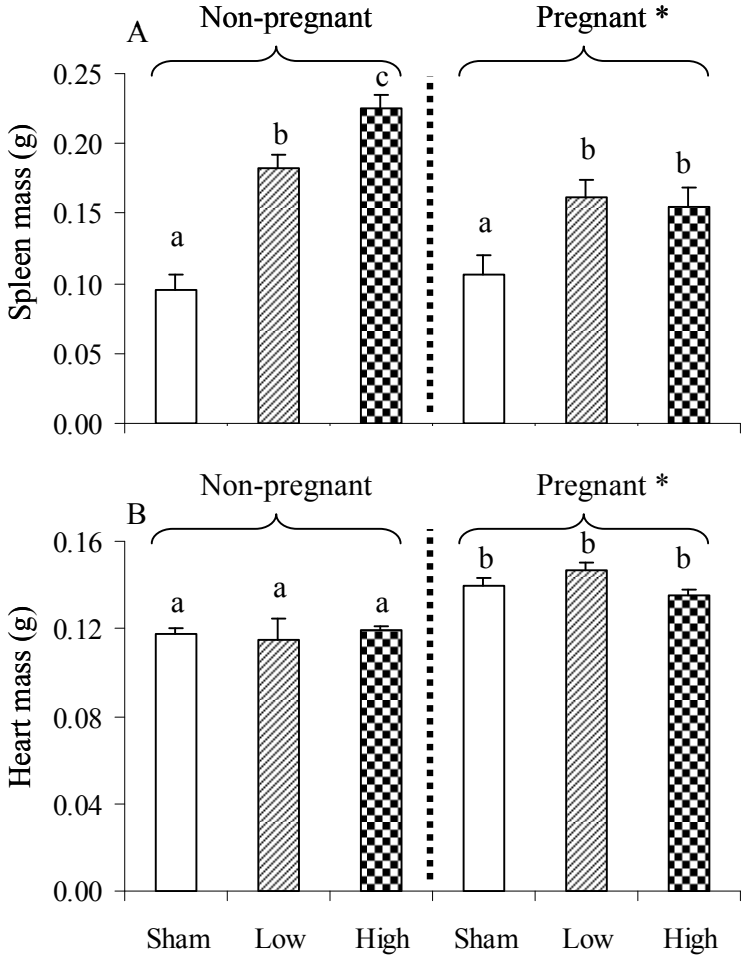

Fig. 4. Interaction effects of pregnancy and *Heligmosomoides bakeri* dose on spleen mass (A) (main effect of pregnancy state: $F_{1,60.6} = 8.09$, $P = 0.006$; main effect of dose: $F_{2,60.8} = 31.7$, $P < 0.0001$; pregnancy state * dose: $F_{2,61.8} = 5.99$, $P = 0.0042$); and heart mass (B) (main effect of pregnancy state: $F_{1,64} = 95.43$, $P < 0.0001$; main effect of dose: $F_{2,64} = 1.07$, $P = 0.3495$; pregnancy state * dose: $F_{2,64} = 3.84$, $P = 0.0265$). Different lowercase letters represent significant differences across all groups, based on multiple comparisons; * represents a significant difference between pregnant and non-pregnant mice.

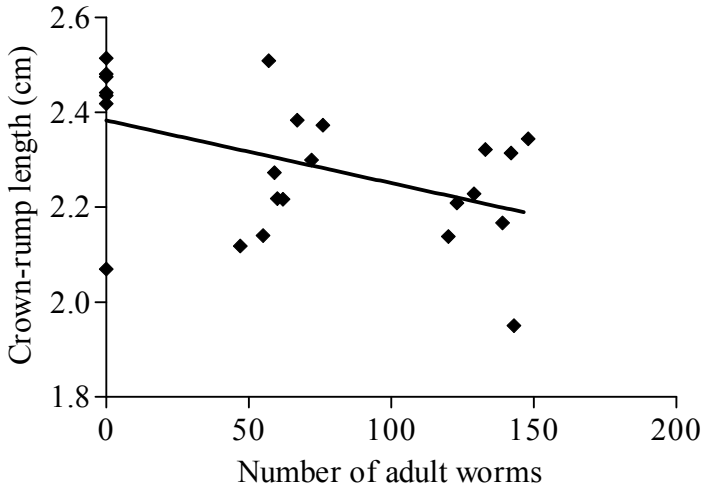

Fig. 5. Relationship between number of adult *Heligmosomoides bakeri* and foetal crown-rump length (mean of all pups in litter). $y = 2.3833 - 0.0013x$, $r^2 = 0.23$, $P = 0.0172$.

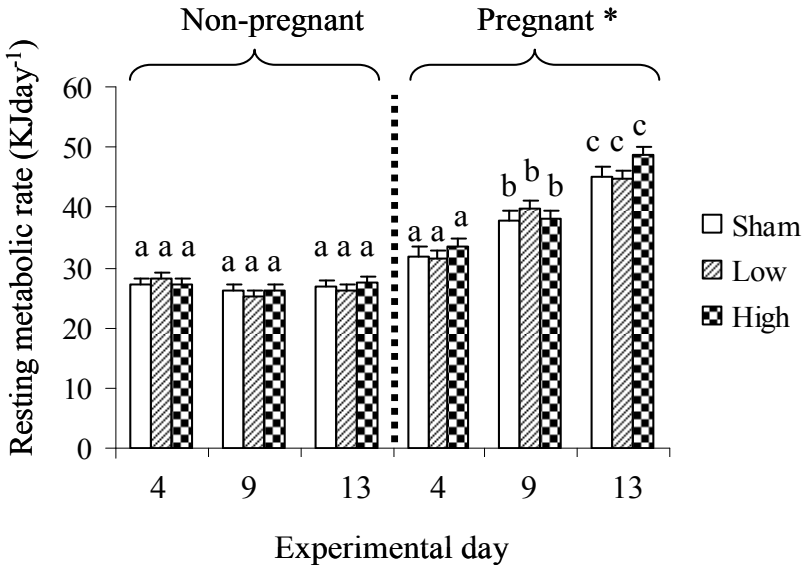

Fig. 6. Resting metabolic rate (RMR) (adjusted for baseline RMR) for non-pregnant and pregnant mice either uninfected (sham), or infected with low or high dose of *Heligmosomoides bakeri*. Main effect of pregnancy state: $F_{1,65} = 245.44$, $P < 0.0001$; main effect of dose: $F_{2,61.8} = 0.68$, $P = 0.5123$; main effect of time: $F_{2,107} = 73.24$, $P < 0.0001$; interaction of pregnancy state * time: $F_{2,107} = 83.1$, $P < 0.0001$; interaction of dose * time: $F_{4,107} = 1.65$, $P = 0.166$; interaction of pregnancy state * dose: $F_{2,61.1} = 0.33$, $P = 0.7235$; interaction of pregnancy state * dose * time: $F_{6,130} = 1.07$, $P = 0.3817$. Different lowercase letters represent significant differences across all groups, based on multiple comparisons; * represents a significant difference between pregnant and non-pregnant mice.

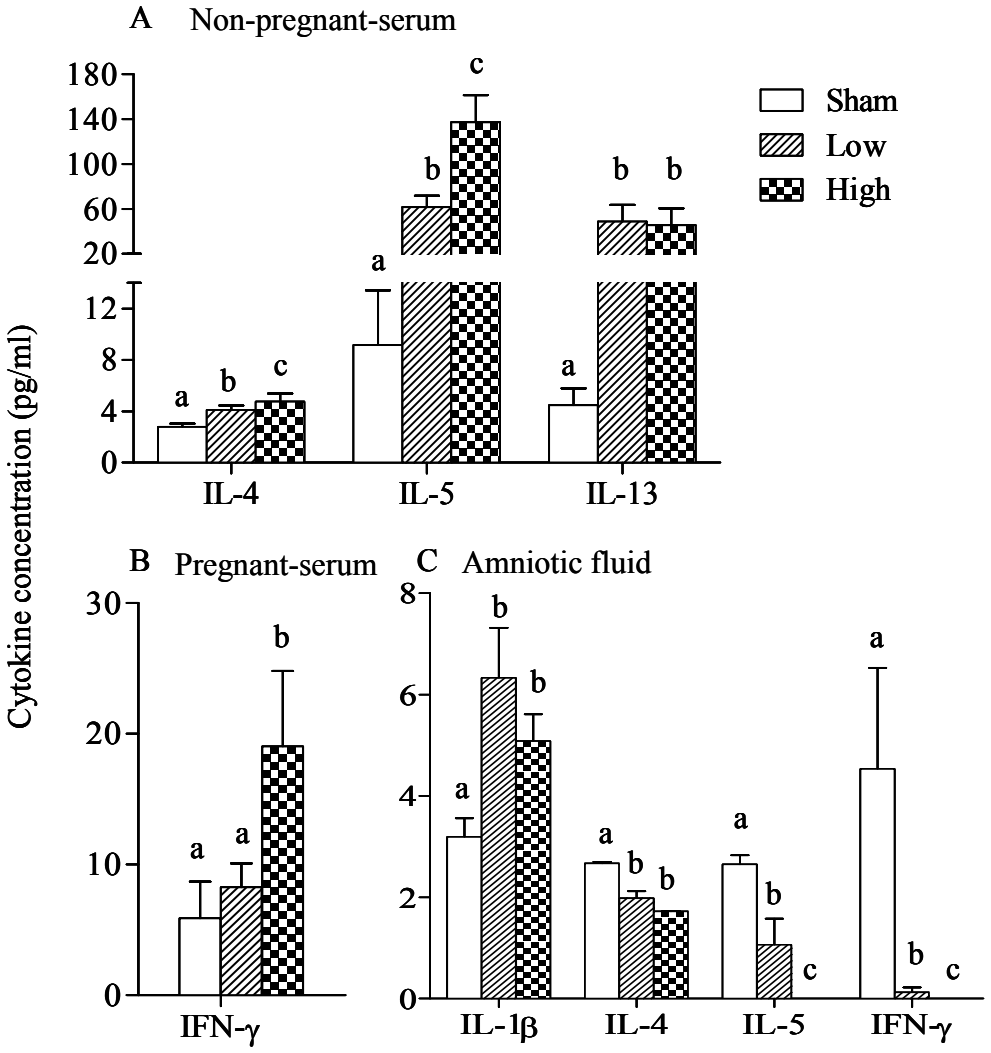

Fig. 7. Effect of infection dose on cytokine profile in serum from non-pregnant (A) and pregnant mice (B), and in amniotic fluid (C). Different lowercase letters represent significant differences between infection doses ($P < 0.05$).

CONNECTING STATEMENT I

In Chapter III, I demonstrated that physiological responses during concurrent nematode infection and pregnancy not only reduced maternal femur bone area, but also impaired foetal linear skeletal growth. We suggested that elevated IFN-γ in maternal serum and IL-1β in amniotic fluid were associated with the above effects on bone. Having developed an animal model for stunting, we were interested in further understanding the mechanisms of stunting and impaired bone mineralization especially during periods of increased energy demand. This formed the basis for the study in Chapter IV to investigate the impact of PD and GI nematode infection during late pregnancy and lactation on RMR, maternal body composition and bone mineralization, and neonatal growth, and to relate these responses to changes in concentrations of corticosterone, leptin and pro-inflammatory cytokines (IL-1β, IL-6). In Chapter IV, we chose to alter the host energy budget by (1) decreasing dietary protein nutrient which reduced energy availability, (2) imposing nematode infection that was expected to increase energy expenditure (Kristan and Hammond 2001), and to combine PD and GI nematode infection with late pregnancy and lactation, as they are known to co-occur in nature (Steketee 2003). Furthermore, besides pro-inflammatory cytokines, we examined a broad spectrum of other markers that included hormones and chemokines in order to provide a more comprehensive investigation of mechanisms underlying changes in RMR, bone mineralization and immune response.

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CHAPTER IV

PROTEIN DEFICIENCY AND NEMATODE INFECTION DURING PREGNANCY AND LACTATION REDUCE MATERNAL BONE MINERALIZATION AND NEONATAL LINEAR GROWTH IN MICE

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Abstract

Using a 2 X 2 factorial design, we investigated the combined impact of protein deficiency and gastrointestinal nematode infection during late pregnancy and lactation on resting metabolic rate (RMR), body composition and bone mineralization, neonatal growth, and the regulatory hormones (corticosterone, leptin, IGF-1) and pro-inflammatory cytokines (IL-1β, IL-6) that may drive these processes. Pregnant CD1 mice, fed either a protein sufficient (PS, 24%) or protein deficient (PD, 6%) isocaloric diet, were infected four times either with 0 (sham) or 100 *Heligmosomoides bakeri* larvae beginning on d 14 of pregnancy. Dams were killed on d 20 post-partum and pups on d 2, d 7, d 14 and d 21. Diet and infection had largely independent effects. PD elevated corticosterone and up-regulated leptin concentration in maternal serum which was associated with reduced food intake leading to lower body mass, RMR and body temperature. Infection reduced food intake, but elevated maternal serum IL-1β and IL-6, and had no effect on corticosterone, leptin, RMR or body temperature. PD decreased maternal bone area (BA) and bone mineral content (BMC). Infection lowered maternal bone mineral density (BMD), consistent with elevated IL-1β and IL-6. The elevated serum IL-1β and lower IGF-1 in pups of PD dams and lower serum leptin and IGF-1 in pups of infected dams were both consistent with the lower pup body mass and shorter crown-rump length. This mouse model provides a novel framework to study the impact of diet and nematode infection on bone.

KEY WORDS: Bone, nematode infection, protein deficiency, pro-inflammatory cytokines, resting metabolic rate.

Introduction

Nutritional requirements for metabolizable protein increase substantially in late gestation and lactation (1), but increased food intake is often not sufficient to meet the demand (2), necessitating energetic, morphological and physiological adjustments, such as reduced energy expenditure (3) and plasticity in size and function of internal organ systems (4) and tissues including bone (5). Bone is under complex regulation involving hormones such as glucocorticoids (GCs) that inhibit bone formation and induce resorption (6, 7), leptin that either inhibits or promotes bone formation (8-10), soluble local growth factors e.g. insulin-like growth factor (IGF)-1 that stimulates bone growth (11) and pro-inflammatory cytokines including IL-1β and IL-6 that inhibit bone formation and induce resorption (12, 13). Other mediators that govern the cross-talk between osteoblasts and osteoclasts are osteoprotegerin (OPG) a bone formation marker, and receptor activator for nuclear factor κ B ligand (RANKL), a bone resorption marker (14).

Both protein deficiency (PD) and gastrointestinal (GI) nematode infection, conditions that co-exist in nature (15), modulate several of these hormones and cytokines (16). During PD, elevated GCs contribute to the induction of a hypermetabolic state and loss of lean mass (17). Approximately one third of bone mass is protein (18). PD decreases not only total bone, lumbar and hip bone mass (19), bone mineral density and strength (20), but also plasma osteocalcin and IGF-1 (21). Only a few studies have looked at effects of GI nematode infection on bone. Nematode infections in sheep reportedly cause resorption of trabecular bone and reduce bone formation (22). In mice, nematode infection during pregnancy decreased femur bone area (BA) and reduced linear growth of the foetus (5). Finally, both PD and GI nematode infection affect resting metabolic rate (RMR) (3, 23).

The objectives of this research were (i) to characterize the impact of PD and GI nematode infection during late pregnancy and lactation on RMR,

maternal body composition and bone mineralization, and neonatal growth, and (ii) to relate these responses to changes in concentrations of corticosterone, leptin and pro-inflammatory cytokines (IL-1β, IL-6), using the mouse - *Heligmosomoides bakeri* [previously named *Heligmosomoides polygyrus*; see Cable *et al.* (24)] model system.

Materials and Methods

Experimental design and diets

We employed a 2 x 2 factorial design that combined 2 levels of dietary protein (PS and PD) with 2 doses [Sham: 4 x 0 third stage larvae (L_3); infected: 4 x 100 ± 3 L_3] of a trickle infection in mice with *Heligmosomoides bakeri* (Nematoda). All pregnant and lactating dams were fed a commercial purified pelleted diet, either protein sufficient (PS) (24% protein, TD.90017) or a protein deficient (PD) (6% protein, TD.90016) (Harlan Teklad, Madison, WI) (**Table 1**) from d 14 of pregnancy through lactation. Food intake was recorded every third day (d) beginning on d 15 of pregnancy. Maternal body mass and rectal body temperature (measured to within 0.1^0C using a thermometer - Microtherma 2T, model TW2-193; Braintree Scientific, Braintree, MA) were recorded every third day beginning on d 14 of pregnancy.

Major maternal outcomes were food intake, body mass, body composition, bone area, mineral content and density, body temperature, RMR, and serum concentrations of corticosterone, leptin and pro-inflammatory cytokines (IL-1β, IL-6). Reproductive outcomes included litter size, pup crown-rump length, pup body mass, and concentrations of corticosterone, leptin, IL-1β, IL-6, and IGF-1 in pup serum, and concentration of corticosterone, leptin, IL-1β and IL-6 in ingested milk.

Mice and experimental protocol

Forty-eight, 8-9 week-old primiparous, timed-pregnant outbred CD1 mice (Charles River, Quebec, Canada) were received on d 13 of pregnancy and

housed individually in Nalgene cages (Fisher Scientific, Montreal, Canada) with stainless steel covers in a temperature controlled mouse room (22-25^{0}C) with a 14:10 h light:dark cycle. Mice were acclimatized by feeding commercial pelleted mouse chow (22/5 Rodent diet 8640, Harlan Teklad, Madison, WI) for one day before being randomly assigned to the two diet and two infection treatment groups (12 dams/group). The experiment was staggered into four sets over a period of 4 months. *H. bakeri* infective 3rd-stage larvae (L_3) were obtained by faecal culture of stock parasites. Dams were infected with L_3 on d14 of pregnancy and d 2, d 9 and d 16 post-partum (PP). Dams were necropsied on d 20 PP. A single pup randomly selected from each litter was necropsied on d 2, d 7, d 14 and d 21. At necropsy, dams and pups were anaesthetized using Ketamine/Xylazine (100/10 mg/kg body mass) intraperitoneally (IP), then exsanguinated following cardiac puncture, between 0600-1000 h, when serum corticosterone level is at its lowest point in the diurnal cycle (25). In addition to pup serum, milk from pup stomachs was collected on d 2, d 7 and d 14, processed and stored at -20^{0}C for later analysis of hormones and cytokines. The small intestine of the dams (d 20 PP) was excised and the number of adult *H. bakeri* in the lumen and 4th-stage larvae (L_4) in the serosal musculature was recorded. All procedures were approved by the McGill Animal Care Committee according to guidelines of the Canadian Council on Animal Care (26).

Resting metabolic rate (RMR)

RMR was measured at room temperature (24 ± 1°C) during the light phase (between 0800h and 1700h) on d 17 of pregnancy, when L_4 would first be within the serosal mucosa, and on d 15 PP, when lactation peaks (27) and mature adult worms would be present in the intestinal lumen. RMR was assessed using an open-flow respirometry system connected to a paramagnetic oxygen analyser (Qubit Systems Research, Kingston, Ontario) as previously described (5).

Maternal body composition and bone measurements

We used the dual-energy X-ray absorptiometry (DXA) technique (Model # QDR 4500A, version 12.5; Hologic Inc., Waltham, MA) to measure maternal body composition (lean and fat mass) and bone area (BA), bone mineral content (BMC) and bone mineral density (BMD) on d 19 PP (4 days post peak lactation). Dams were weighed, immobilized by anaesthesia Ketamine/Xylazine (100/10mg/kg body mass) IP, followed by whole body and high resolution regional scans as previously described (5). The mean intra-individual coefficients of variation for each mouse using the triplicate scans were 3.2% for whole body BMC, 1.9% for BMD, 1.6% for lean mass, and 10.8% for fat.

Litter size, pup body mass and crown-rump length

Litter size was recorded at birth and on d 2, d 7, d 14 and d 21. Mean pup body mass was recorded at birth and from individual pups randomly selected from each litter on d 2, d 7, d 14 and d 21. Pup crown-rump length (measured from the top of the head to the base of the tail) was determined on d 2, d 7, d 14 and d 21, and was used as an index of axial skeletal growth.

Collection of milk from pup stomachs

Milk was collected from pup stomachs after nursing from dams. The stomach contents (50-250 µl) were transferred to 1.8 mL eppendorf tubes (Fisher Scientific, Canada) by squeezing the curdled mass through a small incision made in the fundal area of the stomach wall. Milk samples were homogenized (1:2 dilution) in a solution of protease inhibitors (1 mL/1 mg of USP pancreatin; Protease Inhibitor Cocktail, P2714, Sigma-Aldrich, ON, Canada) on ice, using RNase-free pestle (Kimble-Chase; NJ). The homogenates were centrifuged at 2000 rpm at 4°C for 10 min, after which the supernatant (milk 'serum') was collected and frozen at -20°C (28) for subsequent analyses.

Assessment of hormones and cytokines in serum and milk

Corticosterone was measured in maternal serum on d 20 PP, pup serum on d 21 and pup milk on d 2. IGF-1 was measured in pup serum on d 7, leptin in pup milk on d 7 and pup serum on d 14. We measured RANKL and leptin (bone resorption markers), and OPG and osteocalcin (bone formation markers) in maternal serum on d 20 PP and in pup serum on d 14. We determined the concentration of IL-1β and IL-6 in maternal serum on d 20 PP and in pup serum on d 21, and IL-1β and IL-6 in pup milk on d14.

Assessment of hormones and cytokines was performed using the multiplex bead array immunoassay with the Luminex 200^{TM} xMAP analyzer (Luminex Corp., Austin, TX). We determined the concentration of IL-1β and IL-6 (Millipore, Billerica, MA, # MPXMCYTO-70K); leptin, OPG and osteocalcin (Millipore, Billerica, MA, # MBN1B-41K); RANKL (Millipore, Billerica, MA, # MBN-41K-1RANKL); corticosterone (Millipore, Billerica, MA, # RSH69K) and IGF-1 (Millipore, Billerica, MA, # RMIGF187K). Assays were performed in duplicate following manufacturer's user manual for each kit and as previously described (5).

Statistical analysis

We employed a 2 X 2 factorial design with main effects of diet (PS vs PD) and and infection (sham vs infected) and where appropriate Bonferroni post hoc comparison were done except those noted below. Data were checked for normality and homogeneity of variance and transformed when necessary but only non-transformed means are reported. Pregnancy and lactation periods were treated separately during analysis. Litter size was not equilibrated at parturition.

Maternal body mass, body temperature and food intake were analysed using repeated-measures ANOVA. For RMR, analysis of covariance (ANCOVA), with whole body mass and litter size as covariates was used. For all dam parameters, food intake and litter size, where appropriate, were

included as covariates. A paired *t*-test was used to compare RMR between pregnancy and lactation periods within PS and PD mice. Number of worms recovered was computed as adult worms + L_4 and compared between PS and PD dams. Pup body mass and crown-rump length were analyzed using repeated-measures with maternal food intake as a covariate.

Regression analysis was used to determine the relationship between whole body mass and RMR. Spearman correlation analyses were used to compare RMR against food intake on d 17 of pregnancy and d 15 PP; RMR on d 15 PP against litter size; mean food intake against litter size during lactation; maternal serum corticosterone against body mass (d 20 PP), BMC and lean mass; maternal serum leptin (d 20 PP) against food intake (d 17 PP) and fat mass; and maternal serum IL-1β, IL-6 against RANKL and osteocalcin. We also correlated pup body mass and crown-rump length against pup serum IGF-1 on d 7; pup body mass and crown-rump length against pup serum leptin on d 14; and pup body mass against pup serum IL-1β on d 21.

Unless otherwise indicated, values are presented as least square means ± S.E.M. Most analyses were performed using Proc Mixed model in SAS (v. 9.1; SAS Institute Inc., Cary, NC, USA) and P values < 0.05 were considered statistically significant. Only statistically significant interactions are reported.

Results

Confirmation of infection

All experimental mice that were infected with *H. bakeri* developed mature infections, and no control mice became infected. The number of worms recovered on d 20 PP was higher in PD dams (230 ± 11 worms) compared to PS dams (183 ± 11 worms) ($P = 0.0001$).

Maternal protein deficiency and infection reduced food intake

During late pregnancy, food intake was lower in PD compared to PS mice (**Fig. 1**), but was similar between sham and infected mice (**Fig. 1**). Throughout lactation, food intake initially increased, stabilized between d 8-11 PP, and then increased during peak lactation (d 14-17 PP) in PS mice (Fig. 1). In contrast, food intake remained low throughout lactation in PD mice (Fig. 1). Infection reduced the litter-size adjusted mean food intake during peak lactation (d 14-17 PP) (Fig. 1). In all mice combined, food intake was correlated with litter size during the lactation period (d 2-17 PP) ($r = 0.53$, $P < 0.0001$).

Maternal protein deficiency but not infection reduced body mass and body temperature

Both PS and PD mice gained body mass during late pregnancy (**Fig. 2*A***) but body mass increased less rapidly in PD mice. After parturition, body mass was relatively stable in PS mice until d 16 PP, whereas body mass gradually declined over the first 10 days and remained low throughout the duration of lactation in PD mice (Fig. 2*A*). *H. bakeri* infection had no effect on body mass during late pregnancy or lactation.

Body temperature was unaffected by diet or time during late pregnancy (**Fig. 2*B***) but PD mice had consistently lower body temperature than PS mice during lactation (Fig. 2*B*), especially on d 10 PP and d 13 PP when temperature further declined (Fig. 2*B*). *H. bakeri* infection had no detectable effect on body temperature.

Maternal protein deficiency but not infection reduced resting metabolic rate (RMR)

RMR was lower in PD dams (PD sham 51.3 ± 3.2 kJ/day; PD infected 50.1 ± 3.2 kJ/day) compared to PS dams (PS sham 54.2 ± 3.2 kJ/day; PS infected 57.8 ± 3.2 kJ/day) ($P = 0.0321$) on d 17 of pregnancy, and RMR adjusted for litter size ($P = 0.0068$) was also lower in PD dams (PD sham 31.2

± 2.6 kJ/day; PD infected 31.8 ± 2.7 kJ/day) compared to PS dams (PS sham 47.7 ± 2.8 kJ/day; PS infected 48.5 ± 2.6 kJ/day) ($P < 0.0001$) on d 15 PP. *H. bakeri* infection had no effect on RMR at either time point. RMR was greater during pregnancy (d 17 of pregnancy) compared to lactation (d 20 PP) both for PS mice ($t = 2.72$, $P = 0.0123$) and PD mice ($t = 9.9$, $P < 0.0001$). Linear regression analysis including all mice (n = 48) revealed a poor marginally significant positive relationship between RMR and whole body mass on d 17 of pregnancy ($r^2 = 0.08$, $P = 0.0526$), but a highly significant positive relationship on d 15 PP ($r^2 = 0.62$, $P < 0.0001$). RMR was positively correlated with food intake on d 17 of pregnancy ($r = 0.41$, $P = 0.0041$) and d 15 PP ($r = 0.79$, $P < 0.0001$). Both body mass ($\beta = 0.48$) and litter size ($\beta = 0.46$) were strong predictors of RMR on d 15 PP ($P < 0.0001$ for both).

Maternal protein deficiency and infection reduced bone mineralization

Diet had a stronger impact than infection on variables of maternal whole body composition. On d 19 PP, PD dams had lower whole body mass, lean and fat mass, whole body BA, and BMC compared with PS mice (**Table 2**). In addition, PD dams had lower lumbar BA, BMC and BMD, lower femur BA and BMC, and lower tibia BA (Table 2). Infected mice had lower BMD compared to sham, but infection had no effect on whole body mass, lean mass, fat mass or percent fat, BA or BMC (Table 2). Interestingly, *H. bakeri* infection decreased lumbar BA, BMC and BMD, decreased femur BA and BMC, and decreased tibia BA and BMD (Table 2). Litter size was a significant covariate for fat mass ($P = 0.0002$), BA ($P = 0.0378$), BMC ($P = 0.0028$) and BMD ($P = 0.0116$).

Maternal protein deficiency reduced litter size and pup survival, whereas both maternal PD and infection reduced pup body mass and crown-rump length

Neither maternal diet nor infection had an effect on litter size at birth (mean litter size = 11 ± 1 pups) but in PD mice, litter size declined (by 35.6%) between d 2-7 PP due to cannibalism (data not shown). During lactation, litter

size was consistently larger in PS compared to PD mice across time ($P < 0.0001$) (data not shown).

At birth pup body mass was similar for all treatment groups but on d 7, d 14 and d 21 (**Fig. 3*A***), pups from PD sham and infected dams had lower body mass compared to pups from PS sham and infected dams respectively. Crown-rump length was consistently shorter in pups from PD compared to pups from PS dams across all time-points (**Fig. 3*B***). Crown-rump length was also shorter in pups from infected compared to pups from sham dams, but only on d 14 and d 21 (Fig. 3*B*).

Maternal protein deficiency and infection elevated corticosterone and leptin concentrations in lactating dams that is reflected in pup milk but not in pup serum

PD but not infected dams had higher serum corticosterone concentrations on d 20 PP (**Table 3**). Maternal serum corticosterone was negatively correlated with BMC ($r = -0.3$, $P = 0.0472$), body mass on d 20 PP ($r = -0.4$, $P = 0.003$), and lean mass on d 19 PP ($r = -0.4$, $P = 0.0061$).

Corticosterone concentration in pup milk on d 2 was higher in pups of PD infected dams compared with other experimental groups (Table 3). Neither maternal diet nor infection had a detectable effect on corticosterone concentration in pup serum on d 21 (mean = 110.5 ± 24.2 nmol/L).

Infection had no effect on maternal serum leptin concentration on d 20 PP. However, PD dams had higher serum leptin on d 20 PP compared with PS dams (**Table 3**), and milk collected on d 7 PP from pups of PD dams also had higher leptin concentration compared with milk from pups of PS dams (Table 3). In contrast, serum leptin concentration was lower in pups of PD and infected dams on d 14 compared with pups of PS and sham dams, respectively (Table 3). Maternal serum leptin (d 20 PP) was negatively correlated with food intake

(d 17 PP) (r = -0.4, P = 0.0023) but positively correlated with fat mass (d 19 PP) (r = 0.4, P = 0.0083). Pup serum leptin was positively correlated with pup mass (r = 0.4, P = 0.0021) and crown-rump length on d 14 (r = 0.5, P = 0.0017).

Maternal protein deficiency elevated OPG and lowered RANKL, whereas infection elevated OPG and marginally lowered osteocalcin in maternal serum

RANKL concentration was lower in PD compared to PS dams on d 20 PP (**Table 3**), whereas OPG concentration was elevated in PD infected dams compared with other experimental groups (Table 3). Serum osteocalcin concentration was similar between PD and PS dams, but osteocalcin was borderline decreased (P = 0.0521) in infected compared to sham dams (Table 3). The concentrations of OPG, RANKL and osteocalcin in pup serum were unaffected by maternal PD and *H. bakeri* infection (data not shown).

Maternal infection but not protein deficiency elevated IL-1β, IL-6 in maternal serum, maternal PD elevated IL-1β in pup serum and pup milk, whereas both maternal PD and infection lowered IGF-1 in pup serum

Infected dams had higher IL-1β and IL-6 concentrations in serum compared with sham (uninfected) dams on d 20 PP (**Table 3**), whereas IL-1β and IL-6 were unaffected by diet. There were positive relationships between maternal serum RANKL and IL-1β (r = 0.3, P = 0.0518) and IL-6 (r = 0.4, P = 0.0144) and a negative relationship between serum IL-6 and osteocalcin (r = -0.3, P = 0.0258).

Neither maternal diet nor infection influenced concentration of IL-1β in milk on d 14. Maternal infection had no effect on IL-6 concentration in milk, but milk from pups of PD dams had higher IL-6 concentration compared to milk from pups of PS dams (Table 3).

On d 21, pups from PD dams had higher serum IL-1β concentration compared to pups from PS dams (Table 3) but maternal infection had no effect on IL-1β concentration and neither maternal diet nor infection altered IL-6 in pup serum (data not shown).

On d 7, pups from PD mice had lower serum IGF-1 compared with pups from PS mice (Table 3), and similarly, pups from infected dams had lower IGF-1 compared with pups from sham dams (Table 3). IGF-1 in pup serum was correlated with pup body mass (r = 0.9, P < 0.0001) and pup crown-rump length (r = 0.9, P < 0.0001) on d 7.

Discussion

We investigated the combined impact of PD and GI nematode infection during late pregnancy and lactation on maternal RMR, body composition and bone mineralization, and neonatal growth, and the regulatory hormones (corticosterone, leptin, IGF-1) and pro-inflammatory cytokines (IL-1β, IL-6) that drive these processes. Four important findings were observed: (i) PD reduced maternal body mass and RMR as previously observed (29), whereas infection had no effect on body mass and RMR, (ii) lower bone BA, BMC and BMD were associated with elevated corticosterone and leptin in PD dams, whereas lower BMD was associated with elevated IL-1β and IL-6 in *H. bakeri* infected dams, (iii) maternal PD elevated corticosterone in milk, pup serum IL-1β and IL-6, whereas both PD and infection lowered leptin and independently lowered IGF-1 concentration leading to decreased body mass and linear growth of pups.

Consistent with our previous study (5), *H. bakeri* infection had no effect on food intake during pregnancy, but decreased food intake during lactation, although this decrease had no effect on body mass, RMR and body temperature. Importantly however, infection impaired the elevation of food intake that is necessary around peak lactation (d 14-16 PP), which interestingly coincided

with when the effect of maternal infection on both pup body mass and crown-rump length was first manifested (d 14). Contrary to expectation, infection did not increase corticosterone concentration. In contrast, PD decreased RMR and body temperature but elevated corticosterone which was associated with a lower lean mass (supported by our DXA data) that has been linked previously with a corticosterone-mediated catabolic response (30). However this catabolic response was not associated with elevated IL-1β and IL-6, key proteolytic cytokines (31), whose synthesis can be also suppressed by GCs (32).

Maternal activation of bone remodeling is a well-known phenomenon that occurs during increased demand for calcium for milk production (33). Consistent with other studies in rats (20) and humans (19), PD resulted in lower maternal whole body BA as well as lumbar and femur BA and BMC. Lumbar, femur and tibia bones consist of trabecular-rich areas with a high metabolic turnover (33) and are often the first to meet neonatal calcium demands if calcium is undersupplied during lactation. Interestingly, whole body BMD and most regional bone parameters were also reduced in response to infection, consistent with our previous findings where *H. bakeri* decreased femur BA (5), and in studies in sheep where GI nematode infection caused thinning of the trabecular structure and reduced bone formation (22). Although both PD and infection reduced all lumbar parameters, they had no effect on femur BMD and tibia BMC, suggesting subtle, but important differences in bone metabolism among bone regions (34) in response to PD and *H. bakeri* infection.

We suggest that the key mediators for bone remodeling during PD and infection in the maternal compartment were elevated corticosterone, leptin and pro-inflammatory cytokines (IL-1β and IL-6). GCs decrease BMC and BMD (35) either by inducing osteoblast apoptosis (36) thus inhibiting bone formation (6) or stimulating the activity of mature osteoclasts (37) and inducing bone resorption (7), effects mostly mediated via the OPG-RANKL system (14). As well, leptin which is up-regulated by GCs *in vivo* (38) also affects OPG-

RANKL balance by either inhibiting RANKL (39) or increasing OPG (40), a pattern evident in our PD dams. In healthy animals, the catabolic effects of corticosterone on bone occur together with a fall in OPG and rise in RANKL (41). However, this pattern is not evident during pathological conditions such as osteoporosis where both OPG and RANKL are higher (42) and in juvenile idiopathic arthritis where OPG is higher and RANKL lower (43). Consistent with these observations, we observed a higher concentration of serum OPG and lower RANKL in PD compared to PS dams, and a higher concentration of OPG in infected compared to uninfected dams. However, RANKL was similar in infected compared to uninfected dams in our study. Two observations may help to explain these non-classical pathways for markers of bone remodeling under pathological conditions. First the serum RANKL test kit is an enzyme immunoassay designed to measure only soluble, uncomplexed RANKL and not membrane-bound form of RANKL (44), and it is possible that during PD more RANKL is bound to its receptor and part to OPG, its decoy receptor (43) such that less of RANKL is available in soluble form. The second reason is that PD and GI nematode infection can up-regulate other biochemical pathways, such as leptin (16, 45) which can increase OPG (40) or inhibit RANKL (39). There is need for further research to elucidate additional mechanisms through which bone metabolism may be modulated under pathological conditions.

In our study, infection independently modified bone metabolism, but unlike PD, through pathways involving pro-inflammatory cytokines IL-1β and IL-6. IL-1β inhibits bone formation *in vivo* (13) and induces resorption (46), and IL-6 stimulates osteoclastogenesis leading to resorption (47). Our results in the infected dams are consistent with these effects of IL-1β and IL-6 in inhibiting bone formation/inducing resorption. Expression of RANKL is up-regulated by IL-1β (46), and induction of RANKL in osteoblasts by IL-6-type cytokines is the key event leading to their pro-resorption action (48). This is supported by the positive correlations between IL-1β and RANKL, and between IL-6 and RANKL in maternal serum. Our data support earlier suggestions that

increased OPG production in infected mice may reflect a compensatory mechanism for overproduction of RANKL, IL-1β and IL-6 in order to mitigate the RANKL, IL-1β and IL-6-mediated bone resorption, or could be secondary to a decreased clearance as a result of infection (42, 43).

Effects of PD and infection on bone metabolism were not confined to the maternal system but were evident in the offspring through reduced body mass and crown-rump length. We observed independent inhibitory effects of maternal PD and *H. bakeri* on pup body mass and crown-rump length, with maternal PD exerting a more dramatic effect on crown-rump length, evident on d 2 compared to d 14 for infection. Four factors could explain the lower pup crown-rump length and body mass in our study. First, corticosterone in milk can be transferred to pups (49) and can inhibit the growth hormone (GH)/IGF-1 axis (50). Our data show elevated corticosterone in milk and lower pup serum IGF-1. Second, leptin which peaks around d 10-14 PP in rodents (51) stimulates release of GH (52), which in turn induces synthesis of IGF-1 in cartilage (53) that is critical in promotion of chondrocyte proliferation and maturation during embryonic and neonatal bone development (11). In addition, leptin also enhances longitudinal growth by affecting the proliferation, hypertrophy, and calcification of the growth-plate cartilage (53) and skeletal bone growth (10). These effects were supported by the positive association between IGF-1 and both crown-rump length and body mass on d 7, and between pup serum leptin and crown-rump length on d 14 in our study. Therefore the observed lower leptin in pups from PD and infected dams is consistent with the lower pup crown-rump length and body mass. Third, pro-inflammatory cytokines including IL-1β and IL-6 can be transferred from maternal system to neonates via milk (54) and are known to inhibit both IGF-1 (55) and the growth plate chondrocyte dynamics (56). We observed higher IL-6 in milk, higher IL-1β in serum and lower IGF-1 in serum of pups from PD dams. Collectively, these results outline four pathways through which maternal PD and GI nematode infection can impair linear growth and lower body mass in pups.

In conclusion, our findings are novel in several ways. First, PD and infection had independent effects on bone metabolism in the maternal and neonatal systems. We showed that elevated corticosterone and leptin in PD dams resulted in decreased BA and BMC, whereas *H. bakeri* infection elevated two pro-inflammatory cytokines IL-1β and IL-6 that resulted in lower BMD. Second, the elevated transfer of corticosterone and IL-6 through the milk in PD infected dams appeared to contribute to lower IGF-1 and reduced crown-rump length in pups who did not experience PD and infection directly. Therefore neonatal growth may be modulated by pro-inflammatory cytokines. We propose that the *H. bakeri* – pregnancy – lactation model may provide a useful animal system for studying bone stunting effects common during GI nematode infection in children.

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TABLE 1 Composition of experimental diets

Ingredient	Protein-sufficient (*g/kg*)	Protein-deficient (*g/kg*)
Casein[1]	276.0	69.0
DL-Methionine	3.0	0.9
Sucrose	391.8	571.8
Corn Starch	200.0	200.0
Corn Oil[2]	51.8	53.9
Cellulose[3]	32.62	57.82
Vitamin Mix, Teklad (40060)[4]	10.0	10.0
Ethoxyquin, antioxidant	0.01	0.01
Mineral Mix, Ca-P Deficient (79055)[5]	13.37	13.37
Calcium Phosphate, dibasic	15.3	21.6
Calcium Carbonate	6.1	1.6
Energy, *kJ/g*	15.9	15.9

[1] Edible lactic acid casein, New Zealand Milk Products, Inc.

[2] Columbus Foods Company, Inc.

[3] Solka-floc®, International Fiber Corporation

[4] Vitamin mix (mg/kg diet): retinyl palmitate, 10.9; vitamin C, 991; cholecalciferol, 0.055; *d,l*-α tocopheryl acetate, 121; menadione, 49.6; biotin, 0.4; choline, 1406; folic acid, 2; niacin, 99; calcium pantothenate, 61; riboflavin, 22; thiamine, 20; vitamin B-6, 18; vitamin B-12, 0.03; para-aminobenzoic acid 110; inositol, 110.

[5] Mineral mix (mg/kg diet): K, 3610; Na, 1020; Cl, 1570; Mg, 510; Cu, 5.82; Fe, 36.12; Zn, 34.50; Mn, 58.56; I, 0.21; Mo, 0; Se, 0.11; Cr, 2.0.

TABLE 2 Effect of diet and *Heligmosomoides bakeri* infection on DXA variables in dams on d 19 post-partum[1]

	PS Sham (n = 12)	PS Infected (n = 12)	PD Sham (n = 12)	PD Infected (n = 12)	2-way ANOVA, *P*-values[2] D	I	D x I
Whole body							
Mass, (*g*)[3]	35.4 ± 1.1	37.4 ± 1.1	26.2 ± 1.1	26.1 ± 1.1	< 0.0001	NS	NS
Lean mass, (*g*)	30.7 ± 0.8	32.2 ± 0.8	21.2 ± 0.8	21.1 ± 0.8	< 0.0001	NS	NS
Fat mass, (*g*)	4.8 ± 0.5	5.1 ± 0.5	3.5 ± 0.5	3.6 ± 0.5	0.006	NS	NS
Percent fat, (*%*)	13.4 ± 1.3	13.6 ± 1.2	14 ± 1.2	14.5 ± 1.3	NS	NS	NS
BA, (cm^2)[3]	11.1 ± 0.3	11.5 ± 0.3	8.5 ± 0.3	8.6 ± 0.3	< 0.0001	NS	NS
BMC, (*g*)[3]	0.80 ± 0.04	0.79 ± 0.04	0.64 ± 0.04	0.59 ± 0.04	0.0001	NS	NS
BMD, (g/cm^2)[3]	0.075 ± 0.003	0.072 ± 0.003	0.075 ± 0.003	0.068 ± 0.003	NS	0.033	NS
Lumbar Spine							
BA, (cm^2)	0.61 ± 0.01	0.54 ± 0.01	0.47 ± 0.01	0.44 ± 0.01	< 0.0001	< 0.0001	NS
BMC, (*g*)	0.078 ± 0.003	0.066 ± 0.003	0.060 ± 0.003	0.048 ± 0.003	< 0.0001	< 0.0001	NS
BMD, (g/cm^2)	0.129 ± 0.004	0.120 ± 0.004	0.123 ± 0.004	0.111 ± 0.004	0.003	0.0001	NS

Femur							
BA, (cm^2)[4]	0.40 ± 0.02	0.33 ± 0.02	0.31 ± 0.02	0.27 ± 0.02	< 0.0001	< 0.0001	NS
BMC, (g)	0.028 ± 0.002	0.024 ± 0.002	0.022 ± 0.002	0.019 ± 0.002	< 0.0001	0.012	NS
BMD, (g/cm^2)	0.070 ± 0.004	0.074 ± 0.004	0.069 ± 0.004	0.068 ± 0.004	NS	NS	NS
Tibia							
BA, (cm^2)	0.53 ± 0.01	0.51 ± 0.01	0.45 ± 0.01	0.43 ± 0.01	< 0.0001	0.019	NS
BMC, (g)[3]	0.012 ± 0.001	0.013 ± 0.001	0.014 ± 0.001	0.012 ± 0.001	NS	NS	NS
BMD, (g/cm^2)[4]	0.031 ± 0.004	0.029 ± 0.003	0.029 ± 0.003	0.022 ± 0.004	NS	0.046	NS

[1]Values are Lsmeans ± SEM, n= 12, food intake and litter size were entered as covariates for analyses ($P < 0.05$) NS, Not significant.

[2]Main effects for our statistical analysis included diet (D) and Infection (I) and their interaction D x I.

[3]Data were not normal but had homogeneity of variance; effects were verified by Kruskal-Wallis analysis of ranks and found to be significant for diet (χ^2, $P < 0.0001$ whole body mass; χ^2, $P < 0.0001$ whole body BA; χ^2, $P = 0.0001$ whole body BMC) and for infection (χ^2, $P = 0.033$ whole body BMD).

[4]Data were log transformed [$\log_{10}(y + 10)$] to achieve normality.

[5]Abbreviations: BA, bone area; BMC, bone mineral content; BMD, bone mineral density; DXA, dual-energy X-ray absorptiometry.

TABLE 3 Effect of diet and *Heligmosomoides bakeri* infection on concentration of hormones, cytokines and markers of bone metabolism[1]

	PS Sham (n = 12)	PS Infected (n = 12)	PD Sham (n = 12)	PD Infected (n = 12)	2-way ANOVA, *P*-values[2] D	I	D x I
Dam serum, d 20 PP[3]							
Corticosterone, (*nmol/L*)[4,5]	251 ± 80	156 ± 80	318 ± 80	537 ± 80	0.001	NS	NS
Leptin, (*μg/L*)	0.4 ± 0.2	0.4± 0.2	0.8 ± 0.2	1.0 ± 0.2	0.008	NS	NS
OPG (*μg/L*)[6]	0.9 ± 0.2[b]	0.9 ± 0.2[b]	1.0 ± 0.2[b]	1.3 ± 0.2[a]	0.001	0.035	0.029
RANKL, (*μg/L*)[6]	0.02 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.0001	NS	NS
Osteocalcin, (*μg/L*)	45 ± 7	38 ± 7	50 ± 7	43 ± 7	NS	0.052	NS
IL-1β, (*μg/L*)[5,6]	0.01 ± 0.004	0.02 ± 0.004	0.001 ± 0.004	0.01 ± 0.004	NS	0.020	NS
IL-6, (*μg/L*)[5,6]	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.04 ± 0.01	NS	0.002	NS
Pup serum							
IGF-1, d 7, (*μg/L*)[6]	55 ± 8	34 ± 8	13 ± 8	8 ± 8	< 0.0001	0.012	NS

Leptin, d 14, (*μg/L*)	2.2 ± 0.2	2.1 ± 0.2	2.1 ± 0.2	1.4 ± 0.2	0.015	0.008	NS
IL-1β, d 21, (*μg/L*)[5,6]	0.015 ± 0.009	0.004 ± 0.009	0.03 ± 0.009	0.021 ± 0.009	0.026	NS	NS
Pup milk							
Corticosterone, (*nmol/L*)[4,5]	19 ± 7[b]	19 ± 7[b]	22 ± 7[b]	69 ± 7[a]	0.0003	0.001	0.001
Leptin, d 7, (*μg/L*)	0.18 ± 0.08	0.01 ± 0.08	0.83 ± 0.08	0.49 ± 0.08	< 0.0001	0.003	NS
IL-6, d 14, (*μg/L*)[5,6]	0.002 ± 0.002	0.002 ± 0.002	0.003 ± 0.002	0.006 ± 0.002	0.034	NS	NS

[1]Values are Lsmeans ± SEM, n= 12. Values with different superscripts are significantly different using Bonferroni post hoc multiple comparisons, where food intake and litter size were entered as covariates for analyses in dams ($P < 0.05$) NS, Not significant.

[2]Main effects for our statistical analysis included diet (D) and Infection (I) and their interaction D x I

[3]PP = post-partum

[4]MW for corticosterone = 346.46

[5]Data were log transformed [$\log_{10}(y + 10)$] to achieve normality.

[6]Abbreviations: OPG, osteoprotegerin; RANKL, receptor activator for nuclear factor κ B ligand; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IGF-1, insulin-like growth factor 1.

	D	I	T	D x T	I x T	D x I x T
Pregnancy	P = 0.005	NS	P < 0.0001	P = 0.011	NS	NS
Lactation	P < 0.0001	P = 0.001	P < 0.0001	P < 0.0001	P = 0.002	NS

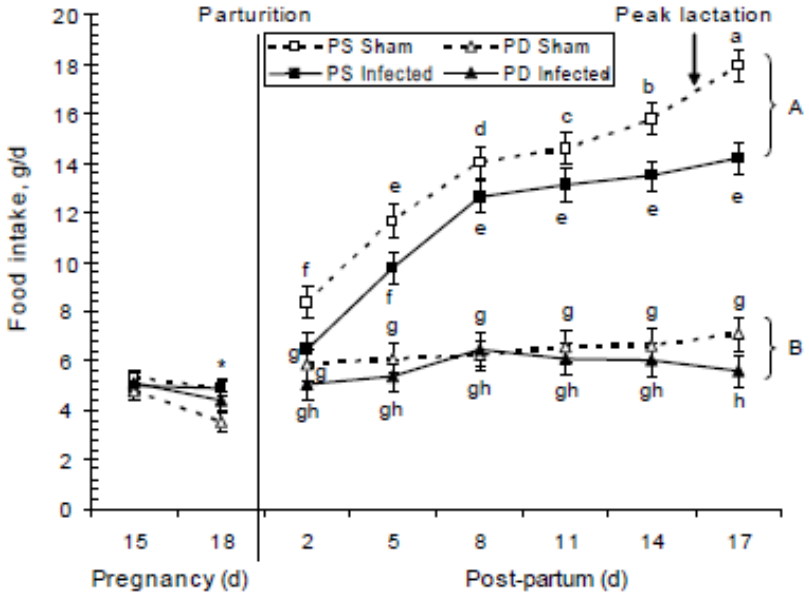
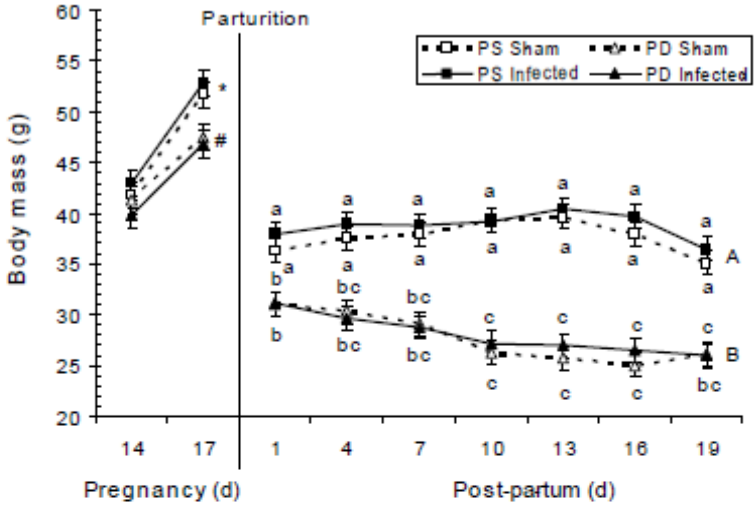


Fig. 1. Effect of diet and *Heligmosomoides bakeri* infection on food intake during pregnancy and during lactation after adjusting for litter size, n = 12 per group, (P = 0.0003). D = Diet, I = Infection, T = Time. An asterisk (*) represents a significant difference in food intake between d 15 and d 18 of pregnancy in both sham and infected PD dams. Different upper case letters represent differences for diet, different lowercase letters represent differences within a group across time during lactation within groups (P < 0.05). Values are Lsmeans ± SEM

A

Body mass	D	I	T	D x T	I x T	D x I x T
Pregnancy	P = 0.0004	NS	P < 0.0001	P < 0.0001	NS	NS
Lactation	P < 0.0001	NS	P < 0.0001	P < 0.0001	NS	NS



B

Body temp	D	I	T	D x T	I x T	D x I x T
Pregnancy	P = 0.036	NS	P = 0.001	NS	NS	NS
Lactation	P < 0.0001	NS	NS	P < 0.0001	P < 0.0001	NS

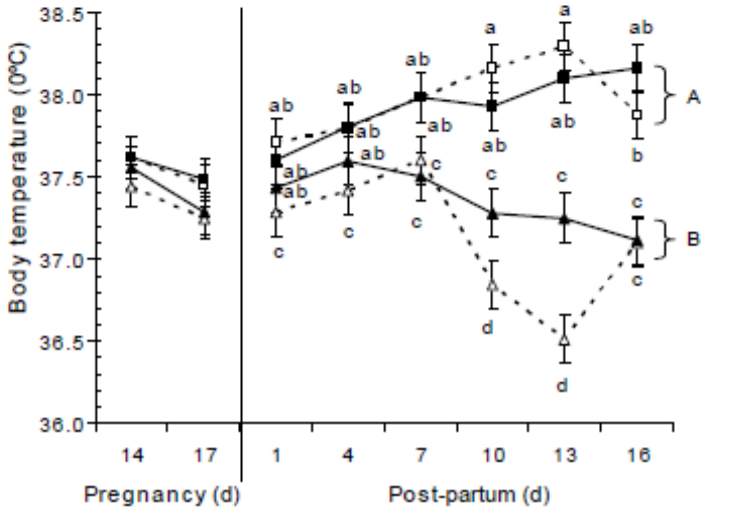


Fig. 2. Effect of diet on maternal body mass (*A*) and body temperature (*B*) across time during pregnancy and lactation, n = 12 per group. D = Diet, I = Infection, T = Time. An asterisk (*) and number sign (#) represent significant differences in body mass between d14-d17 of

pregnancy in PS and PD dams respectively. Different upper case letters represent differences for diet, different lowercase letters represent differences within a group across time during lactation within groups ($P < 0.05$). Values are Lsmeans ± SEM

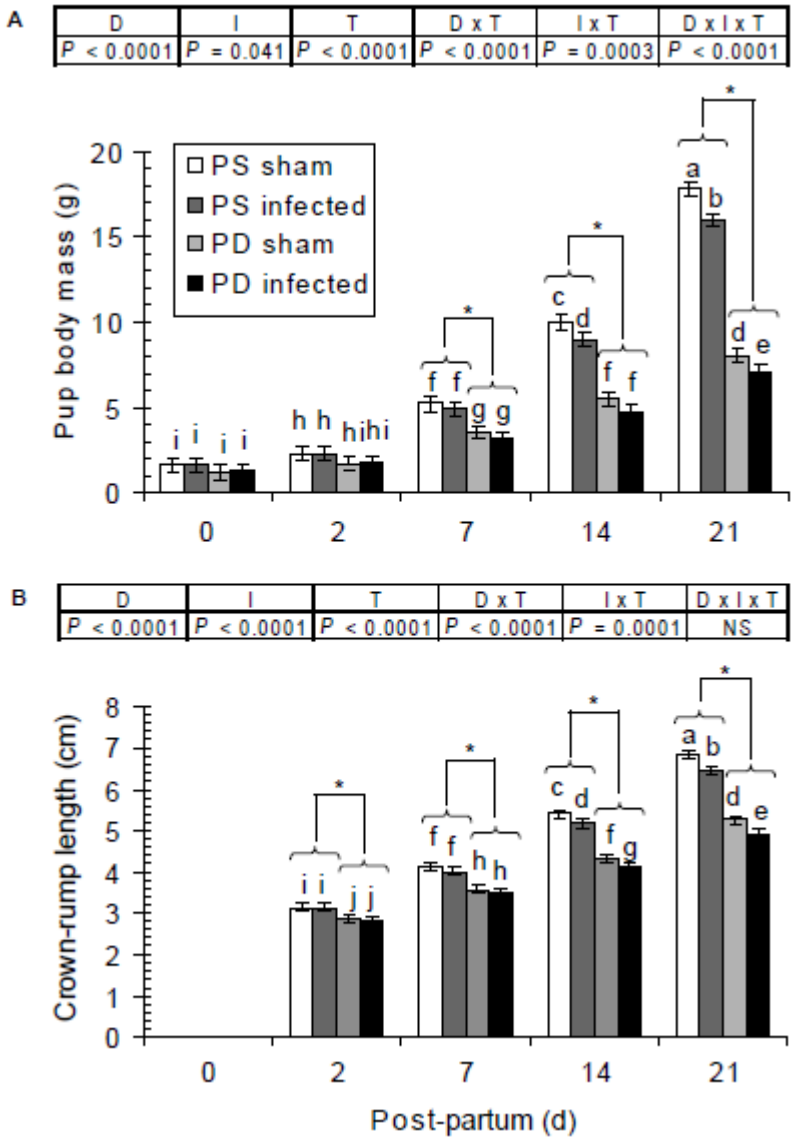


Fig. 3. Interaction effect of maternal diet and *Heligmosomoides bakeri* infection on pup body mass (*A*) and (*B*) pup crown-rump length (cm) across time, n = 12 per group. D = Diet, I = Infection, T = Time. An asterisk (*) represents a significant difference between PS and PD; different lowercase letters represent differences among groups within a time-point and across time ($P < 0.05$). Maternal food intake was included as a covariate in the analysis; values are Lsmeans ± SEM.

CONNECTING STATEMENT II

The study in Chapter IV showed that PD elevated worm burden, and maternal PD and infection reduced pup body mass and linear growth. The elevated corticosterone in milk, serum IL-1β and lower leptin concentration in pups from PD and infected dams were associated with the impaired neonatal growth. These effects were particularly intriguing considering that pups were not subjected to direct diet or infection treatments, and indicated that the negative impacts of maternal infection and PD may not be confined to bone tissue but may involve maternal and neonatal lymphoid organs. PD is known to result in breakdown of immunity to GI nematodes in lactating rodents (Houdijk *et al.* 2005), whereas maternal conditions (e.g. undernutrition, GI nematode infection) during reproductive period and maternal effort (e.g. nursing, brooding) can affect offspring immunocompetence and survival (Saino *et al.* 1997; Kristan 2002). Therefore, in Chapter V, we sought to confirm that PD during pregnancy and lactation impairs maternal immune response to trickle *H. bakeri* infection, and investigated whether PD and *H. bakeri* infection during pregnancy and lactation would impair development of neonate immune system. We investigated the intriguing possibility that impaired neonatal immune development may occur perhaps through the altered profile of maternal hormones and pro-inflammatory cytokines that are transferred from the stressed dam to the pup via milk.

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CHAPTER V

IMPACT OF PROTEIN DEFICIENCY AND GASTROINTESTINAL NEMATODE INFECTION ON DEVELOPMENT OF THE NEONATAL IMMUNE SYSTEM IN MICE

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ABSTRACT

Neonatal immune development begins in pregnancy and continues during lactation. Because conditions of malnutrition and parasite infections are often encountered simultaneously, we explored the intriguing possibility that protein deficiency (PD) and a chronic gastrointestinal (GI) nematode infection in the dam could impair neonatal immune development. Beginning on d14 of pregnancy, pregnant mice were fed protein sufficient (PS, 24%) or protein deficient (PD, 6%) isocaloric diets and infected weekly with either 0 (sham) or 100 *Heligmosomoides bakeri* larvae. A single pup per litter was killed on d2, d7, d14 and d21 post-partum (PP) and dams were killed on d20 PP. As expected, infection increased Th2 cytokines (IL-4, IL-5) and eotaxin in serum of PS dams, whereas lower milk VEGF was observed in PD infected dams. PD infected dams had elevated corticosterone and leptin compared with PS infected dams which was associated with lower IL-13 and increased faecal egg output and worm burden. Evidence of impaired neonatal immune system included reduced lymphoid organ mass in pups induced by both maternal infection and PD, whereas the percentage of T cells and T:B cell ratio in the spleen was increased only by maternal PD. These changes were associated with elevated corticosterone and IL-6 in maternal milk, and lower serum leptin and IGF-1 in pups from PD infected dams. Together, these results show that specific pro-inflammatory cytokines, corticosterone and leptin are up-regulated during nematode infection and PD and their combined effects impair neonatal immune development.

Keywords: Protein deficiency, nematode infection, lactation, corticosterone, leptin, thymus, spleen, lymphocytes.

INTRODUCTION

Immune system ontogeny begins from early to mid gestation in humans and pigs, and from mid to late gestation in mice (51). In mice, thymus organogenesis begins on embryonic day 10.5 from the endodermal layer of the pharyngeal pouch (24), whereas spleen development begins just before embryonic day 11.5 with condensation of the mesenchyme underlying the dorsal mesogastrium epithelium (46). Progenitor T cells first begin to migrate to the thymus from the early sites of hematopoiesis around day 11 of gestation (39) while the development of thymic γδ and αβ T cells, and B cells in foetal liver and bone marrow, occur during the last quarter of the gestation period (34, 51). The first set of mature CD4 or CD8 expressing T cells appears 1-2 days before birth in mice (62); thus mice are born with a small number of mature lymphocytes and expansion of the peripheral lymphocyte pool mainly occurs postnatally (31). Despite their low frequency, these T cells can be primed to produce cytokines such as IFN-γ (31), IL-2 and IL-4 (1). However, cytokine production in neonates is generally low, which may in part be compensated by cytokines from breast milk (25).

Although there is substantial evidence on the effects of PD on the adult immune function, little work has been done on the effect of maternal dietary PD and infection on neonatal immune development. Effects of PD on host immunity in adults are generally inhibitory, and include reduced homing of mucosal lymphocytes to the gut (50), reduced cytokine production by both Th1 (16) and Th2 cells (35), reduced numbers of memory cells (56) and depressed blood eosinophilia and IgG1 (10). Consequently, PD prolongs survival of the gastrointestinal (GI) nematode *Heligmosomoides bakeri* (35), increases its fecundity (38) and may increase susceptibility to incoming larva in mice (10). In addition, short-term protein malnutrition (4-7 days) has been shown to increase T cells in spleen, B cells in the Peyer's patches and T:B cell ratio in the spleen, but reduce B cells in spleen and IgA in small intestine in adult mice (48). In weanling mice, PD results in cessation of growth of lymphoid tissues

and a reduction in cell numbers associated with corticosteroid-induced lympholysis (9). There is also evidence for in utero sensitization to *Ascaris lumbricoides* in newborns of mothers with ascariasis (28). Offspring of *Dipetalonema viteae, Acanthocheilonema viteae* and *Trichinella spiralis*-infected pregnant rats and mice are immunologically less responsive to parasite antigens, are more susceptible or have reduced survival than the offspring of uninfected mothers (30, 67, 72). However, some protection has been reported in offspring of *H. bakeri* infected dams upon re-infection with the same parasite at day 35 post-partum (41). Collectively, these observations support the hypothesis that pre- and postnatal exposure to PD and helminth infection may influence the development of the neonate immune system.

PD often coexists with GI nematode infections throughout life (40), including the reproductive period. Although the prenatal and antenatal periods are thought to be one of high susceptibility to diet-induced immunologic changes, there are no studies on the effects of dietary PD and nematode infection during pregnancy and lactation on neonatal immune development. The objectives of the present study were (i) to confirm that PD during late pregnancy and lactation in mice would impair maternal immune response to trickle infection with *Heligmosomoides bakeri* [previously named *Heligmosomoides polygyrus*; see Cable *et al.* (11)], (ii) to test whether PD and/or *H. bakeri* infection increases the transfer of corticosterone and pro-inflammatory cytokines in milk, and (iii) to determine whether this impairs development of neonate immune system by reducing mass of lymphoid organs, total cells and lymphocyte proportions. Here, we demonstrate that (i) maternal PD impaired immune responses induced by infection as evidenced by lowered serum interleukin (IL)-13 and higher worm burden and faecal egg output (EPG) in infected lactating dams, (ii) this was associated with elevated corticosterone and leptin both in maternal serum and pup milk, as well as elevated IL-6 in pup milk in response to maternal PD and infection, and (iii) elevated cytokines in milk led to reduced leptin and IGF-1 in pup serum which

were associated with delayed impaired lymphoid tissue development in response to both maternal infection and PD, but elevated T cell populations in the pups induced by maternal PD.

MATERIALS AND METHODS

Mice and Diets. Forty-eight, 8-9 week-old female primiparous pregnant outbred CD1 mice at day 13 of pregnancy (Charles River, QC, Canada) were housed individually in Nalgene cages (Fisher Scientific) with stainless steel covers in a temperature controlled mouse room (22–25 ^{0}C) with a 14:10 h light:dark cycle. All pregnant mice were fed commercial purified pelleted diet (Harlan Teklad), either protein sufficient (PS) (24% protein, TD.90017) or a protein deficient (PD) (6% protein, TD.90016) (60) from day 14 of pregnancy through lactation. The 24% control level is considered adequate for most strains of mice and not excessive for any (58). Food and water were provided to the mice *ad libitum*.

Experimental design and Infection. PS and PD pregnant dams were either trickle infected with *H. bakeri* (4 x 100 ± 3 L_3) suspended in water or given a sham dose (4 x 0 L_3) of 100 µl of water. As it was impossible to process all mice at the same time, the experiment was staggered into four sets over a period of 4 months. Pregnant mice were acclimatized by feeding commercial pelleted mouse chow (22/5 Rodent diet 8640, Harlan Teklad) for 1 day before being randomly assigned to the 2 diet and 2 infection treatment groups (12 mice per group).

L_3 obtained by faecal culture of stock parasites maintained in outbred CD1 mice (Charles River) were counted in 10 sham doses for accuracy before infecting the mice. The same larval culture was used for all trickle infections within each set of mice. Day of parturition was considered as day 0 of postnatal life. Dams were infected with 100 parasite larvae weekly, on pregnancy day 14 and days 2, 9 and 16 post-partum (PP).

At necropsy, dams and pups were anaesthetized using Ketamine/Xylazine (100/10 mg/kg body weight) intraperitoneally (IP), then exsanguinated by cardiac puncture. Maternal necropsies were carried out on d20 PP, sera was collected and frozen at -20^{0}C for analyses of cytokines, chemokines and hormones. Mice were killed between 0600-1000 hours, covering the time when serum corticosterone concentration is at its lowest point in the diurnal cycle (33). Entrance to the animal room was restricted for a 10-hour period before necropsies were performed in order to maintain basal levels of corticosterone. Blood was collected from each mouse by cardiac puncture within the shortest period after anaesthetization following removal of the mouse from its cage. Relative organ mass of thymus and spleen as a percentage of body mass was determined. Identical procedures were used for all mice. Litter size was recorded at birth, on d2, d7, d14 and d21 PP.

The study involved 192 pups, none of which were subjected to direct diet or infection treatments at any time. A single pup randomly selected from each litter was necropsied on d2, d7, d14 and d21 PP; serum and milk from pup stomachs were collected, processed and stored for later analysis of hormones, cytokines and chemokines. Thymus and spleens of pups were isolated, weighed and processed to analyse lymphocyte proportions on d7 and d21 PP by flow cytometry. Relative pup thymus and spleen organ masses as a percentage of body mass were determined on d2, d7, d14 and d21 PP. All procedures were approved by the McGill Animal Care Committee according to guidelines of the Canadian Council on Animal Care (13).

Parasitological indicators. Egg production by *H. bakeri* was calculated as number of eggs per gram (EPG) using total number of eggs determined by a modified McMaster technique (66) using 3 h stool collections from dams, on d5 and d18 PP. At necropsy the small intestine was excised. The duodenum (10 cm immediately distal to the pyloric sphincter) was divided into three equal length sections (proximal, mid and distal) and the remaining

small intestine was divided equally into the jejunum and ileum. The numbers of adult *H. bakeri* in the lumen and 4th-stage larvae (L_4) in the serosal musculature for each section was determined by examining intestines that were slit open onto a glass plate and scanned using a dissection microscope.

Collection of milk from pup stomachs. Milk was collected from pup stomachs after nursing from dams, a method shown to be superior to obtaining milk samples by pumping when comparing rodents that vary in nutritional status (18). The stomach contents (50-250 µl) were transferred to Eppendorf tubes by squeezing the curdled mass through a small incision made in the fundal area of the stomach wall. Milk samples were homogenized (1:2 dilution) in a solution of protease inhibitors (1 ml/1 mg of USP pancreatin; Protease Inhibitor Cocktail, P2714, Sigma-Aldrich, ON, Canada) on ice, using RNase-free pestle (Kimble-Chase; NJ). The homogenates were centrifuged at x 620 g at 4°C for 10 min, after which the supernatant (milk 'serum') was collected and frozen at -20°C (43) for subsequent analyses.

Assessment of hormone, cytokine and chemokine concentrations in serum and milk. Corticosterone was measured in maternal serum on d20 PP, pup serum on d21 PP and pup milk on d2 PP. We determined the concentration of cytokines and chemokines in maternal serum on d20 PP, pup serum on d21 PP and pup milk on d14 PP, and we also measured leptin in maternal serum on d20 PP and in pup serum on d14 PP. IGF-1 was measured in pup serum on d7 PP.

Hormones, cytokine and chemokine concentrations were measured using the multiplex bead array immunoassay with the Luminex 200^{TM} xMAP analyzer (Luminex Corp., Austin, TX). Concentrations of corticosterone, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, interferon-gamma (IFN)-γ, tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), eotaxin, monocyte chemotactic protein (MCP)-1, leptin, and IGF-1

were assayed in duplicate following manufacturer's instructions (Millipore) (59).

Flow cytometry analysis. Thymic and splenic single-cell suspensions were obtained by mashing pup organs through 70-μm pore size nylon cell strainers (BD Biosciences). Red blood cells (RBCs) were lysed in ACK buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM NaEDTA (pH 7.2); 1 ml/tube, 2 min at room temperature). Live cell numbers were determined after staining dead cells using 0.4% Trypan blue (Invitrogen) and counting using a Neubauer hemocytometer. 10^6 cells per sample were washed in FACS buffer [1x PBS, 0.1% BSA], incubated with 10 μl of FcR Block (clone 2.4G2 BD Pharmingen) for 15 min on ice, and stained using the following mAbs: fluorescein isothiocyanate (FITC)-conjugated anti-CD3ε, phycoerythrin (PE)-conjugated anti-CD4 (L3T4), PE-Cy7-conjugated anti-CD8α (Ly-2), allophycocyanin (APC)-conjugated anti-CD25α (IL-2Ra, p55), APC-AlexaFluor® 750-conjugated anti-CD44 (Pgp-1, Ly-24), and PE-Cy5.5-conjugated anti-CD45R (B220) antibodies, all from eBioscience. Isotype controls included Armenian Hamster IgG-FITC, Rat IgG2b-PE, Rat IgG2a-PE-Cy7, Rat IgG1-APC, Rat IgG2b-APC-Alexa Fluor® 750 and Rat IgG2a-PE-Cy5.5. Cells were washed, fixed in 3% paraformaldehyde, and analysed by flow cytometry (BD FACSAria™ I) based on a minimum of 100,000 events. Flow cytometry data were analysed using the FlowJo software version 7.5. Analysis of lymphocyte populations from pup lymph nodes was not possible due to insufficient cell numbers, even after pooling the tissues.

Statistical analysis. Analyses were performed using a one-way ANOVA with post-hoc Tukey's adjustment to account for multiple comparisons. Data were checked for normality and homogeneity of variance and transformed when necessary. Transformed data are indicated where appropriate; however, only non-transformed means are reported. Analyses included adjustment for litter size where appropriate. The impact of infection

was examined by the contrast, within diet treatments, between infected and uninfected dams or their pups. The impact of diet was examined by contrasting, within infection treatments, between PS and PD dams or their pups.

Spearman correlation analyses were used to compare maternal serum IL-5 against eotaxin; maternal serum corticosterone on d20 PP against corticosterone in pup milk on d2 PP; corticosterone in pup milk on d2 PP against relative thymus and spleen mass on d2 PP; and leptin in pup serum on d7 PP against relative thymus and spleen mass on d7 PP. We also correlated pup serum IL-4 against spleen B-cell proportion on d21 PP; and the CD4+:CD8+ ratio in the thymus against the CD4:CD8 ratio in the spleen on d7 PP and on d21 PP in pups to assess whether the CD4+:CD8+ in the spleen segregates independently from this same phenotype in the thymus.

Unless otherwise indicated, values are presented as least square means ± S.E.M. Most analysis were performed using Proc Mixed model in SAS (v. 9.1; SAS Institute Inc., Cary, NC, USA) and P values < 0.05 were considered statistically significant. Only statistically significant interactions are reported.

RESULTS

Protein deficiency during lactation impaired maternal Th2 immunity and promoted parasite survival. Trickle infection with *H. bakeri* induced splenomegaly in PS dams ($P < 0.0001$) (Table 1) as well as a Th2 cytokine response as evidenced by higher IL-4 ($P = 0.003$) and IL-5 ($P < 0.0001$) and eotaxin ($P = 0.003$) on d20 PP (Table 2). As expected, maternal serum IL-5 was positively correlated with eotaxin ($r = 0.45$, n = 48, $P = 0.0014$). PD suppressed these responses, as evidenced by lower relative mass of thymus ($P = 0.0378$) and spleen ($P < 0.0001$) in infected PD dams compared with infected PS dams (Table 1). Furthermore, PD reduced IL-13 in both sham ($P = 0.0438$) and infected ($P = 0.0516$) dams (Table 2). Our hypothesis that

worm development would be enhanced and expulsion delayed in PD compared to PS dams was supported by the lower number of L_4 recovered (after adjusting for litter size) (Fig. 1A), by the more anteriorad location of worms in PD dams (Fig. 1B), and by the higher EPG both on d5 PP (Fig. 1C) and on d18 PP (after adjusting for litter size) (Fig. 1D).

Maternal infection and protein deficiency induced an elevation in corticosterone and leptin concentrations in lactating dams, which was reflected in pup milk but not in pup serum. In order to determine whether the stresses of maternal infection and PD would stimulate the hypothalamic-pituitary-adrenal (HPA) axis, we measured corticosterone in maternal serum on d20 PP. Although infection alone did not alter corticosterone concentrations, superimposition of PD led to a 3.4-fold elevation of serum corticosterone among infected dams ($P = 0.0047$) (Table 2). Infection led to an increase in corticosterone concentration in pup milk, but only among the pups from PD dams on d2 PP ($P < 0.0001$) (Table 3). Corticosterone in pup milk on d2 PP was positively correlated with corticosterone in maternal serum on d20 PP ($r = 0.39$, n = 48, $P = 0.0059$), suggesting possible earlier maternal transfer of corticosterone via milk. In contrast, neither maternal infection nor PD altered corticosterone concentrations in pup serum, as measured on d21 PP (Table 4).

Leptin in maternal serum was elevated in response to PD, but only in infected dams ($P = 0.054$) (Table 2), and the same pattern was seen in pup milk on d7 PP (Table 3). However, leptin concentration showed the opposite response in pup serum when measured on d14 PP when it was lower in pups of PD infected compared to pups of PS infected dams ($P = 0.0019$) (Table 4).

Maternal protein deficiency reduced serum IGF-1 in pups of uninfected and infected dams. The concentration of IGF-1 was measured only in pup serum on d7 PP. PD reduced the concentration of IGF-1 in pups

from infected dams ($P = 0.0036$) (Table 4) and uninfected dams ($P < 0.0001$) (Table 4).

Maternal protein deficiency lowered milk VEGF but increased milk IL-6 and serum eotaxin in pups of infected dams. We detected IL-1β, IL-4, IL-6, eotaxin and VEGF in pup milk on d14 PP and in pup serum on d21 PP. In addition, IL-5 and IL-13 were detected in pup serum on d21 PP. Pups from PD infected dams had higher milk concentration of IL-6 ($P = 0.0187$) but lower concentration of VEGF ($P = 0.0162$) compared to pups from PS infected dams on d14 PP (Table 3). The only observed difference in cytokines in pup serum was for eotaxin which was elevated on d21 PP in pups from PD infected compared to PS infected dams ($P = 0.0527$) (Table 4).

Pup thymus relative mass and total numbers of cells, but not lymphocyte proportions, were reduced in response to maternal protein deficiency. In order to determine whether maternal PD and/or infection would impair neonatal immune development, we measured changes in the mass of thymus on d7, d14 and d21 PP, and/or total cells including the composition of lymphocyte subpopulations in thymus on d7 and d21 PP. Maternal PD reduced the relative thymus mass in pups from uninfected dams, but only on d14 PP ($P = 0.0005$) (Fig. 2A). Relative thymus mass on d2 PP was negatively correlated with corticosterone in pup milk on d2 PP ($r = -0.42$, n = 48, $P = 0.0028$). In addition, the correlation between relative thymus mass on d7 PP and pup serum leptin on d7 PP approached significance ($r = 0.26$, n = 48, $P = 0.0711$). Maternal PD also reduced the total number of cells in the thymus on d7 PP in pups from both uninfected ($P < 0.0001$) and infected PS dams ($P = 0.0018$) (Fig. 3A), but no diet effects were detected on d21 PP (Fig. 3B). Maternal infection had no impact on the relative mass (Fig. 2A) or total number of cells (Fig. 3A and 3B).

Consistent with ontogeny of immune cell populations over time, there were higher proportions for CD44+CD25- ($P = 0.0002$), CD4+ ($P = 0.023$) and CD8+ ($P = 0.0029$), but lower proportion for CD44-CD25- ($P = 0.0005$) and CD4:CD8 ratio ($P < 0.0001$) in the pup thymus on d21 PP compared to d7 PP (Fig. 4). Interestingly, maternal PD did not change the proportion of the various cell subpopulations or the CD4+:CD8+ ratio in the pup thymus on d7 or d21 PP (Fig. 4). Furthermore, maternal infection had no impact on cell populations in the pup thymus on d7 or d21 PP (Fig. 4A and 4B).

Pup spleen relative mass and total number of cells were reduced and T cell populations were elevated in response to maternal protein deficiency. We examined whether maternal PD and/or infection would impair neonatal immune development through changes in the mass of spleen on d7, d14 and d21 PP, and/or total cells including the composition of lymphocyte subpopulations in spleen on d7 and d21 PP. PD reduced relative spleen mass on d7, d14 and d21 PP in pups from both sham and infected dams ($P < 0.0001$ for all comparisons) (Fig. 2B), and reduced the total number of spleen cells on d21 PP, regardless of infection status of the dams (sham comparison: $P = 0.0004$; infected comparison: $P = 0.0001$) (Fig. 3D).

We also determined the total number of cells in the spleen of pups across time. The total number of spleen cells was lower in pups of infected PD compared with uninfected PD dams on d7 PP ($P < 0.0001$) (Fig. 3C), whereas relative spleen mass of the PS pups was elevated by maternal infection on d21 PP ($P = 0.0114$) (Fig. 2B). The total number of cells in the spleen were higher on d21 PP compared to d7 PP in pups from PS sham ($P = 0.0048$) and PS infected dams ($P < 0.0001$) (Fig. 3C vs 3D).

Cell populations in the pup spleen differed between d7 and d21 PP. Compared with d7 PP, there were higher proportions for CD3+ ($P < 0.0001$), CD4+ ($P < 0.0001$) and CD8+ ($P < 0.0001$) T cells, B cells ($P < 0.0001$) and

T:B cell ratio (P = 0.0104), but lower proportion for CD4:CD8 ratio (P = 0.0007) on d21 PP (Fig. 5). Whereas the proportion of CD3+ T cells and the proportion of B220+ (B cells) were higher on d21 PP compared to d7 PP in pups from PS sham (P = 0.0107; P = 0.0007 respectively), PD sham (P < 0.0001; P < 0.0001 respectively) and PD infected dams (P < 0.0001; P = 0.0062 respectively) (Fig. 5), the proportion of both cell populations remained unchanged in pups from PS infected mice. The proportion of CD4+ T cells was higher on d21 PP compared to d7 PP in pups from PD sham (P < 0.0001) and PD infected dams (P < 0.0001) (Fig. 5). Finally, there was a positive correlation between the ratio of CD4+:CD8+ T cells in the thymus and CD4+:CD8+ T cells in the spleen on d7 PP (r = 0.38, n = 48, P = 0.0084), but no significant correlation on d21 PP.

Maternal diet modestly affected the percentage of lymphocytes in the spleen of on d7 PP compared to d21 PP. On d7 PP, spleens of pups from PD sham dams had higher percentage of CD3+CD4+CD8+ double positive (DP) T cells (P = 0.0361) and T:B cell ratio (P = 0.0214) compared to pups from PS sham dams (Fig. 5). On d21 PP, spleens of pups from PD sham dams had higher percentage of CD3+ (Total T cells) (P = 0.0017), CD4+ (P = 0.0009) and CD8+ (P = 0.0333) T cells compared to spleens of pups from PS sham dams (Fig. 5). Similarly, spleens of pups from PD infected dams had higher percentage of CD3+ (P = 0.0007) and CD4+ (P = 0.0001), but not CD8+ T cells compared to spleens of pups from PS infected dams on d21 PP (Fig. 5). The spleen T:B cell ratio was higher in pups from PD sham (P = 0.0436) and PD infected (P = 0.0194) compared to PS sham and PS infected dams on d21 PP, respectively (Fig. 5). Neither maternal PD nor infection had an effect on CD4+:CD8+ ratio in the spleen on either d7 or d21 PP. Spleen B-cell proportion was positively correlated with pup serum IL-4 on d21 PP (r = 0.41, n = 48, P = 0.004).

DISCUSSION

Most of the previous research on neonatal immune development has focused on evaluation of response of offspring born to mothers that had been infected, either to parasite antigen or direct infection (30, 41, 67). The present study investigated for the first time the impact of the stress imposed by maternal PD and infection directly on the early development of the neonatal immune system. We have previously shown that PD in the presence of a GI nematode infection in pregnant dams impaired foetal (59) and neonatal linear growth (60). Therefore, here we explored whether these growth stunting effects would be evident in other developing tissues such as the neonatal immune system. Our results showed that PD during late pregnancy and lactation not only impaired maternal immune response to *H. bakeri* infection but also affected neonatal immune development in three important ways: (i) spleen and thymus mass were reduced; (ii) total cell populations were reduced and (iii) the proportions of several T cell populations were increased in spleens of pups from uninfected and infected dams that had been fed PD diets. Our results also showed that these developmental changes were linked to elevations in corticosterone, leptin and the pro-inflammatory cytokine IL-6 in mother's milk that, in turn, were collectively associated with lower leptin and IGF-1 in pup serum.

Protective immunity against GI nematodes, including the common murine nematode *H. bakeri*, is mediated by the Th2 cytokines IL-4 and IL-13, as well as IL-3, -5, -9 and -10 (22, 70). In our study, both PS and PD infected dams displayed the typical elevation in Th2 cytokines (IL-4 and IL-5), as well as eotaxin. Eotaxin induces chemotaxis in eosinophils (73), which also play an important role in resistance against GI nematodes (63). In addition, eotaxin's correlation with IL-5 was consistent with the role of IL-5 in the up-regulation of MHC class II molecules on eosinophils (29). Collectively these cytokines act to reduce the number of larvae surviving the tissue phase of development in

the wall of the small intestine, to arrest the development of larvae in the intestinal wall, and to reduce the survival of adult worms in the gut lumen (8).

A suppression of the Th2 immune response, either directly by PD or indirectly through elevation of corticosterone and leptin, was evident. The lower number of L_4 and higher percentage of adult worms recovered and delayed expulsion of adult worms in PD dams is consistent with these mechanisms and with previous research in PD mice (35). In addition, the more anteriorad location of adult *H. bakeri* in PD dams suggested that fewer worms were displaced from their preferred location in the proximal duodenum (6), indicating an inhibition in the expulsion process during PD. Interestingly, in support of this, we observed that PD lowered the IL-13 response in infected dams. IL-13 is important not only in the activation of the signal transducer and activator of transcription 6 (STAT6) pathway that is critical for the Th2 immune response (2), but also in the inflammation-induced hypercontractility of murine smooth muscle cells that facilitates worm expulsion (2, 70). The elevated EPG and worm burden, as well as the lower IL-13 in PD infected dams, may also be related to immunosuppressive effects of the elevated corticosterone (5) and the lower lymphoid tissue masses observed in this study. It has been suggested that enhanced secretion of corticosterone may explain increased per capita egg production during *H. bakeri* infection (37). In addition, corticosterone may reduce immunocompetence by inducing the up-regulation of leptin (52), which is known to impair functional immunity during *H. bakeri* infection (69).

The effects of PD and *H. bakeri* infection on the immune system were not only confined to the maternal system, but were also evident in pups despite the fact that pups were not subjected to direct diet or infection treatments. We had hypothesized that impaired neonatal immune development may be mediated both in utero and postnatally via milk, through factors such as glucocorticoids (GCs) (12) and pro-inflammatory cytokines such as IL-6 (57)

that are known to be elevated during PD (33) and chronic GI nematode infection (17, 19). Increased GCs cause thymic atrophy (15), induce programmed cell death (PCD) of immature thymocytes during intra-thymic T cell selection (4), and apoptosis of thymic T cells (26) and apoptosis in B cells (44). IL-6 is known to induce thymic atrophy at higher concentrations (27), whereas chronic oral administration of leptin to dams has been shown to downregulate endogenously produced leptin in neonates (64). Leptin plays an important role in immune development, and has been shown to prevent starvation-induced thymic atrophy in mice (53), through either increased thymopoiesis (32) or inhibition of apoptosis (49). Furthermore lower leptin concentration is linked to thymocyte depletion (65). In addition, both corticosterone and IL-6 can inhibit the growth hormone (GH)/IGF-1 axis (7, 47) which may result in lower mass of lymphoid organs. Therefore, we suggest that the reduction in mass of thymus and spleens of pups and the changes in total number of cells attributable to maternal PD and *H. bakeri* may be related to lower serum leptin and IGF-1 in pups induced by elevated transfer of corticosterone and pro-inflammatory cytokine IL-6 through milk.

Interestingly, maternal PD increased CD3+CD4+CD8+ T cells on d7 PP in the spleen in pups. Although CD4+CD8+ double positive (DP) T lymphocytes are unconventional and rarely described, they have been reported in peripheral blood in humans, rodents, swine, chicken and monkeys [reviewed in (76)]. Specifically, murine peripheral DP cells have been described as a subset of intraepithelial lymphocytes (IELs) (76) and are known to be involved in inflammatory processes such as inflammatory bowel disease (IBD) (54). Two observations may explain the elevated spleen CD4+CD8+ T cells in pups of PD dams. First, elevated DP T cells induced by maternal PD may be associated with PD-induced alterations in the migratory response of thymocytes, with abnormal export of immature thymocytes to the spleen, some of them having bypassed the normal selective selection process. However, it has been suggested that peripheral DP T cells in rodents represent mature

lymphocytes which likely have important effector functions such as cytokine secretion (76). Second, elevated DP T cells in spleen may be associated with increased inflammation in the pup attributable to maternal PD. PD is known to elevate systemic inflammation (45), and the elevated eotaxin in serum of pups from PD infected dams on d21 PP was consistent with this phenomenon.

Maternal PD also increased both CD4+ and CD8+ T cells on d21 PP in the spleen in pups, regardless of maternal infection status. These results are in accord with previous research that demonstrated increased spleen T cells and T:B cell ratio in adult BALB/c mice subjected to protein malnutrition (48). PD has been reported to increase the expression of the co-stimulatory signal CD28 on T helper cells in the spleen (48), important for the activation of naive T cells and for the prevention of anergy (14). Given the existing evidence for in utero sensitization to ascariasis (28), it is possible that elevated T cells in the spleen of pups from PD infected dams may reflect enhanced transplacental transfer of parasite antigens over and above that which may occur in response to infection of PS dams. However, we note that IL-2, an inducer of T cell proliferation (39), and Th2 cytokines (IL-4, IL-5 and IL-13) were not elevated in serum of pups from PD infected dams. Alternatively, the increase in T cells may be associated with the effects of elevated transfer of corticosterone via milk in response to PD during maternal infection, consistent with the elevated eotaxin in serum of pups from PD infected dams that may be an indication of elevated stress (36). Although the effects of corticosterone on immune development are generally inhibitory (26), elevated corticosterone transferred via milk in pups of PD infected dams may paradoxically be involved in rescuing immature thymocytes from programmed cell death (PCD) during intrathymic T cell selection (4). Consequently, the elevation of splenic total T cells, CD4+ and CD8+ T cells in maternal PD may be associated with enhanced developmental transition and eventual export to the spleen. However, if this 'corticosterone-associated rescue' was to hold, we would have expected to see some effect on differentiating T cells in the thymus (39). This

phenomenon needs further research. On the other hand, the positive correlation between IL-4 and proportion of splenic B cells at d21 PP was likely associated with the role of IL-4 in isotype switching (3).

The CD4+:CD8+ ratio is an important indicator for the immune status. However, it was not surprising that this ratio in the thymus and spleen of pups was unaffected by either maternal diet or infection, because studies in mice (74) and humans (61) evidenced that a low CD4+:CD8+ ratio in protein energy malnutrition (PEM) is confined to the blood, and is irrelevant to the thymus or spleen (42). Additional evidence also points to the stability of the CD4+:CD8+ ratio in the spleen in diverse weanling murine models of wasting PEM known to produce profound immunoincompetence (75). It is noteworthy that the ratio of CD4+:CD8+ T cells in the thymus and periphery do not always correlate (55). For instance, depletion of peripheral CD4+ T cells does not lead to increased production of CD4+ cells by the thymus (20), and other factors such as genes that act exclusively on mature T cells may also affect the ratio of CD4:CD8 T cells in the periphery (55). The fact that the thymus CD4+:CD8+ ratio did not correlate with spleen CD4+:CD8+ ratio at d21 PP (data not shown) is consistent with other findings and supports the suggestion that CD4+:CD8+ in the thymus segregates independently from this same phenotype in peripheral organs such as the spleen (55), at least as animals age (since it was correlated at d7 PP). Therefore, our results indicate that the ratios of CD4+:CD8+ in the organ in which most αβ T cells are generated, the thymus, may only predict ratios in the periphery during the early, but not necessarily during the late neonatal period.

Impaired neonatal lymphoid organ development in pups from PD infected dams may also be associated with low milk VEGF concentration. VEGF is a critical regulator of organ and body size in neonatal mice (23). VEGFR-1 and VEGFR-2 receptors are present in the neonatal intestinal luminal epithelium (71), where they signal the need for new capillary

angiogenesis and vascular permeability (21). Therefore the lower milk VEGF concentration in pups from PD infected dams may contribute to impaired development and morphology of the neonatal gut, resulting in reduced nutrient absorption and affecting neonatal growth, including development of the neonatal immune system. However, elevated corticosterone concentration in milk may ameliorate adverse effects induced by low milk VEGF. GCs are known to induce a range of structural and functional changes that activate digestive function postnatally in the gut such as increased enterocyte proliferation, decreased wall thickness, and induction of digestive enzyme activities (68).

In summary, maternal PD impaired immune function during trickle *H. bakeri* infection, impaired development of lymphoid organs and modified lymphocyte proportions in pups. Immune development in pups from PD and infected dams was also associated with reduced mass of lymphoid organs, probably driven by elevated transfer of maternal corticosterone and IL-6 in milk, and lower leptin concentration in serum which may have down-regulated IGF-1 and blunted lymphoid tissue growth. Our findings on the effects of maternal PD and infection on immune development are novel considering that significant maturation of immune organ systems occurs in late gestation for most species (51), and may thus be prone to programming stimuli during this period. The *H. bakeri* – pregnancy – lactation model may provide a useful animal system for studying the impact of PD and GI nematode infection during the neonatal period.

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TABLE 1. Effect of diet and *Heligmosomoides bakeri* infection on relative maternal thymus and spleen mass on day 20 post-partum

	PS Sham (n = 12)	PS Infected (n = 12)	PD Sham (n = 12)	PD Infected (n = 12)	SEM	Overall *P*-value[1]
Thymus	0.18[a]	0.18[a]	0.16[ab]	0.14[b]	0.02	0.0194
Spleen	0.47[a]	0.81[b]	0.31[c]	0.46[ad]	0.03	< 0.0001

[1] Different lowercase letters represent significant differences across treatment groups, $P < 0.05$

TABLE 2. Effect of diet and *Heligmosomoides bakeri* infection on concentration of hormones, cytokines and chemokines in maternal serum on day 20 post-partum

	PS Sham (n = 12)	PS Infected (n = 12)	PD Sham (n = 12)	PD Infected (n = 12)	Overall *P*-value[1]
Corticosterone‡ (*ng/ml*)	86.7 ± 27.9[ab]	54.4 ± 27.9[a]	110.3 ± 27.9[ab]	185.5 ± 27.9[b]	0.0084
Leptin (*pg/ml*)	453.4 ± 187.4[ab]	371.1 ± 187.4[a]	845.8 ± 195.1[b]	976.9 ± 187.4[b]	0.013
IL-1β‡ (*pg/ml*)	6.6 ± 3.8[ab]	17.1 ± 3.8[a]	1 ± 3.8[b]	9.9 ± 3.8[ab]	0.0337
IL-2‡ (*pg/ml*)	5.8 ± 4.2	10 ± 4.4	2.3 ± 4.2	17.7 ± 4.2	0.0876
IL-4‡ (*pg/ml*)	6.2 ± 3.7[a]	20.8 ± 3.7[b]	2.5 ± 3.7[a]	14.6 ± 3.7[b]	< 0.0001
IL-5‡ (*pg/ml*)	2.8 ± 3.7[a]	24.3 ± 3.7[b]	3.6 ± 3.7[a]	29.5 ± 3.7[b]	< 0.0001
IL-6‡ (*pg/ml*)	9.2 ± 7.9	24.3 ± 7.9	11.5 ± 7.9	37.5 ± 7.9	0.0247[2]
IL-10‡ (*pg/ml*)	8.7 ± 5.3[ab]	22.3 ± 5.3[a]	1.8 ± 5.3[b]	14.5 ± 5.3[ab]	0.0093
IL-13‡ (*pg/ml*)	189.1 ± 90.5[a]	391.2 ± 90.5[a]	87 ± 90.5[b]	298 ± 90.5[b]	0.0006

IFN-γ (*pg/ml*)	4.1 ± 5.1	14.2 ± 5.1	9.2 ± 5.1	14.7 ± 5.1	0.4066
TNF-α (*pg/ml*)	5.1 ± 2.4	5.9 ± 2.4	3.8 ± 2.4	6.8 ± 2.4	0.6659
MCP-1 (*pg/ml*)	19.7 ± 14.6	10.9 ± 14.6	37.4 ± 14.6	27.8 ± 14.6	0.619
Eotaxin‡ (*pg/ml*)	530.6 ± 198.9[a]	1176.2 ± 198.9[b]	500.6 ± 198.9[a]	1437.9 ± 198.9[b]	< 0.0001
VEGF (*pg/ml*)	3.9 ± 0.9	4.6 ± 0.9	2.7 ± 0.9	4.9 ± 0.9	0.1774

[1] Different lowercase letters represent significant differences across treatment groups

[2] No significant multiple comparison effects detected

‡ Data were log transformed [$\log_{10}$ (y + 10)] to achieve normality.

TABLE 3. Effect of maternal diet and *Heligmosomoides bakeri* infection on concentration of hormones, cytokines and chemokines in pup milk

	PS Sham (n = 12)	PS Infected (n = 12)	PD sham (n = 12)	PD Infected (n = 12)	Overall *P*-value[1]
Pup Milk, d2 PP					
Corticosterone (*ng/ml*)	6.5 ± 2.3[a]	6.5 ± 2.3[a]	7.6 ± 2.3[a]	24 ± 2.3[b]	< 0.0001
Pup Milk, d7 PP					
Leptin (*pg/ml*)	206.2 ± 113.9[ab]	2.8 ± 123[a]	672.1 ± 132.7[b]	628.1 ± 142.7[b]	0.0013
Pup Milk, d14 PP					
IL-1β (*pg/ml*)	1.2 ± 3.2	0.3 ± 3.4	1.9 ± 3.6	4.2 ± 3.9	0.896
IL-4 (*pg/ml*)	3.3 ± 2.2	4.3 ± 2.2	4.8 ± 2.2	5.7 ± 2.2	0.2454
IL-6‡ (*pg/ml*)	2 ± 1.6[a]	1.5 ± 1.6[a]	2.5 ± 1.6[ab]	6.5 ± 1.6[b]	0.0173

Eotaxin (*pg/ml*)	96.6 ± 41.1	129.5 ± 41.1	164.7 ± 41.1	188.5 ± 41.1	0.4149
VEGF (*ng/ml*)	17.7 ± 3.6[ab]	24.4 ± 3.6[a]	6.5 ± 3.6[b]	8.8 ± 3.6[b]	0.0029

[1] Different lowercase letters represent significant differences across treatment groups

‡ Data were log transformed [$\log_{10}$ (y + 10)] to achieve normality.

We did not detect IFN-γ, IL-2, IL-5, IL-7, IL-10, IL-12p70, IL-13, TNF-α or MCP-1 in pup milk on d14 PP.

TABLE 4. Effect of maternal diet and *Heligmosomoides bakeri* infection on concentration of hormones, cytokines and chemokines in pup serum

	PS Sham (n = 12)	PS Infected (n = 12)	PD sham (n = 12)	PD Infected (n = 12)	Overall *P*-value[1]
Pup serum, d7 PP					
IGF-1, (n*g/ml*)	55 ± 8[a]	33.7 ± 8[b]	13.2 ± 8[c]	8.3 ± 8[c]	< 0.0001
Pup serum, d14 PP					
Leptin (*pg/ml*)	2216.4 ± 285.5[a]	2194.6 ± 310.3[a]	1897.6 ± 336.3[a]	556.7 ± 363.3[b]	0.0009
Pup serum, d21 PP					
Corticosterone (*ng/ml*)	41.1 ± 16.6	61.1 ± 16.6	36.4 ± 16.6	14.7 ± 16.6	0.2813
IL-1β‡ (*pg/ml*)	14.1 ± 10.4	8.6 ± 11	24.2 ± 11.6	8.4 ± 12.3	0.5939
IL-4‡ (*pg/ml*)	2.8 ± 0.9	2.6 ± 0.9	2 ± 0.9	1.8 ± 0.9	0.0344[2]
IL-5 (*pg/ml*)	1.8 ± 0.9	1.3 ± 0.9	0.5 ± 0.9	1.2 ± 0.9	0.6219
IL-6 (*pg/ml*)	2.8 ± 1	1.8 ± 1	2.3 ± 1	2.4 ± 1	0.6824

IL-13 (*pg/ml*)	282.7 ± 70.5	239.9 ± 72	298.3 ± 73.6	275.6 ± 75.3	0.8471
Eotaxin (*pg/ml*)	153.7 ± 35[a]	200.1 ± 35.8[a]	233.7 ± 36.6[ab]	315.9 ± 37.5[b]	0.0051
VEGF (*pg/ml*)	0.5 ± 0.5	0.4 ± 0.5	2.1 ± 0.6	0.3 ± 0.6	0.0878

[1] Different lowercase letters represent significant differences across treatment groups

[2] No significant multiple comparison effects detected

‡ Data were log transformed [$\log_{10}$ (y + 10)] to achieve normality.

We did not detect IFN-γ, IL-2, IL-7, IL-10, IL-12p70, TNF-α or MCP-1 in pup serum on d21 PP.

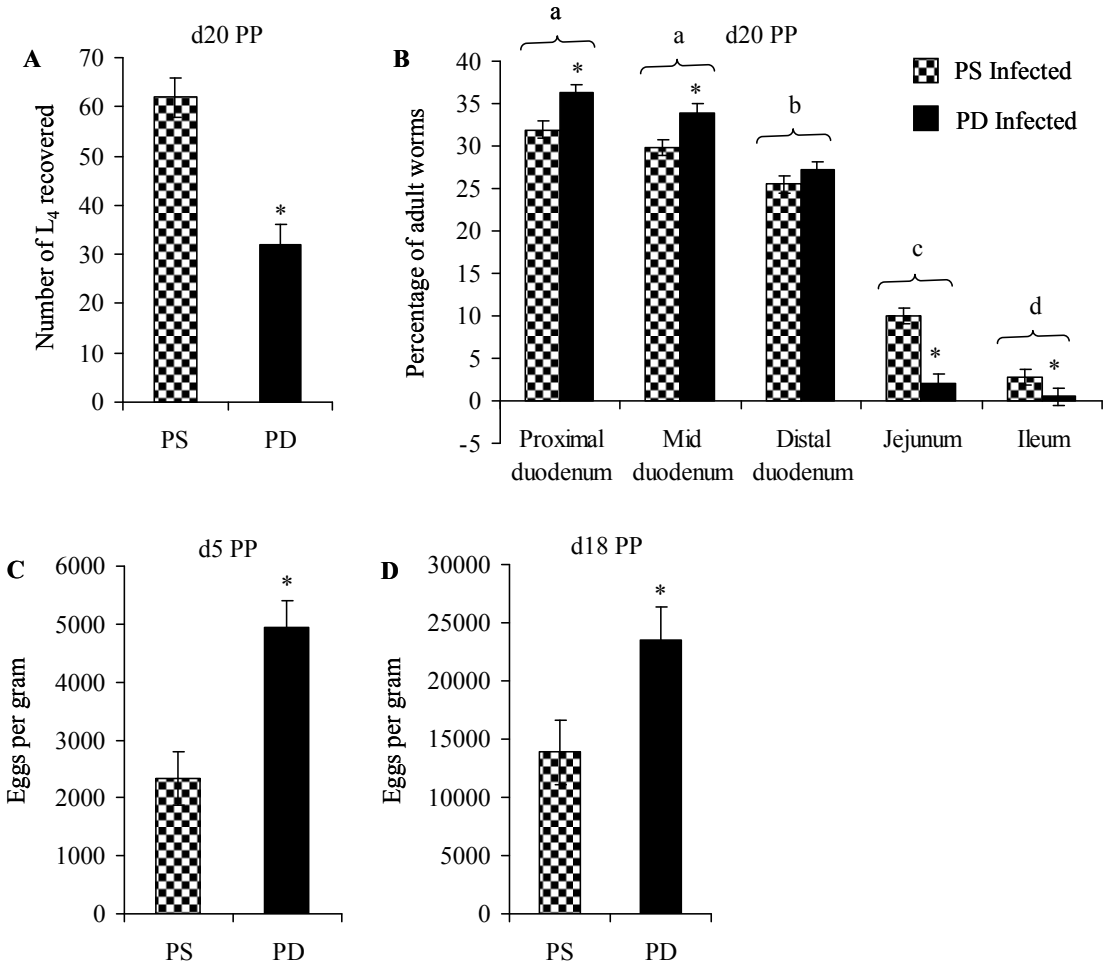

FIG. 1. (A) Effect of diet on *Heligmosomoides bakeri* larvae (L_4) recovered on day 20 post-partum (PP) ($P = 0.0001$). (B) Effect of diet on percentage distribution of *Heligmosomoides bakeri* along the small intestine on day 20 PP (diet x location: $P < 0.0001$). Effect of diet on *Heligmosomoides bakeri* eggs per gram (EPG) on day 5 PP (C) ($P < 0.0001$); and on day 18 PP (B) ($P < 0.0001$). Difference between PS and PD infected dams is represented by *; different lowercase letters represent significant differences across regions, and difference between PS and PD infected dams within a region is represented by *, ($P < 0.05$).

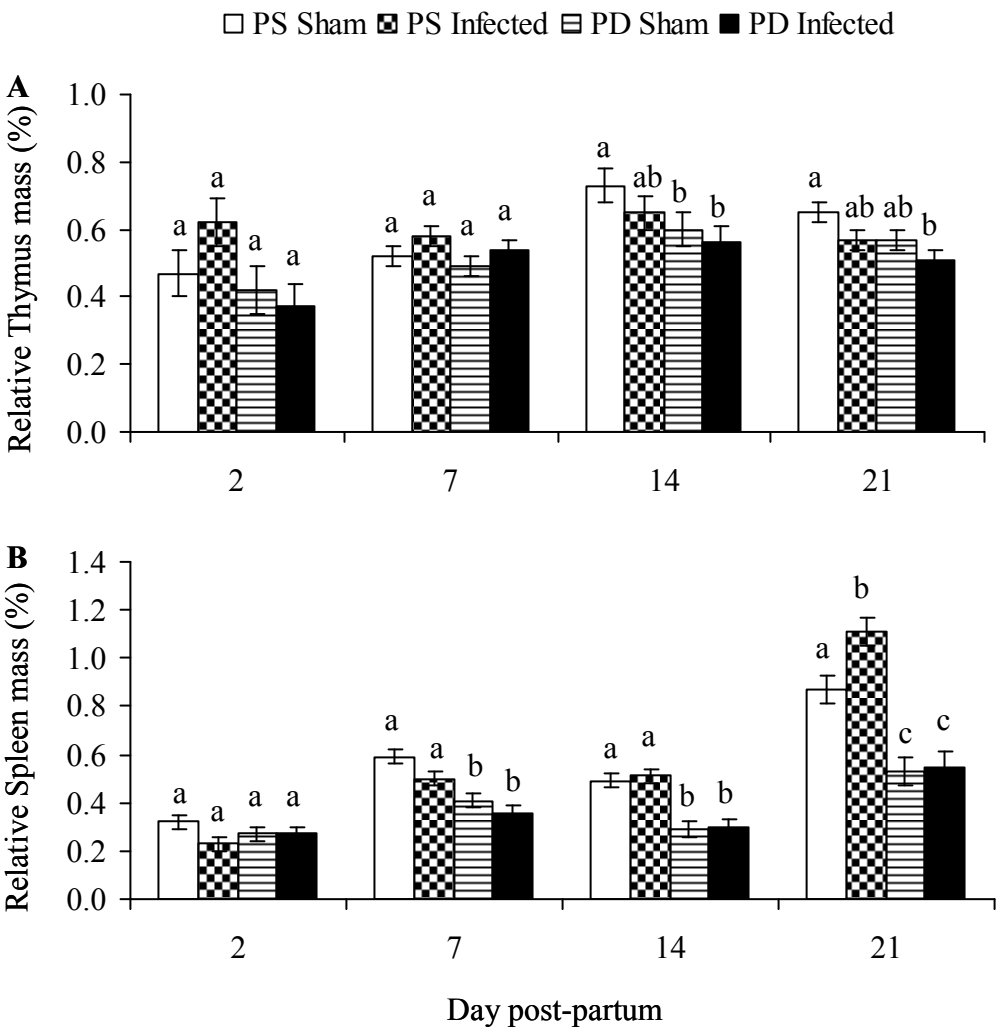

FIG. 2. Effect of maternal diet and maternal *Heligmosomoides bakeri* infection on (A) relative thymus mass (as a percentage of body mass) and (B) spleen mass (as a percentage of body mass) on days 2, 7, 14 and 21 post-partum (PP). Difference between treatment groups within a time-point is represented by different lowercase letters ($P < 0.05$).

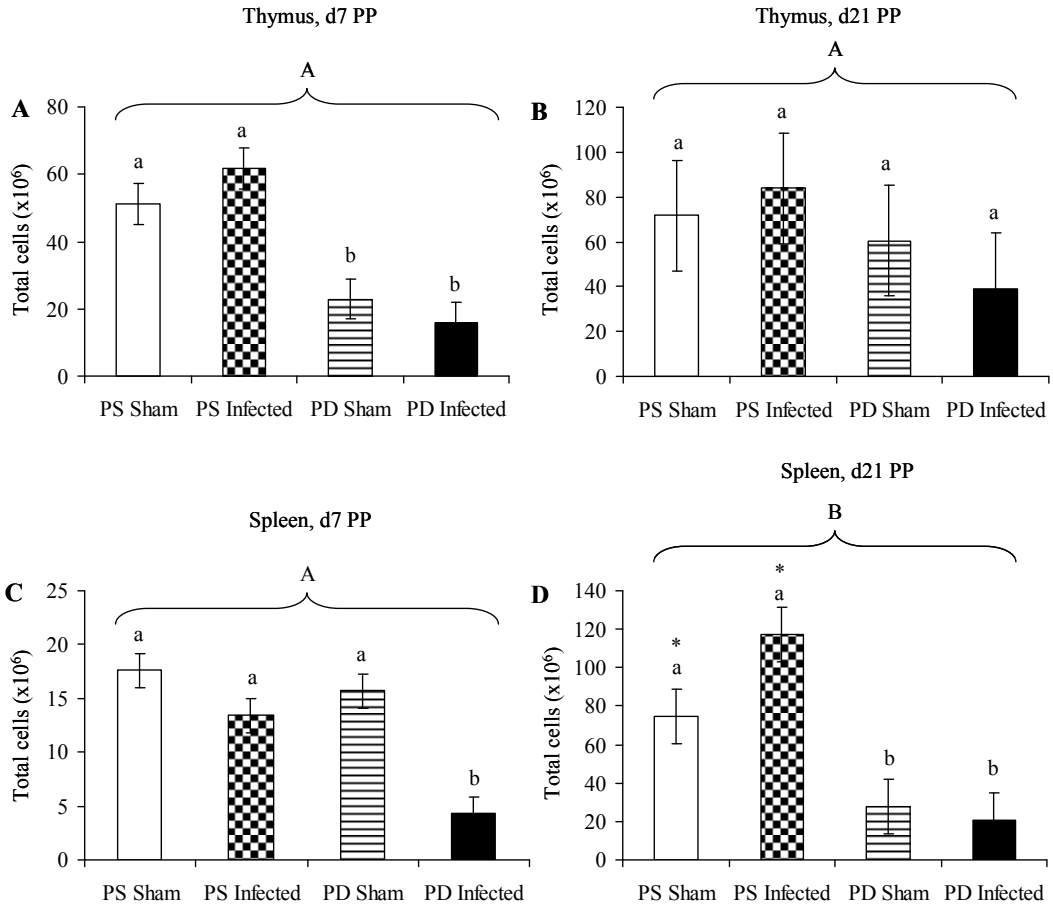

FIG. 3. Effect of maternal diet and maternal *Heligmosomoides bakeri* infection on total cells ($x10^6$) in the thymus (A, B) and spleen (C, D) on days 7 and 21 post-partum (PP). Difference between treatment groups is represented by different lowercase letters; different uppercase letters represent significant effect of time between day 7 and 21 PP; difference between day 7 and 21 PP within a treatment group in each tissue is represented by * ($P < 0.05$).

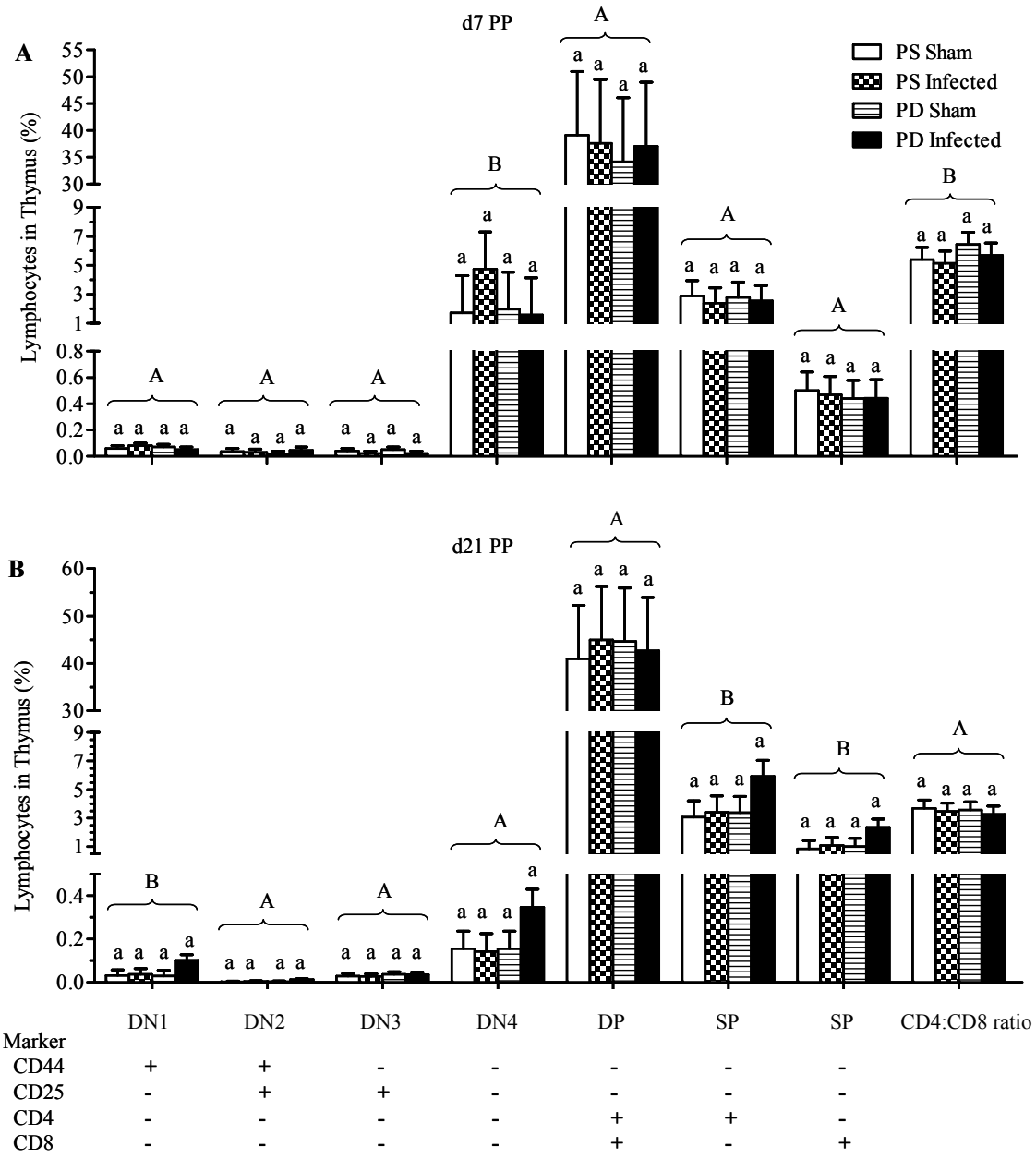

FIG. 4. Effect of maternal diet and *Heligmosomoides bakeri* infection on percentage proportions of T cells in the thymus (A) on day 7 and (B) day 21 post-partum (PP). DN = Double negative; DP = Double positive; SP = Single positive. Difference in cell proportions between treatment groups is represented by different lowercase letters; different uppercase letters represent significant effect of time between day 7 and 21 PP ($P < 0.05$).

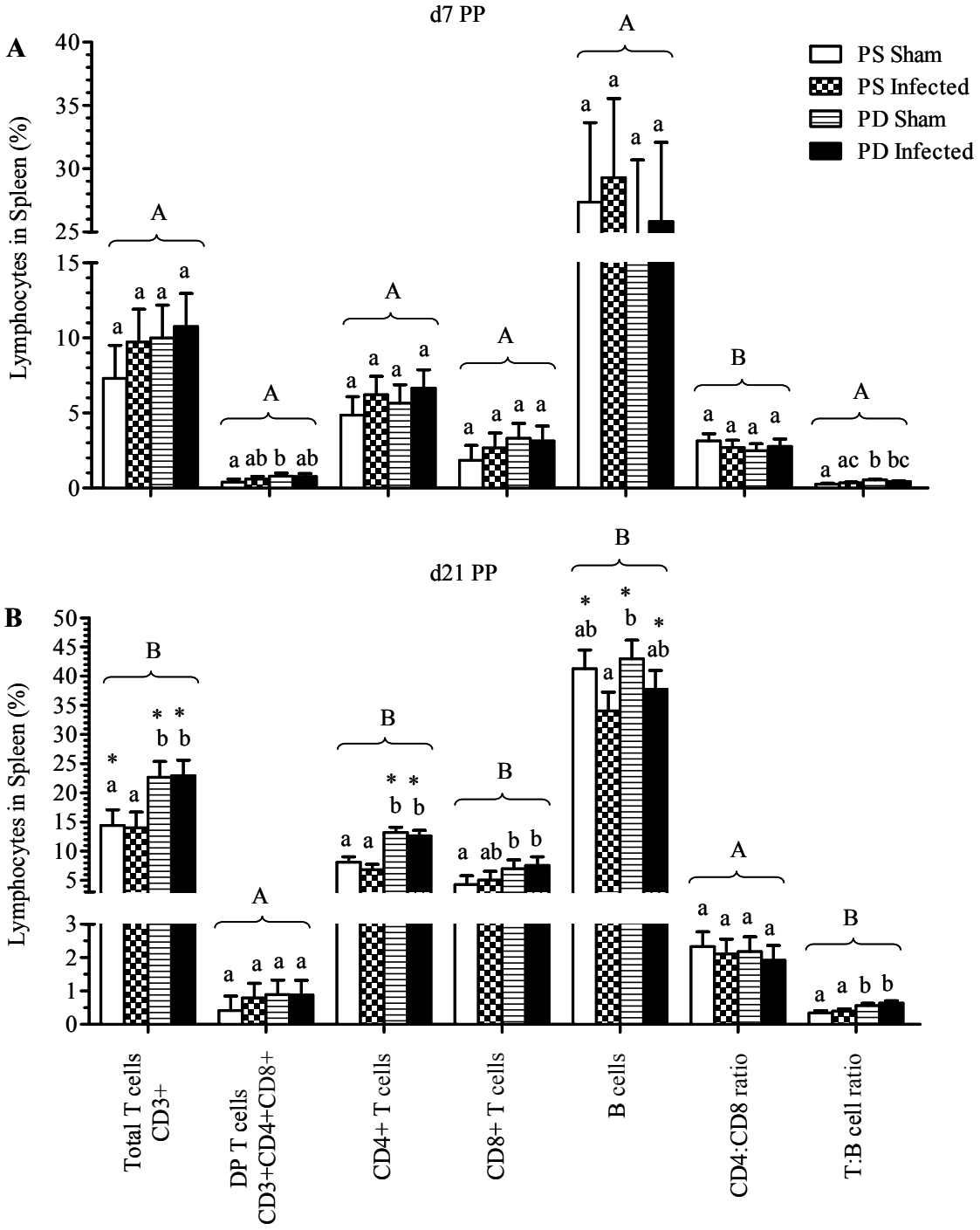

FIG. 5. Effect of maternal diet and *Heligmosomoides bakeri* infection on percentage proportions of T and B cells in the spleen (A) on day 7 and (B) day 21 post-partum (PP). DN = Double negative; DP = Double

positive; SP = Single positive. Difference in cell proportions between treatment groups is represented by different lowercase letters; difference between day 7 and 21 PP within a treatment group is represented by *; different uppercase letters represent significant effect of time between day 7 and 21 PP ($P < 0.05$).

CHAPTER VI

GENERAL DISCUSSION

The research presented in this thesis demonstrated that concurrent nematode infection and pregnancy induce physiological responses involving changes in pro-inflammatory cytokines IFN-γ and IL-β that reduce bone area and impair linear growth in the murine dam and foetus, respectively. Moreover, superimposition of PD on nematode infection during pregnancy and lactation resulted in reduced maternal bone mineralization and neonatal linear growth, with PD further reducing RMR. These responses were associated with changes in corticosterone, leptin, and pro-inflammatory cytokines (IL-1β and IL-6) in serum and milk. Furthermore, PD impaired immune function against *H. bakeri* infection which appeared to be associated with elevated corticosterone and leptin and lower IL-13. Combined maternal PD and *H. bakeri* infection elevated corticosterone and IL-6 in milk, but reduced pup serum leptin and IGF-1, and together these were associated not only with impaired lymphoid tissue development, but also elevated T cell proportions in the spleens of pups.

One of the primary interests for the research in this thesis was to examine the phenotypic plasticity as a means through which animals cope with different stresses within their environments. We hypothesized that pregnancy and infection would elevate RMR (Chapter III), whereas PD would reduce RMR (Chapter IV) in dams. As expected, pregnancy elevated RMR and PD reduced RMR. The decrease in RMR during PD was accompanied by a reduction in body temperature. Surprisingly trickle *H. bakeri* infection had no effect on RMR, both in the studies in Chapter III and IV. Instead infected dams lowered their body temperature in Chapter III, but this decrease in body temperature was not observed in Chapter IV. *H. bakeri* primary infection in the absence of pregnancy has been reported to elevate RMR (Kristan and Hammond 2000; Kristan and Hammond 2001), but not always (Kristan 2002;

Kristan and Hammond 2004; Kristan and Hammond 2006). Considering that up-regulation of immune responses is energetically demanding (Buttgereit *et al.* 2000), infection was expected to increase RMR. More so, we presumed that a trickle infection with a parasite should elicit a stronger immune response that would presumably be costly. It appears that a reduction in core body temperature was important in mitigating increased energy demands associated with infection in Chapter III. We can speculate that in Chapter IV, the effect of infection on RMR was modest and was likely absorbed within the "safety margins", thereby not invoking a reduction in body temperature as an energy saving mechanism. Morphological compensatory mechanisms such as changes in organs involved in immune responses may represent part of the "safety margin" and may have ameliorated any significant change in RMR associated with *H. bakeri* infection. Alternatively, the absence of an effect of trickle *H. bakeri* infection on RMR may support an earlier suggestion that host energy budgets are complex, and simple effects such as stimulation and maintenance of a costly immune response and pathology may not easily explain the impact of parasites on host energy budget (Scantlebury *et al.* 2007). On the other hand, studies on the effect of PD on RMR have been equivocal (Hartsook *et al.* 1973; Gurr *et al.* 1980). We expected that RMR would reduce during PD likely associated with loss in body mass attributable to either reduced food intake (Pine *et al.* 1994) or the corticosterone-mediated catabolic response (Hall-Angeras *et al.* 1990) that occur during PD. Body mass is a strong predictor of RMR (Odiere *et al.* 2010), and its reduction, accompanied by lower body temperature during PD was therefore consistent with the lower RMR in our study. A reduction is body temperature can reduce RMR (McNab 1970; Kristan and Hammond 2006), but in addition, the reduction in body temperature during PD may also have served to mitigate inflammation (Deng *et al.* 2003). Changes in RMR, body temperature and body mass during pregnancy and PD highlighted the importance of phenotypic plasticity in function and morphology.

The limitation of our RMR studies was the fact that RMR was determined at room temperature (22–25^{0}C) instead of the thermoneutral temperature for mice (30 ± 1^{0}C) (Hart 1971), which may overestimate RMR due to adaptive thermogenesis. We note however that in standard laboratory environments mice are housed at 20-24 ^{0}C (Gordon 1993) and maintenance temperature for CD1 mice at Charles River is 21 ± 1 ^{0}C (http://www.criver.com/SiteCollectionDocuments/CD1-MICE.pdf). We were concerned that temperatures higher than 25^{0}C may have affected the temperature-sensitive implantation process which occurs at night (2200-2300 h) of day 4 (Das *et al.* 1994) in mice (for the study in Chapter III), or affected the rapid foetal development that occurs during the late pregnancy (Renfree *et al.* 1975) (for the study in Chapter IV). As these effects on RMR emerged, we were convinced that phenotypic plasticity and energy repartitioning may also occur in other compartments such as bone tissue or immune system. Recently, there has been increased interest in the role that bone plays in endocrine energy homeostasis (Wolf 2008). Moreover, physiological changes associated with pregnancy, infection and PD were envisaged to influence bone metabolism both in the maternal, foetal and neonatal compartments.

We hypothesized that a shift in cytokine profiles in infected pregnant mice towards Th1 would modify maternal bone remodeling and impair foetal linear skeletal growth (Chapter III). We observed higher femur bone area and mineralization among pregnant mice, while infection lowered maternal femur bone area which was associated with elevated IFN-γ in maternal serum of heavily infected pregnant mice. In addition, infection reduced foetal crown-rump length which was associated with higher amniotic fluid IL-1β. We further hypothesized that maternal PD and infection would reduce bone mineralization and impair neonatal linear skeletal growth (Chapter IV). We showed that both PD and infection lowered maternal bone mineralization, but through different mechanisms. Lower mineralization during PD was associated with elevated corticosterone and leptin, whereas it was consistent with elevated

serum IL-1β and IL-6 during infection. The elevated serum IL-1β, lower IGF-1 and leptin in pups of PD and infected dams were consistent with the shorter crown-rump length. Contrary to common misperception that bone is a static and compact tissue, bone tissue undergoes constant remodeling to repair micro-damaged sections and maintain skeletal integrity (Raisz 1999). This process is regulated by a suite of hormones, cytokines and chemokines (Nguyen *et al.* 1991; Defranco *et al.* 1992; Pogoda *et al.* 2006). Given that approximately one third of bone mass is protein, and essential amino acids are necessary for new bone matrix synthesis (Heaney 1998), we expected that PD would directly reduce bone mineralization. Not only did our findings show this, but they also showed that lower bone mineralization was associated with up-regulated corticosterone and leptin that are known to decrease bone mineralization and inhibit bone formation, respectively (Karabelyos *et al.* 1998; Pogoda *et al.* 2006). On the other hand, infection also reduced bone mineralization, but through pathways involving elevated pro-inflammatory cytokines (IL-1β and IL-6) that induce bone resorption (Nguyen *et al.* 1991; Manolagas 1998). This impact of GI nematode infection on bone is intriguing, especially since few studies have looked at this phenomenon, despite the known stunting effects associated with chronic GI nematode infections in children (Payne *et al.* 2007). This indicates that up-regulation of the immune system during infection may be associated with changes in bone mineralization.

Although immunity against GI nematode infections is Th2-mediated (Maizels and Yazdanbakhsh 2003), susceptibility to chronic infection is propagated by Th1 pro-inflammatory cytokines (Hayes *et al.* 2004) that may also act directly on markers of bone resorption. For instance, IL-1β and IL-6 stimulate osteoclastogenesis by increasing the expression of RANKL while decreasing OPG expression (Nakashima *et al.* 2000). In contrast, cytokines inhibiting osteoclastogenesis, such as IL-13 and transforming growth factor-β1 suppress the expression of RANKL but enhance expression of its inhibitor OPG (Nakashima *et al.* 2000). RANKL is an important co-stimulation

molecule involved both in T cell-dendritic cell communication and in dendritic cell survival (Anderson *et al.* 1997; Bachmann *et al.* 1999). Given that RANKL is also produced by activated T cells (Yeung 2004), it is therefore plausible that activated T cells during GI nematode infections may be an additional source for RANKL. Indeed, it has been suggested that T cells may provide the link between inflammation and bone loss (Yeung 2004). Furthermore, leptin which inhibits bone formation (Pogoda *et al.* 2006) is known to be elevated during inflammation (Barbier *et al.* 1998), and to promote T cell activity *in vitro*, shifting the immune response towards a predominant Th1 (Lord *et al.* 1998). Our results on bone markers in Chapter IV add to the body of evidence that shows that these markers behave differently from the classical pathway during pathological conditions (Yano *et al.* 1999; Masi *et al.* 2004). It is known that during pathological conditions, molecules produced by activated cells (e.g. infiltrating leukcocytes) shift the balance between osteoblastic and osteoclastic activities (Bolon *et al.* 2002). We have also shown that leptin has differential effects on bone metabolism in young animals compared to adults. Therefore, our findings suggest that the up-regulation of immune responses also influenced bone metabolism.

We further explored whether shifts in cytokine profiles in infected pregnant mice would promote nematode survival (Chapter III), and whether concurrent maternal PD and infection during late pregnancy and lactation would impair the maternal immune response and development of neonate immune system (Chapter V). In Chapter III, we hypothesized that combined pregnancy and *H. bakeri* infection would elevate pro-inflammatory cytokines beyond the level required for parturition (Romero *et al.* 1989; Orsi *et al.* 2006), leading to a shift towards Th1 immunity that would result in elevated worm burden. Consistent with this, we observed elevated IFN-γ in maternal serum of heavily infected pregnant mice and lower IL-4, IL-5 and IL-13 that resulted in higher worm burden. In Chapter V, we hypothesized that PD during infection would impair Th2 immune function, leading to elevated worm burden. We

observed lower IL-13, increased faecal egg output and worm burden in PD infected dams, which was associated with elevated corticosterone and leptin. We further hypothesized that maternal PD and infection would impair neonatal immune development through the altered profile of maternal hormones and pro-inflammatory cytokines that are transferred from the stressed dam to the pup via milk. Both maternal infection and PD reduced lymphoid organ mass in pups, but the percentage of T cells and T:B cell ratio in the spleen was increased only by maternal PD. These changes were associated with elevated corticosterone and IL-6 concentration in milk, and lower pup serum leptin and IGF-1 in pups from PD infected dams.

A substantial body of evidence shows that a temporal loss of acquired immunity to nematode parasite infection occurs around parturition and during lactation, which results in a marked increase in faecal egg output and worm burden (Barger 1993; Houdijk *et al.* 2003; Normanton *et al.* 2007). However, few studies have looked at the cytokine profiles in order to corroborate this breakdown of acquired immunity, though several workers have more or less implicitly assumed this when interpreting observed cases of breakdown of immunity. Our research provides evidence that the elevated worm burden in pregnant mice was indeed accompanied by a decrease in Th2 cytokines (Chapter III). The observed elevation of IFN-γ may reduce worm expulsion by suppressing Th2 responses (Urban *et al.* 1993), and down-regulating intestinal epithelial cell turnover (Cliffe *et al.* 2005). Presumably, this breakdown of immunity associated with a shift towards Th1 may have served to reallocate energy resources to the developing foetus. In Chapter V, the elevated EPG, worm burden and lower IL-13 in PD infected dams were likely related to immunosuppressive effects of corticosterone (Auphan *et al.* 1995), that is elevated during PD (Hill *et al.* 1995).

The impact of prenatal stress on immune system in the offspring is complex and varies depending on the stage of pregnancy at which the animal is stressed, the sex of the offspring, the species, and, indeed, what immune compartment and response one considers (Shanks and Lightman 2001). The

thymus is a central component of the immune system responsible for production and education of new T cells throughout life (Haynes *et al.* 2000). Thymopoiesis is highly susceptible to damage by physiologic stressors (Hick *et al.* 2006) and this can contribute to modifications in lymphocyte proportions. Effects of elevated maternal GCs on foetal and neonatal immune development are largely inhibitory. For instance, prenatal exposure to betamethasone used to accelerate foetal lung maturation was associated with mild decreases of subpopulations of T-cells in premature infants (Lazzarin *et al.* 1986). In addition, a long term decrease in the number of T lymphocytes was reported in a small number of premature infants who received high-dose hydrocortisone soon after birth (Gunn *et al.* 1981). We were able to detect modifications in cell proportions in the spleen of pups, but not the thymus. These effects were likely mediated by elevated corticosterone and IL-6 in milk, and lower pup serum leptin. However, instead of a decrease in T cell proportions, we observed an elevation of T cells in the spleen of pups from PD dams, consistent with previous research (Manhart *et al.* 2000). We suggested that this elevation of T cells may be associated with enhanced proliferation of these cells in the spleen, an important site not only for T-cell activation (Teixeira-Carvalho *et al.* 2002) but also proliferation (Tischendorf 1985). Given the existing evidence for in utero sensitization to ascariasis (Guadalupe *et al.* 2009), it is possible that elevated T cells in the spleen of pups from PD infected dams may reflect enhanced transplacental transfer of parasite antigens.

Leptin appears to be an important mediator for early immune development. Primary lymphoid tissues, such as the thymus, contain deposits of adipose tissue capable of producing leptin (Pond 2000; Sempowski *et al.* 2000). Leptin has been postulated to provide a survival signal to developing CD4+CD8+ DP thymocytes (Howard *et al.* 1999), and leptin-deficient *ob/ob* mice exhibit chronic thymic atrophy and decreased numbers of total thymocytes and splenocytes (Howard *et al.* 1999). Leptin is not only an important mediator for immune development (Hick *et al.* 2006; Mansour *et al.* 2006), but also enhances immunological recovery following re-feeding of

malnourished infants (Palacio *et al.* 2002). The lower serum leptin concentration in pups from PD and infected dams in our study was consistent with previous study where plasma leptin levels were lower in 21-day old pups from undernourished than from well-nourished mice (Chisari *et al.* 2001). Moreover, maternal PD appears to modulate the makeup of the pool of maternal cytokines passing through the mammary glands, resulting in elevated IL-6 in milk. The exact mechanism through which different cytokines are filtered across the mammary glands still needs to be elucidated.

We take cognisance of the fact that in Chapters IV and V, data from the same experiment was analysed using two different statistical methods. We used a 2 X 2 Factorial in the study reported in Chapter IV whereas a one-way ANOVA was used for the study in Chapter V. In Chapter IV, we were more interested in the effects of diet and infection separately (main effects) and together (their interaction effect). In Chapter V, because of the important immunological responses associated with infection, we felt it more appropriate not to focus on main effects that would have for instance lumped uninfected mice together with infected mice, but rather to treat the four different treatment groups independently. We were more interested in looking at the pup as an experimental unit, and making comparisons among PS sham, PS infected, PD sham and PD infected dams. Because of this different approach in analysis of data from the same experiment, we had more statistical power with the 2 X 2 Factorial than with the one-way ANOVA. Although the 2 X 2 Factorial has more inherent advantages, we feel confident that our approach was justified.

Findings from our studies have several implications. First, impaired functional immunity against nematode infection during PD adds to the body of evidence supporting the immunonutrition strategy in the control parasitic infections (Koski and Scott 2001; Kyriazakis and Houdijk 2006). This is especially important given the rapidly developing drug resistance to almost every new chemotherapeutic agent (Jackson and Coop 2000; Gasbarre *et al.* 2009). Second, our studies are in accord with the "Barker hypothesis" in

showing that adverse intra-uterine and immediate post-natal environments are strong programming stimuli associated with adverse health disorders in adult life (Barker 1998). Although poor growth in utero and early post-partum may be compensated by catch-up growth later (Forsen *et al.* 2000; Hales and Barker 2001), catch-up growth is also associated with reduced quality and quantity of life (Hales *et al.* 1996; Ozanne *et al.* 2004). In a broader context, effects such as stunting are associated with poor cognitive function in late childhood (Mendez and Adair 1999), short stature is associated with an increased risk of cardiovascular and respiratory mortality (Davey-Smith *et al.* 2000; Martyn *et al.* 1996) and birth complications (Kappel *et al.* 1987). Third, our data suggest that leptin administration may be an important therapeutic strategy towards promoting thymus replenishment in order to ameliorate impaired immune development associated with protein malnutrition in neonates. Fourth, we succeeded in creating a mouse model to study bone remodeling and stunting during concurrent infection and reproduction.

Following our research, future studies in the context of protein deficiency and/or nematode infection can further explore the cellular mechanisms through which pro-inflammatory cytokines, leptin and corticosterone modulate bone metabolism and growth either in concert or independent of IGF-1 at the tissue level (instead of systemic indicators), using histology and immunohistochemistry. Second, it is important to corroborate DXA data with markers of bone metabolism such as OPG and RANKL. In this regard, the method of quantitation for RANKL and the specimen used need to be considered. It has been suggested that there may be some functional differences between membrane-bound RANKL (as measured by flow cytometry on single cell suspensions) and soluble RANKL (measured using ELISA assays on serum) which may lead to conflicting data (Yeung 2004). Future research may benefit by using reverse transcription polymerase chain reaction (RT-PCR) to quantitate messenger RNA production that will measure message precursors for both soluble and membrane-bound variants, e.g. from

peripheral blood mononuclear cells (PBMCs) or from bone marrow mononuclear cells (BM MNCs). Future research may also benefit by looking at additional growth factors such as bone morphogenetic proteins (BMPs) that have an important role during embryonic development on the embryonic patterning and early skeletal formation.

In conclusion, we investigated the effect of maternal GI nematode infection during pregnancy and PD in the maternal, foetal and neonatal compartments. We showed that pro-inflammatory cytokines, corticosterone and leptin up-regulated during nematode infection and PD modify bone metabolism, and impair neonatal linear growth and immune development. Of note was the fact that both the foetal and neonatal systems were impacted even though they were not exposed to direct *H. bakeri* infection or PD diet treatments. We succeeded in creating an animal model that we believe provides a novel framework to study the impact of diet and nematode infection on growth and development.

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