THE INTERACTION OF DIETARY PROTEIN AND ZINC DEFICIENCIES WITH *HELIGMOSOMOIDES POLYGYRUS* INFECTION IN MICE

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September, 1994

A Thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the
degree of Masters of Science

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Sociology of	0340
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Asian	0305
Canadian (English)	0352
Canadian (French)	0355
English	0593
Germanic	0311
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Romance	0313
Slavic and East European	0314

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Philosophy	0422
Religion	
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Gerontology	0351
History	
General	0578
Ancient	0579
Medieval	0581
Modern	0582
Black	0328
African	0331
Asia, Australia and Oceania	0332
Canadian	0334
European	0335
Latin American	0336
Middle Eastern	0333
United States	0337
History of Science	0585
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General	0615
International Law and Relations	0616
Public Administration	0617
Recreation	0814
Social Work	0452
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Theory and Methods	0344
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Urban and Regional Planning	0999
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General	0473
Agronomy	0285
Animal Culture and Nutrition	0475
Animal Pathology	0476
Food Science and Technology	0359
Forestry and Wildlife	0478
Plant Culture	0479
Plant Pathology	0480
Plant Physiology	0817
Range Management	0777
Wood Technology	0746
Biology	
General	0306
Anatomy	0287
Biostatistics	0308
Botany	0309
Cell	0379
Ecology	0329
Entomology	0353
Genetics	0369
Limnology	0793
Microbiology	0410
Molecular	0307
Neuroscience	0317
Oceanography	0416
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Radiation	0821
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Zoology	0472
Biophysics	
General	0786
Medical	0760

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Paleontology	0418
Paleozoology	0985
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Physical Geography	0368
Physical Oceanography	0415

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Health Sciences	
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Chemotherapy	0992
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Hospital Management	0769
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Immunology	0982
Medicine and Surgery	0564
Mental Health	0347
Nursing	0569
Nutrition	0570
Obstetrics and Gynecology	0380
Occupational Health and Therapy	0354
Ophthalmology	0381
Pathology	0571
Pharmacology	0419
Pharmacy	0572
Physical Therapy	0382
Public Health	0573
Radiology	0574
Recreation	0575
Speech Pathology	0460
Toxicology	0383
Home Economics	0386

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Biochemistry	0487
Inorganic	0488
Nuclear	0738
Organic	0490
Pharmaceutical	0491
Physical	0494
Polymer	0495
Radiation	0754
Mathematics	0405
Physics	
General	0605
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Astronomy and Astrophysics	0606
Atmospheric Science	0608
Atomic	0748
Electronics and Electricity	0607
Elementary Particles and High Energy	0798
Fluid and Plasma	0759
Molecular	0609
Nuclear	0610
Optics	0752
Radiation	0756
Solid State	0611
Statistics	0463

Applied Sciences

Applied Mechanics	0346
Computer Science	0984
Engineering	
General	0537
Aerospace	0538
Agricultural	0539
Automotive	0540
Biomedical	0541
Chemical	0542
Civil	0543
Electronics and Electrical	0544
Heat and Thermodynamics	0348
Hydraulic	0545
Industrial	0546
Marine	0547
Materials Science	0794
Mechanical	0548
Metallurgy	0743
Mining	0551
Nuclear	0552
Packaging	0549
Petroleum	0765
Sanitary and Municipal	0554
System Science	0790
Geotechnology	0428
Operations Research	0796
Plastics Technology	0795
Textile Technology	0994

PSYCHOLOGY

General	0621
Behavioral	0384
Clinical	0622
Developmental	0620
Experimental	0623
Industrial	0624
Personality	0625
Physiological	0989
Psychobiology	0349
Psychometrics	0632
Social	0451

Suggested short title:

Protein and zinc restrictions and
Heligmosomoides polygyrus infection

ABSTRACT

The effects of single and combined dietary protein and zinc restrictions on the outcome of primary and challenge infections with the intestinal nematode *Heligmosomoides polygyrus* in mice were examined using a 3 X 2 factorial design that combined three levels of dietary protein (24% - control; 7% - marginal; 3% - low) with 2 levels of dietary zinc (60 mg/kg - control; 3 mg/kg - marginal). Protein and zinc restrictions, at these levels, produced independent effects on final worm burdens. While mice fed both marginal and low protein diets, and marginal zinc diets had significantly higher worm burdens in a primary infection, the response to a challenge infection was only impaired in animals fed the low protein diet. Eosinophilia was significantly reduced by zinc restriction in the primary infection and by the lowest level of protein restriction in the challenge infection. The magnitude of the serum IgG1 concentration was significantly lowered by protein restriction in both the primary and challenge infections. The impaired response to a challenge immunizing protocol in the animals fed the 3% protein diet, along with the reduced eosinophilia and IgG1 response, indicates a negative effect of protein deficiency on the host immune response to an intestinal nematode infection.

RÉSUMÉ

Les effets indépendents et combinés des déficiences en protéines et en zinc sur la survie du nématode intestinale *Heligmosomoides polygyrus* dans la souris furent étudiés en utilisant des rations contenant 24, 7 ou 3% de protéines combinés avec 60 ou 3 mg/kg de zinc. Les restrictions en protéines et en zinc à ces niveaux produisirent des effets indépendents sur la survie du parasite. Le degré d'infestation par les vers était plus élevé dans les souris recevant 7% et 3% de protéines et dans les souris recevant 3 mg/kg de zinc lors d'une première infection. Parcontre, lors d'une réinfection, seulement les souris recevant 3% de protéines avaient un degré d'infestation plus élevé. Le nombre des éosinophiles dans le sang était réduit chez les souris recevant 3 mg/kg de zinc lors d'une première infection et chez les souris recevant 3% de protéines lors d'une réinfection. La concentration des IgG1 dans le serum était réduite chez les souris recevant 3% de protéines lors d'une première infection et lors d'une réinfection. L'incapacité des souris recevant 3% de protéines de réduire le degré d'infestation lors d'une réeinfection, ainsi que leur niveaux moins élevé des éosinophils et des IgG1 dans le sang, indiquent qu'une déficiance en protéines affaiblit la réponse immunitaire de l'hôte face a un nématode intestinale.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr. Kristine Koski, Dr. Marilyn Scott and Dr. Mary Stevenson for their advice and guidance without which this thesis could not have been completed.

I especially thank Ph.D. students Christine Gagnon and Hai Ning Shi, whose friendship and support have truly made all the difference. Hai Ning Shi also deserves a special thanks for teaching me all of the parasitological and immunological techniques necessary for this research; his kindness and patience were always greatly appreciated. The participation of Stephanie Pearl in feeding the animals and of April Matsuno in extracting the tibia bone was very helpful and appreciated.

I dedicate this thesis to my sister Francine, who was with me throughout, and who somehow, was always able to make me laugh.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I. LITERATURE REVIEW

I. INTRODUCTION

Of the various diseases affecting humans, intestinal parasitic infections are among the most common and persistent globally (Pawlowski, 1984). Current estimates suggest that soil-transmitted helminths infect more than 1 billion people and that more than two million clinical cases occur each year (Pawlowski, 1984; WHO, 1987). The prevalence is highest in developing countries, especially where poverty coupled with a lack of sanitation and a low level of education and health care prevails (Pawlowski, 1984). Furthermore, parasitic infections are chronic and tend to co-occur with malnutrition in the same individuals (Crompton, 1986).

It is well established that malnutrition and parasitic diseases are closely interrelated. In their extensive review of the literature, Scrimshaw et al. (1968) classified the interaction between infection and nutrition as being synergistic, antagonistic or neutral. Among the intestinal parasites, a synergistic response was most often observed. That is, malnutrition and infection tended to be mutually aggravating. Based on more than 64 studies grouped according to types of nutritional deficiency in laboratory animals, it was found that starvation, or deficiency of protein and specific amino acids, vitamin A, thiamin, riboflavin, folic acid, iron, calcium or phosphorus all caused the host to be more susceptible to helminth infections. Exceptions were seen with vitamin E deficiency which was antagonistic with *Trichinella spiralis* infection, and vitamin with D deficiency which produced variable and inconclusive results (Scrimshaw et al., 1968).

This concept of interaction set forth by Scrimshaw et al. (1968) more than twenty years

ago has formed a conceptual framework for the study of the interrelationship between nutrition and parasitic diseases. However, more recently, some researchers have suggested that the nature of the interaction may not be as simple as that described by Scrimshaw and co-workers (1968). Solomons and Keush (1981) proposed that, rather, a complex and changing relationship may prevail at different times in the course of an infection or in subsequent encounters with the same agent. Indeed, parasitic diseases appear to be linked in a complex three sided interrelationship with both host nutritional status and host immunity (Beisel, 1982). Nutritional deficiencies can impair host immune function, while both the nutritional status and the immune response of the host can exert, by uncertain mechanisms, an enhancing or suppressing effect on the course of a parasitic disease; on the other hand, parasitic infections can alter both the nutritional and the immunologic status of the host (reviewed by Beisel, 1982).

Although the detrimental effects of intestinal helminth infections on host nutritional status in human populations have been extensively reviewed (Stephenson, 1987), the effect of nutritional status on intestinal helminth survival and transmission is less documented and remains poorly understood (Bundy and Golden, 1987). Since the review of Scrimshaw et al. (1968), better controlled experimental animal studies examining the effects of specific nutrient deficiencies on intestinal helminths have accumulated, with the majority focusing on protein or zinc because of their strong association with host immune function. These studies will form the focus of this literature review.

II. EFFECT OF PROTEIN AND ZINC DEFICIENCIES ON OUTCOME OF INTESTINAL HELMINTH INFECTIONS

A. EFFECT OF PROTEIN DEFICIENCY

1. Human studies

Although numerous studies have suggested that several species of intestinal helminths can have a negative effect on the protein status of the host (reviewed by Solomons and Keush, 1981; Stephenson, 1987), the converse, the effects of protein malnutrition on the perpetuation of intestinal helminths in humans have been less studied. Some studies conducted in the 1970's (i.e. Jose and Welch, 1970; Purtilo et al., 1976) have found an association between malnutrition and parasitic load, but the experimental designs were not adequate to test the hypothesis that the protein deficient host is more susceptible to acquire heavier infections. Moreover statistical analysis was rarely done.

For example, in a large scale survey of PCM in Australian aboriginal children, Jose et al. (1970) observed a rapid increase in parasite load occurring between the ages of 12 and 18 months in all children and, although the results were not analyzed statistically, the authors reported that growth retarded children appeared to be infected with heavier loads and with a greater number of species compared to normal children. Since malnutrition and disturbance of bowel function were present before parasites became established, the authors suggested that the normal child had the capacity to remove the majority of infecting organisms whereas a malnourished child had decreased immunity to these organisms.

Different results were observed by Purtilo et al. (1976) who classified the nutritional status

of 63 Brazilian children (aged from 15 to 50 months) according to the Gomez scale of PCM, and found that normal children and those with mild PCM had a greater load of intestinal parasites compared to the more severely malnourished children; the more malnourished children however were several months younger than the other groups and results were not analyzed statistically. Purtilo et al. (1976) also found that all classes of immunoglobulin were high in both malnourished and well nourished children as compared to those published for healthy children. Elevations in IgG, IgA and particularly IgE were positively correlated with the intensity of parasitism, but bore no relationship with the nutritional status of the children. In contrast, eosinophil counts in peripheral blood, which were also positively correlated with parasitism, decreased with increasing severity of PCM.

Dawson et al. (1987) found that the prevalence of *Ascaris lumbricoides* and *Trichuris trichuria* was significantly higher in mildly wasted children than in moderately/severely wasted and normal children. A major drawback of this study is that the intensity of infection was not measured. Nevertheless the authors concluded that mild malnutrition increases, whereas severe malnutrition decreases, susceptibility to helminths as suggested by Bundy and Golden (1987).

2. Experimental animal models

a. *Heligmosomoides polygyrus*

Heligmosomoides polygyrus is an intestinal nematode parasite of small rodents which has been proposed as a model for human hookworm (Bartlett and Ball, 1972). Primary infections with *H. polygyrus* are usually chronic, lasting from 4 weeks to 10 months depending on mouse strain (Ehrenford, 1954; Robinson et al., 1989; Wahid et al., 1989), while host resistance can be

elicited after challenge or repeated infections (Behnke and Robinson, 1985; Behnke and Wakelin, 1977; Prowse et al., 1979) and is manifested by the elimination of young and adult worms from the intestine of the infected host and by a reduction of worm size and fecundity (Behnke, 1987; Monroy and Enriquez, 1992).

In an early study, Bawden (1969) examined the effects of a low quality diet on *H. polygyrus* primary infection in mice. The control diet consisted of a pelleted chow and the low quality diet consisted of white bread. Although the low quality diet was poor in many nutrients other than protein, some interesting effects of diet on worm parameters were observed. Mice receiving the white bread had significantly more larvae encysted in the intestinal wall and significantly more adult worms in the intestinal lumen early in the infection whereas, in contrast, there was no difference in the number of adult worms between the groups later in the infection. Therefore, even though parasite establishment was favoured in the malnourished animals, subsequent parasite survival was decreased. The distribution of the parasite along the gut was also affected by diet such that early in the infection, parasites were restricted to the proximal end of the small intestine in the well fed mice whereas the distribution was more extensive in the restricted mice. This difference in the parasite distribution was no longer observed later in the infection; the parasites in both groups were restricted to the anterior part of the duodenum. The authors suggested that the anterior migration of the nematodes may be essential for their survival and that those who did not reach the anterior location were expelled from the host. They argued that the parasites in the malnourished hosts were more susceptible because of their longer migration route, which would explain their decreased survival. Subsequent work by Sukhdeo and Croll (1981) showed that the site of larval penetration may be linked to bile concentration.

Using better controlled dietary protocols, Brailsford and Mapes (1987) and Slater and Keymer (1988) reported no effect of protein deficiency on *H. polygyrus* primary infection. In both experiments, mice fed isoenergetic diets containing 2% protein compared with either 16 or 20% protein maintained comparably large worm burdens 21 days (Slater and Keymer, 1988) and 30 days (Brailsford and Mapes, 1987) post-infection. Unfortunately, the effect of protein deficiency on the developmental profile of *H. polygyrus* primary infection was not assessed.

Slater and Keymer (1986a) used repeated infections with *H. polygyrus*, a protocol known to stimulate host acquired immunity (Prowse et al., 1979), to study the effects of protein deficiency on the host response to infection. Mice were fed diets containing either 2% or 8% protein and were repeatedly infected with either 5, 10, 20 or 40 larvae every 2 weeks for a period of 12 weeks. Mice fed the low protein diet accumulated adult worms in direct proportion to exposure throughout the experiment. In contrast, mice fed the higher protein diet had a dose dependent reduction in the survival of adult worms between 6 and 12 weeks post-infection. The authors offered two suggestions: either the high protein group developed a dose dependent acquired immunity against the parasite which was impaired in the protein deficient animals or, alternatively, protein deficient mice were more susceptible to parasite induced immunosuppression, since adult worms are able to release immunomodulatory factors which act to immunosuppress the host (Behnke, 1987). Per capita fecundity of female worms was also higher in mice fed the diet containing 2% protein. The authors attributed the increased female fecundity to a more favourable intestinal environment in the deficient hosts or perhaps to a lack of acquired immunity which can act to depress worm fecundity.

Keymer and Tarlton (1991) also observed an effect of dietary protein restriction on the

accumulation of worms following a period of repeated infections with *H. polygyrus*. However, their model was more complex as it included the effect of diet (3% protein vs 16% protein), the effect of strain (resistant NIH mice vs susceptible CBA mice) and the effect of exposure (5 larvae vs 40 larvae per 2 weeks). All three factors had significant effects on final worm burdens 10 weeks after repeated infections, and significant interactions also occurred between all of these factors. Unfortunately, statistical contrasts were not done on all of the effects. From the graphic representation of the data it can be observed that NIH mice fed 16% protein had substantially less worms than NIH mice fed 3% protein, which would support the results of Slater and Keymer (1986a) discussed above. The response in the CBA mice, however, appeared to be in the opposite direction. Although these comparisons were not done statistically, CBA mice fed 3% protein appeared to have fewer worms than CBA mice fed 16% protein. The authors concluded that high exposure and high dietary protein were associated with the development of a strong protective immunity against *H. polygyrus*, but did not interpret the contrasting data observed in the CBA mice. Such interactions between genetic susceptibility and nutritional status would yield important information and should be further investigated.

Slater and Keymer (1988) also studied the influence of dietary protein on the efficacy with which mice could be immunized against re-infection with *H. polygyrus*. Two experiments examined the ability of gamma-irradiated larvae to immunize male CD1 outbred mice, fed either 2%, 4% or 8% protein diets, against a challenge infection. Mice fed 8% protein were 30% protected following immunization, whereas mice fed 2% or 4% protein were not protected. Also, female dry weight and fecundity were significantly reduced by immunization in mice fed either 4% or 8% protein whereas no change in these parameters was observed in mice fed 2% protein.

In a third experiment, inbred high responder NIH female mice were used Immunization with gamma-irradiated larvae resulted in protection in all of the dietary groups; however, protection was greater in mice fed 4%, 8% or 16% protein (80 to 90% protected) than in mice fed 2% protein (40% protected). Interestingly, it was noticed from these results that the threshold below which protein deprivation suppressed immunity was higher in outbred male mice which were not protected at 4% protein intake compared to inbred NIH female mice which were approximately 80% protected when fed 4% protein. Although this difference was attributed to the strain of mice, the sex of the mice could also have played an important role, since male mice are more susceptible than female mice (Dobson, 1978). Therefore the sex and genetics of the host may introduce another level of complexity in the nutrition-parasite interaction. The authors concluded from these experiments that dietary protein can influence the extent to which mice can be immunized against infection with *H. polygyrus*, which was evident with respect to an increase in the number, dry weight and fecundity of worms that survived an infection in protein deficient mice.

Slater and Keymer (1986b) have also studied the influence of dietary protein on the experimental epidemiology of *H. polygyrus*. In contrast to repeated infection experiments, in which the hosts are artificially infected at regular intervals with a known number of parasites, experimental epidemiology studies the population dynamics of a parasite that is transmitted naturally within a population of hosts under experimental conditions. Slater and Keymer (1986b) observed that in colonies of mice receiving a control diet of 8% protein, the prevalence and intensity of infection initially increased with time and then declined, probably as a result of acquired immunity. The prevalence and intensity of infection increased less rapidly within a

closed population of mice fed a 2% protein diet, but continued to rise over the entire experimental period. This continued rise was interpreted as evidence of a negative effect of protein malnutrition on host immunocompetence. Slater (1988) modified the previous experimental protocol on the epidemiology of *H. polygyrus*, by removing cohorts of mice from the experimental colonies at 5 week intervals while simultaneously introducing young naive mice. In each cohort, a larger number of parasites was found to infect mice from a colony fed a 2% protein diet as compared to mice fed a 16% protein diet. In contrast to the study of Slater (1986b), the rate at which naive mice acquired infection was higher in the low protein colony The daily egg output from female worms was also greater for parasites infecting protein deficient mice. These experiments showed that dietary protein deficiency can influence the rate of parasite transmission within a population by increasing the survival of adult parasites and by increasing female fecundity within malnourished hosts.

Although the authors of the previously described studies have suggested that increased *H. polygyrus* burdens and fecundity in protein deficient mice are most likely a result of reduced host immunocompetence, only one study directly measured host immune function. Slater and Keymer (1988) reported that immunization to *H. polygyrus* primed all mice, whether well fed or protein restricted, for an intense production of antibody against larval parasite antigen after a challenge infection. Although a slightly higher titre of IgG was detected in the plasma of mice fed on a 16% protein diet compared to a 2% protein diet, the authors concluded that this was unlikely sufficient to account for the reduced resistance in the protein deficient mice. The development of eosinophilia in immunized mice was significantly delayed and reduced in protein deficient mice following challenge infection, and it was suggested that a reduction in the number

of eosinophils attacking larval worms contributed to the low level of resistance observed in these animals.

b. *Nippostrongylus brasiliensis*

Rats infected with the intestinal nematode *Nippostrongylus brasiliensis* also serve as a model for human hookworm infection. However, unlike *H. polygyrus, N. brasiliensis* causes an acute infection which is normally expelled from the rat's intestine by a complex immune response between 10 to 16 days following a primary infection (Kelly and Dineen, 1972).

Studies focusing on *N. brasiliensis* infections in rats have shown that parasite expulsion was delayed in rats fed a 10% protein diet compared to a 30% protein diet (Bolin et al., 1977). Acquired resistance to reinfection with *N. brasiliensis* was also significantly reduced in rats fed 10% protein (Duncombe et al., 1979). Repletion of the deficient animals by reverting to a complete diet restored their capacity to mount an effective immune response to a secondary infection (Duncombe et al., 1979).

Bolin et al. (1977) observed normal antibody mediated damage to parasite, assessed by histological techniques, in both protein deficient and control rats and, therefore, suggested that the defect in worm expulsion in the protein deficient rats occurred either in the cell-mediated immune response or in one of the other non-specific mediators of worm expulsion. Subsequent studies have shown that impaired *N. brasiliensis* expulsion in protein deficient rats was unlikely to be due to a defect in the cell-mediated or systemic humoral immune systems, as syngeneic transfer of sensitized lymphocytes (Cummins et al., 1978) or transfer of immune serum (Duncombe et al., 1981) did not restore the ability to expel the parasite. However, Duncombe et

al. (1981) observed a defect in the bone marrow components of protein deficient rats. They demonstrated that transfer of either immune or non-immune bone marrow cells restored worm expulsion. Cummins et al. (1987a, 1987b) examined the possibility that the mucosal mast cells (MMC) had been restored by a bone marrow precursor. They found that although MMC hyperplasia was reduced in infected protein deficient rats (Cummins et al., 1987a), the activation of these cells, assessed by the systemic release of mucosal mast cell protease II, was similar in protein deficient and control animals (Cummins et al., 1987b). These authors concluded that the delay in worm expulsion could not be attributed to defective mast cell function, and suggested that protein deficiency may have interfered with the generation of secondary inflammatory mediators or, alternatively, with the tissue response to mediators released by MMC (Cummins et al., 1987b).

c. *Trichuris muris*

Michael and Bundy (1991) studied the effects of mild dietary protein deficiency on helminth population dynamics, in the *Trichuris muris*-mouse model of human trichuriasis. This model is of an acute nematode infection (Behnke, 1987) in which both primary and repeated infections stimulate strong immune responses leading to expulsion of the parasite by 20 days after infection (Wakelin and Lee, 1987).

Michael and Bundy (1991) reported that the initial establishment of *T. muris* was not different between mice receiving a 4% protein or a 16% protein diet, but that subsequent parasite survival was prolonged in the protein restricted mice. Although mean faecal egg output per mouse measured at regular intervals was found to be higher in the protein restricted group, the

mean egg output per female worm measured at the end of the experiment did not differ between the dietary groups, indicating that egg output was directly related to worm burden. This is in contrast to the reports of Slater and Keymer (1986a, 1988) who observed increased *H. polygyrus* fecundity in protein deficient mice. It is not clear whether these different results are related to the magnitude of protein deficiency or to factors associated with the different parasites.

Michael and Bundy (1992) subsequently studied the role of the immune response in the relationship between protein malnutrition and *T. muris* infection in mice. The specific antibody response to *T. muris* antigen (IgG1, IgA and total IgG+IgA+IgM) was more vigourous, both quantitatively (serum titres) and qualitatively (antigen recognition by IgG1), in mice fed a 4% protein diet, even though worm expulsion was delayed in these mice as compared to mice fed a 16% protein diet. It was suggested that the lack of effectiveness of the intact antibody response in the malnourished mice may be due to nutritionally mediated changes in cell-mediated immunity.

B. EFFECT OF ZINC DEFICIENCY

1. Human studies

There is a limited number of studies on the interaction between zinc deficiency and intestinal helminth infections in humans. Bundy and Golden (1987) reported a small but significant inverse correlation between serum zinc levels in Jamaican children and the intensity of *Trichiuris trichura* infection. It was suggested that zinc deficiency in children led to an impaired immune response to infection and, consequently, to higher parasite burdens. These

results are difficult to interpret, however, because 1) serum zinc is an unreliable indicator of zinc status, as it largely reflects current dietary intake and it is affected by several factors such as stress, infection and other nutritional deficiencies (King, 1990), and 2) a significant correlation between nutritional status and parasite status does not necessarily demonstrate a direct effect of nutrition on the host response. The fact that the two variables are correlated could also indicate a negative effect of parasite burden on nutritional status; it is well established that high intestinal parasite loads can have a negative effect on nutrient absorption and on host nutritional status (Stephenson, 1987).

Grazioso et al. (1993) recently participated in a large scale randomized, double blind controlled trial of zinc supplementation in Guatemalan schoolchildren in an attempt to determine whether initial intestinal parasite load was related to zinc status and whether zinc supplementation after drug induced expulsion of the parasites would alter parasitic reinfection. These authors found no significant difference in the initial prevalence and intensity of intestinal helminth infections (*A. lumbricoides* and *T. trichura*) between the zinc supplemented and placebo groups prior to supplementation and, furthermore, parasite status in children having low serum and hair zinc concentrations did not differ from normal children. After anthelmintic treatment and a period of 120-150 days of zinc supplementation, no difference in parasite status was observed between the placebo and supplemented groups. It was concluded that neither zinc status nor oral zinc supplementation had an effect on parasite status of children. Although this study was well designed to test a possible effect of zinc status on parasite load, it was limited by the very low incidence of zinc deficiency in the studied population. For example, only 7% of the children had a plasma zinc concentration characteristic of zinc deficiency.

2. Experimental animal models

a. *Heligmosomoides polygyrus*

Several experiments conducted in our laboratory have examined the effects of zinc restriction on the response of mice to *H. polygyrus* infection.

Minkus et al. (1992) reported no effects of marginal zinc deficiency on worm burden or female parasite fecundity in either a primary or a challenge infection. It was concluded that a marginal dietary zinc restriction with a diet containing 5 mg zinc/kg diet (causing a 40% reduction in plasma zinc but no reduction in tissue zinc) was not sufficient to modify parasite parameters. Also, it was observed that the T-cell mediated antibody response to sheep red blood cells (SRBC) was not impaired by the chosen level of zinc deficiency.

In contrast, Shi et al. (1994a) observed a significant effect of a more severe dietary zinc restriction (0.75 mg zinc/kg diet) on worm burden in a primary infection. Zinc deficient mice had significantly higher worm burdens than control mice 2 and 5 weeks post-infection. The higher worm burdens in zinc deficient mice 2 weeks post-infection, however, may have been due to lower food intake resulting from zinc deficiency, since the worm burdens did not differ from pair-fed controls. At 5 weeks post-infection, worm burdens were significantly higher in zinc deficient mice compared to pair-feds and *ad libitum*-fed control mice, indicating that zinc deficiency prolonged parasite survival in a primary infection. Female parasite fecundity was not affected by zinc deficiency. Functional T-cell impairment was also observed in the zinc deficient mice. Zinc deficient mice had an impaired delayed type hypersensitivity (DTH) response to parasite antigen, lower levels of serum IgG1 and IgE, and lower eosinophilia than control mice. Also, spleen cells from the zinc deficient mice produced low quantities of the cytokines IL-5, IL4

and IFN-gamma. The authors suggested an effect of zinc deficiency on either the function or the numbers of CD4+ T-helper (Th) cells. The marked effects of zinc deficiency on both worm burden and parameters of immune function led to the conclusion that host immunity plays an important role during a primary infection and that primary immune response to *H. polygyrus* is significantly impaired by zinc deficiency.

b. Other intestinal nematodes

El-Hag and colleagues (1989a) studied the effect of zinc deficiency on *N. brasiliensis* infection in rats. These authors found no significant difference in the number or size of worms, measured 3, 7 and 12 days post-infection, and no difference in female worm fecundity measured in utero 7 days post-infection, among rats fed either 3 mg zinc/kg or ad libitum or pair fed 40 mg zinc/kg diets. The zinc deficient rats, however, excreted significantly more parasite eggs over the whole infection time period than did the controls. Given that the groups harboured similar numbers of worms, the authors attributed the higher egg excretion in zinc deficient rats to impaired immunological interference with the process of worm egg production. In a subsequent study, El-Hag at al. (1989b) again found no effect of zinc deficiency on *N. brasiliensis* infection 7 days post-infection. Furthermore, the in vitro response of spleen cells to *N. brasiliensis* antigen was normal in zinc deficient rats. Therefore, zinc deficiency appears to have no effect on the expulsion of *N. brasiliensis* from rats, and the T-cell mediated antibody response to parasite antigen appears to be intact.

The effect of zinc deficiency was also studied in relation to *Trichinella spiralis* (Fenwick et al. 1990a) and *Strongyloides ratti* (Fenwick et al., 1990b) infections in rats. In both studies,

rats fed diets containing 3 mg zinc/kg had delayed expulsion of the parasites when compared to rats fed diets containing 40 mg zinc/kg, and in both cases, impaired expulsion was restored by zinc repletion. In the case of *S. ratti*, parasite establishment was increased by zinc deficiency and subsequent expulsion was delayed. Spontaneous cure, however, was achieved in all the animals by 38 days post-infection. In contrast, initial establishment of *T. spiralis* was not affected by zinc deficiency, but subsequent expulsion was impaired. Furthermore, the distribution of *T. spiralis* was affected by diet such that the parasites were established more proximally in the zinc deficient rats. No difference in *S. ratti* distribution was observed between the dietary groups. Fenwick et al. (1990a, 1990b) suggested that changes in T-cell function induced by zinc deficiency were likely to interfere with the immune response to infection. Although these authors did not directly demonstrate impaired T-cell function, they observed thymic atrophy (both absolute and relative weight reduction) in zinc deficient rats.

In summary, the effects of protein and zinc deficiencies on intestinal helminths have not been thoroughly investigated in human populations and conflicting results have been observed. While Jose et al. (1970) found that growth retarded children were more susceptible to intestinal helminth infections, Purtilo et al. (1975) reported that it was the normal children and those with mild PCM who had the greatest intensity of infection. In contrast, Dawson et al., (1987) found that the prevalence was highest in the mildly wasted children as compared to the normal and moderately/severely wasted children. Studies on zinc deficiency, in one instance have found serum zinc to be negatively associated with an intestinal helminth load (Bundy and Golden, 1987), whereas in another case, have found serum and hair zinc to have no relation with helminth status and zinc supplementation had no effect on the rate of reinfection (Grazioso et al., 1993).

Some of the difficulties in conducting well designed experiments in human populations are due to the high number of uncontrollable variables. For example, concurrent infections and polyparasitism are common. Moreover, initial infecting doses are not measurable and intensity of infection is difficult to measure accurately. Nutritional deficiencies in humans are also complex in respect to their severity, duration, and multiplicity and are generally influenced by concurrent infection (Beisel, 1982).

Protein and zinc deficiencies have been more extensively studied in experimental animal models and, in general, protein deficiency has been shown to impair host resistance to *H. polygyrus*, *N. brasiliensis* and *T. muris*. In the case of *H. polygyrus*, female fecundity was also increased by protein deficiency (Slater and Keymer, 1986a). The effects of zinc are more controversial. Zinc deficiency was found to have no effect on *N. brasiliensis* expulsion, although parasite egg output was highest in zinc deficient animals (El-Hag et al., 1989a). The same level of zinc restriction resulted in impaired *S. ratti* and *T. spiralis* expulsion mechanisms (Fenwick et al., 1990a and 1990b). In the *H. polygyrus*-mouse model, mild zinc deficiency (5 mg zinc/kg diet) had no effect on worm burden and fecundity in both primary and challenge infections (Minkus et al., 1992), whereas a more severe deficiency (0.75 mg zinc/kg diet) resulted in impaired host immune response and higher worm burden in a primary infection (Shi et al., 1994a). Studies being currently conducted in our laboratory have also shown negative effects of a severe zinc deficiency on the response to challenge infection (Shi et al., 1994b).

Although most studies on protein and zinc deficiency have suggested impaired host immune response to intestinal helminths in deficient animals, the precise mechanisms have not been elucidated.

III. PROPOSED MECHANISMS

Bundy and Golden (1987) hypothesized that human host nutritional status influences the whole ecological relationship between the host and parasite. These authors suggested at least three main mechanisms by which host nutritional status might influence helminth parasites; these are nutritionally mediated changes in (a) host immunity, (b) the parasite environment and (c) parasite nutrition.

A. HOST IMMUNITY

Inflammatory responses and immunity to intestinal helminth parasites are T-cell dependent processes, involving an intense eosinophilia, mast cell degranulation and antibody response (Befus, 1986; Chandra, 1984; Wakelin, 1986). Numerous studies have shown that PCM and deficiencies of many individual nutrients such as protein, iron, zinc, copper and vitamin A can exert depressive effects on host immune function (reviewed by Chandra, 1984 and 1988; Gershwin et al., 1985). Since host immunity is one of the key factors influencing outcome of parasitic infections (Wakelin, 1986), nutritionally mediated changes in host immune function may be expected to enhance parasite survival and fecundity (Bundy and Golden, 1987; Chandra, 1984).

B. NUTRITIONALLY MEDIATED CHANGES IN THE PARASITE ENVIRONMENT

Intestinal helminths are thought to have very specific physiological and chemical requirements in their host gut environment (Mettrick and Podesta, 1974). For example, the

digestive enzymes pepsin, trypsin and pancreatin, and bicarbonate are important environmental factors for such processes as hatching, exsheatment and excystation of some helminth species (Chappell, 1982; Noble et al., 1989). Bundy and Golden (1987) suggested that nutritionally mediated changes in the gut may make this environment unsuitable for parasite establishment and maturation.

C. MALNUTRITION OF THE PARASITE

A third mechanism by which host nutritional status may affect intestinal parasites is by interfering with the nutritional needs of the parasite (Bundy and Golden, 1987). Although the nutrient requirements of parasites have not been established, they are thought to be very specific since it has been difficult to define appropriate media to maintain gut helminths in vitro (Douvres and Urban, 1983; Franke and Weinstein, 1983 and 1984; Mauro and Weinstein, 1979). The responses by helminths to changes in the host diet may be direct if the species is feeding off the contents of the intestinal lumen (Crompton, 1987). However, intestinal helminths may be able to obtain from their host, requirements that are not in the host diet (Barrett, 1981) and malnutrition of the host may not necessarily lead to malnutrition of the parasite (Bundy and Golden, 1987).

Bundy and Golden (1987) further hypothesized that these three possible interactions may vary with the nature and severity of nutrient deficiency. These authors speculated that reduced host immunocompetence may be the main determinant of helminth infection in mild/moderately malnourished individuals, which would lead to heavier infections, but that as the host becomes more severely malnourished, gut physiology and parasite malnutrition may become more

important, leading to resolution of infection.

IV. EFFECTS OF PROTEIN AND ZINC DEFICIENCIES ON IMMUNE FUNCTION

The negative effects of malnutrition on host immune function are thought to be a main mechanism by which host malnutrition may potentiate parasitic infections (Bundy and Golden, 1987; Chandra, 1984). In fact, results from experimental studies on the effect of either protein or zinc interaction with intestinal helminth infections have largely suggested an effect of nutritional deficiency on host immune response to infection, although the precise mechanism have not been elucidated. The effects of experimental protein and zinc deficiencies on immune function have extensively studied and reviewed (i.e. Beisel, 1983; Gershwin et al., 1985; Sherman, 1992) and only a brief review of the main observations which may be relevant to intestinal helminths are reported in this section.

A. PROTEIN

The effect of PEM on immune function has been widely studied in humans. Because dietary protein was once thought to be the major nutritional shortage in the third world, and because many individuals with PEM experienced immune dysfunction, it was proposed in the 1960's that protein deficiency resulted in impaired immune function (Gershwin et al., 1985). However, it is now generally considered that PEM results from a combination of insufficient protein, calories, and a wide range of specific nutrients (Gershwin et al., 1985; Latham, 1990). Thus, results from studies conducted in PEM patients may be attributable to a mixture of

nutritional deficiencies. Experimental animal models, however, have been developed to examine protein deficiency in isolation.

1. Effect on lymphoid tissue

Thymic tissue appears to be particularly sensitive to protein deficiency, when initiated in very young animals (Bell et al., 1976; Mittal et al., 1988; Woodward et al., 1992). Spleen and mesenteric lymph nodes are also affected, but to a lesser extent than the thymus (Bell et al., 1976). Ultrastructural morphology analysis has shown that, in one experimental system, both cortical and medullary thymic epithelia lose 90% and 65% of their initial tissue volume, respectively, in severely protein deficient animals (Mittal et al., 1988). It was suggested that such involution could reduce the capacity of the thymus to promote thymocyte development, both through the synthesis of paracrine hormones and through contact-mediated stimuli, since thymic epithelial involution and pathology correlated with low levels of serum thymulin bioactivity (Mittal et al., 1988). In support of this, injecting a thymus hormone extract to protein deficient mice improved cell-mediated immune function (Petro et al., 1982; Watson et al., 1983) and splenic T cell responsiveness to polyclonal mitogens (Watson et al., 1983).

2. Effect on lymphocyte number and function

Profound decreases in lymphocyte numbers in the various lymphoid organs are often reported in protein malnourished animals. Total nucleated cell counts are reduced in thymus, spleen and mesenteric lymph nodes (Bell et al., 1976; Bises et al., 1987; Petro, 1985). More recently, experiments using surface marker analysis of cellular suspensions have also found

decreased lymphocyte numbers in various lymphoid organs (Bises et al., 1987; Woodward and Miller, 1991). Woodward and Miller (1991) estimated the size of the total recirculating lymphocyte pool in protein deficient weanling mice that had a depressed thymus-dependent acquired immune response to SRBC. These authors found a drastic reduction in the recirculating lymphocyte pool size, and the numbers of lymphocytes from the spleen and mesenteric lymph nodes. It was found that the reduction in B cell numbers was proportionately greater than the reduction in T cell numbers and the authors suggested that depression in the T cell dependent antibody response may not be dependent on a decreased number of regulatory T cells relative to B cells as had been previously proposed (Chandra, 1988). Other investigators have found a decreased percentage of T cells and IgA producing B cells within the spleen and mesenteric lymph nodes of protein deficient rats, but total B cells were not measured (Slobodianik et al., 1984).

It has been proposed that T cell dependent immunodepression in PEM results from the reduced ratio of T-helper/inducer to T-suppressor/cytotoxic cell subsets which occurs in the blood of malnourished children (Chandra, 1988; Chandra and Newbern, 1977). Studies in protein deficient animals, however, have not supported this view (Woodward, 1992). Bises et al. (1987) found no difference in the ratio of T-helper/T-suppressor cells in the spleen, and Lopez and Roux (1989) found no change in this ratio in Peyer's patches of protein deficient rats. Woodward and Miller (1991) reported no difference in the CD4+ to CD8+ T cell ratio within the recirculating pool, the spleen or the mesenteric lymph nodes, despite a drastic reduction in total lymphocyte numbers in protein deficient mice, and these authors concluded that depression of thymus dependent immunity in PEM can develop independently of an imbalance of T-helper/T-suppressor

ratio but that the possibility still exists that an imbalance within CD4+ (Th1 and Th2) or CD8+ subsets could influence T cell dependent immunity (Woodward and Miller, 1991).

3. Effect on antibody production

Many studies have examined the effects of protein deficiency on antibody production in experimental animals. Kenny et al. (1968) found that protein deficient rats had decreased antibody titers in response to SRBC immunization together with decreased numbers of antibody-forming cells. Mathur at al. (1972) found that mice fed a low protein diet produced a significantly lower antibody response to an intraperitoneal dose of SRBC. Protein deficiency resulted in a 70-80% reduction in the number of plaque-forming cells. Injection of thymocytes from syngeneic animals at the time of SRBC immunization was able to restore antibody production, indicating that a defect in T-helper cell function may have impaired antibody production in the deficient animals (Mathur et al., 1972). Other investigators have examined the primary and secondary response of protein deficient mice to immunization with alloantigens (Malave and Layrisse, 1976), and found that cells producing IgM alloantibodies were increased by protein deficiency, while the IgG response was markedly depressed. Malave and Pocino (1980) further demonstrated evidence to suggest that the elevated IgM response was due to defective suppressor cell activity.

4. Effect on phagocytes

Several studies have found decreased numbers or activity of phagocytic cells. Reduction in phagocytic activity correlated with increased susceptibility to BCG (Bacille, Calmette, Guérin)

infection in protein deficient animals (Bhuyan and Ramalingaswcami, 1973 and 1974), and a reduction in the number of peritoneal macrophages correlated with impaired clearance of *Esherichia coli* from peripheral blood and spleen (Price and Bell, 1975). Other studies have found that macrophages from protein deficient animals have an impaired ability to eliminate PVP (Coovadia and Soothill, 1976). More recently, Nimmanwudipong et al. (1992) have shown that protein deficient mice had markedly reduced proportion of splenic macrophages that expressed IA antigen, which may indirectly indicate impaired antigen processing and presentation.

B. ZINC

The association between zinc and immune function was first documented with the discovery of human zinc deficiency by Prasad et al. (1963) who observed that zinc deficient adolescent males experienced increased susceptibility to infection. Further evidence for zinc's role in immunity came from studies on the inborn error of metabolism, acrodermatitis enteropathica, a disease which is caused by defective absorption of zinc and in which the major causes of death are often linked to immunodeficiency (Moynahan and Barnes, 1973; reviewed by Sherman, 1992).

1. Effect on lymphoid tissue

Experimental studies have shown a significant depression in several aspects of T-cell-mediated immune response with dietary zinc deficiency. Fraker et al. (1977) first reported that dietary zinc deficiency induced thymic atrophy with preferential involution of the cortex. Luecke et al. (1978) further demonstrated that it was zinc deficiency and not the reduced food intake

associated with feeding a zinc deficient diet that was responsible for atrophy of thymus and spleen. Also, thymulin, a thymic hormone produced by the thymic epithelium is reduced in zinc deficient animals and humans (Dardenne et al., 1984; Iwata et al., 1979).

2. Effect on lymphocyte number and function

Atrophy of lymphoid organs is accompanied by a loss in cellularity. Studies have shown that a 30 day period of zinc deficiency reduced the total number of splenic lymphocytes and macrophages 40 to 50% in young adult mice (Fraker et al., 1986; Wirth et al., 1984). King and Fraker (1991) examined whether such a substantial change in the total numbers of splenocytes was accompanied by alterations in the proportion of T cells to B cells and/or subsets of these cells. They reported that marginally zinc-deficient mice had a normal ratio of T cells to B cells with no notable change in the subsets of lymphocytes making up these two classes. Severely zinc deficient mice demonstrated a 20% increase in the overall ratio of T helper to T suppressor/cytotoxic cells as well as a modest decline in the percentage of B cells (5-8%). Thus it seems that whereas zinc deficiency caused significant reductions in the total numbers of splenic lymphocytes, it caused no significant change in the composition of the splenic lymphocytes except in the most severe deficient cases.

T cell proliferation is also affected by zinc deficiency. In vitro zinc deficiency can be produced by adding EDTA to spleen cell cultures. T-cell proliferation after stimulation with either phytohemagglutinin (PHA) or Concanavalin A (ConA) was decreased in this zinc-deficient system, whereas no change in B cell proliferation in response to lipopolysaccharide (LPS) was found (Zanzonico et al., 1981). T cells from zinc deficient animals also show reduced

proliferation in response to mitogens and specific antigens (Fraker et al., 1982; Hilderbrandt et al., 1982; James et al., 1989).

3. Effect on antibody production

The capacity of mice to mount antibody and cell-mediated immune responses is significantly reduced in zinc deficiency. Antibody production to sheep red blood cells (SRBC), a T cell dependent antigen, is reduced in zinc deficient young adult mice, and parenteral T cell infusions can correct the defects in antibody production (Fraker et al., 1978). Therefore, the defect in antibody production appears to be due to defective T cell helper function. Dietary zinc deficiency in neonatal mice also impairs antibody production to SRBC (Beach et al., 1980a and 1980b; Zwickl and Fraker, 1980), and repletion with zinc results in a normalization of IgM plaque response and an elevation of the IgG response (Zwickl and Fraker, 1980). Beach et al. (1980a and 1980b) found a markedly altered serum immunoglobulin profile in postnatally zinc deprived mice, with no detectable serum IgM, IgG2a, or IgA, and notably elevated levels of IgG1.

4. Effect on phagocytes

In addition to the effects of zinc deficiency on T lymphocyte number and function, studies have suggested that the intracellular concentration of zinc helps to determine the magnitude of oxygen consumption, phagocytic activity, and bactericidal capacities of neutrophils (Beisel, 1986; Chvapil, 1976; Chvapil et al. 1977; Hambidge et al., 1986). Wirth et al. (1984) reported decreased numbers of splenic macrophages in zinc deficient mice, and reduced ability to

phagocytize and kill the intracellular parasite *Trypanosoma cruzi*.

In summary, protein and zinc deficiencies in laboratory animals have been shown to impair several aspects of the immune system. All of the host immune parameters that have been shown to be adversely affected by protein or zinc deficiency are also known to play a role in defense against parasitic infections. Although many studies have examined independently the effects of nutritional deficiencies on immune function and on parasitic infection, very few studies have examined the interaction between both aspects in the same system.

V. STATEMENT OF PURPOSE

Protein deficiency and, in some instances, zinc deficiency have been shown to increase host susceptibility to intestinal nematode infections in several experimental animal models. The effects of combined dietary protein and zinc deficiencies on the outcome of intestinal nematode infection, however, have not been previously studied. Because nutrient deficiencies rarely occur as isolated events in humans, and because PEM and chronic zinc deficiency often co-occur in the same individuals, along with parasitic disease, it is important to investigate whether combined deficiencies of these nutrients would alter the response that has been previously observed during single nutrient deficiencies.

Studies on single protein and zinc deficiencies have largely suggested impaired host immune function as the mechanism by which malnutrition resulted in higher parasite burdens. As compared to single deficiencies, combined protein and zinc deficiencies may have a greater

effect on host immune function and result in a greater level of infection (synergistic effect). Alternatively, combined deficiencies may have greater effects on other determinants of infection such as gut physiology or parasite nutrition, which could result in a lower level of infection (antagonistic effect). Finally, combined deficiencies may have no net effect above the effects of single deficiency and may result in the same level of infection.

The purpose of this study was to determine whether combined dietary protein and zinc deficiencies would have independent or interacting effects on the outcome of primary and challenge infections with *Heligmosomoides polygyrus* in mice. The specific objectives were to determine the effects of protein and zinc restrictions, alone and in combination, using diets containing adequate, marginal and low levels of protein (24%, 7% and 3% protein) with either adequate or marginal levels of zinc (60 mg zinc/kg 3 mg zinc/kg), on parameters of parasite establishment, survival and reproduction, and on some aspects of the host immune response to infection. Both primary and challenge infection protocols were used because they are known to produce a different host response to infection. Upon first exposure to the parasite (primary infection), the host is immunosuppressed by the adult worm and protective immunity is very weak. In contrast, the challenge infection protocol stimulates a more rapid, intense and protective immune response; this protocol mimics, in a short time frame, a response that would occur after a period of repeated exposure to the parasite.

CHAPTER 2. MATERIALS AND METHODS

I. EXPERIMENTAL ANIMAL MODEL:

THE *HELIGMOSOMOIDES POLYGYRUS*-MOUSE MODEL

Heligmosomoides polygyrus (= *Nematospiroides dubius*) is a trichostrongyloid nematode parasite of small rodents. This intestinal nematode infection was introduced as an experimental model in laboratory mice by Spurlock (1943) and has since been widely used as a model for studies on host immunology, immunogenetics, toxicology, pharmacology and epidemiology of gastrointestinal parasitism (Behnke, 1987; Keymer, 1985; Monroy and Enriquez, 1992;). This nematode-mouse system has been proposed as a model of human hookworm disease and nematode infections of veterinary importance by Bartlett and Ball (1972), due to its chronicity during a primary infection, its simple direct life cycle, an incomplete and variable host resistance and the stimulation of host resistance by repeated infections (Bartlett and Ball, 1972).

A. LIFE CYCLE

Parasite eggs are voided in the faeces of an infected host, and after approximately 36 hours and under appropriate conditions of temperature, humidity and lighting, start hatching to release the first stage larvae (L_1) (Figure 2.1). This larval stage is bacterial feeding and undergoes two molts to form ensheathed non-feeding infective third stage larvae (L_3) within 4 days (Bryant, 1973). The L_3 are ingested by a host and, within 24 hours, exsheath in the lumen of the stomach and migrate to the small intestine where they penetrate the mucosa and submucosa of the

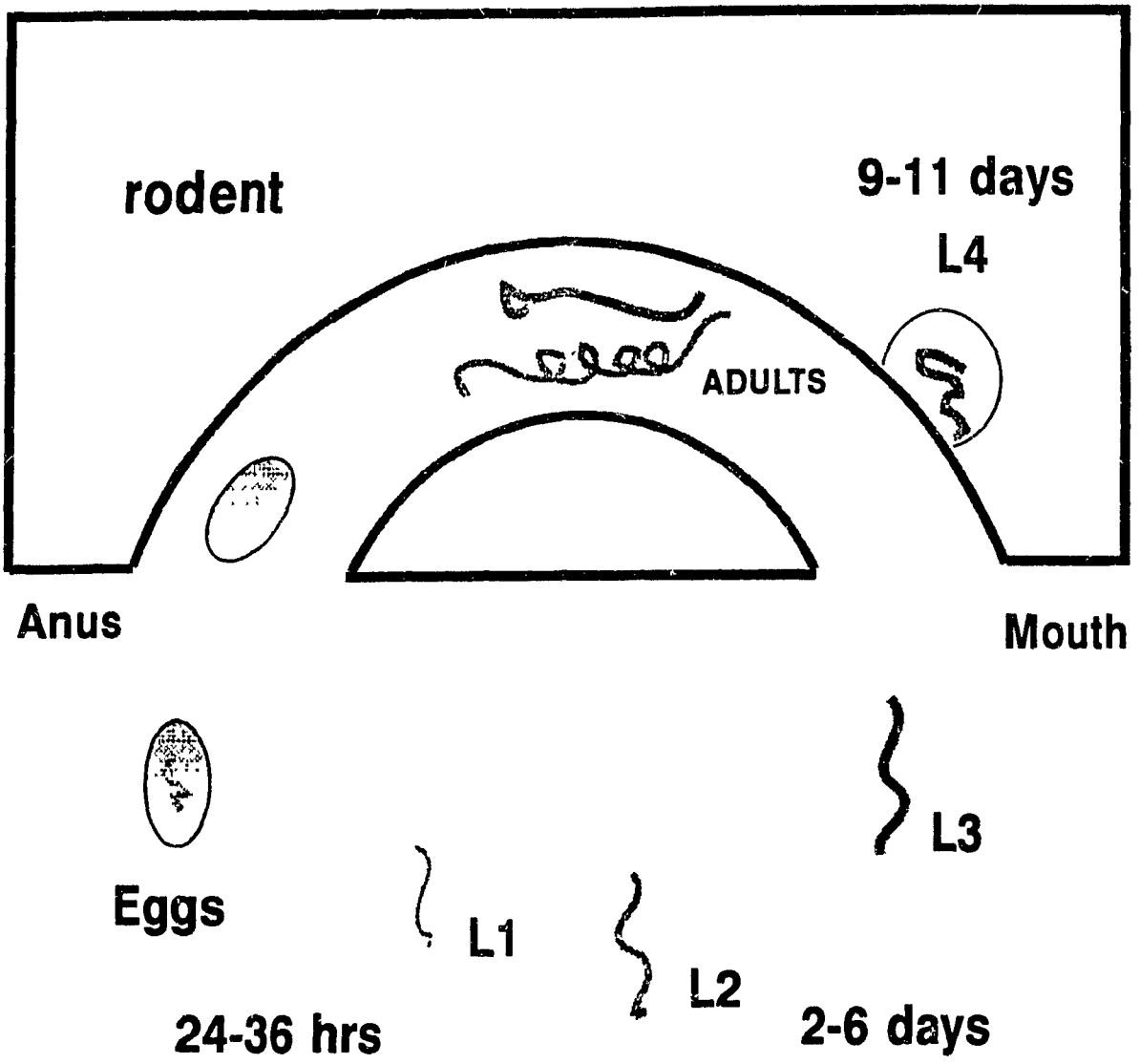

Fig. 2.1 The life cycle of Heligmosomoides polygyrus

duodenum to reach the serosal musculature where they encyst and begin molting (Bryant, 1973; Sukhedeo and Mettrick, 1984). The larvae then undergo 2 molts to a fourth stage larvae (L_4) and to the pre-adult stage, between 1 to 7 days post-infection (Bryant, 1973). After 7-8 days, the pre-adults emerge into the intestinal lumen where they begin an anterior migration towards the first few centimetres of the intestine. Once established, the adults mate and the females start producing eggs. Eggs can be seen as early as 10 days post-infection and by day 14, significant numbers of eggs can be seen in the faeces of infected hosts (Bryant, 1973). The adults may reside in the intestine for up to 10 months in a primary infection, depending on mouse strain, before senescence occurs and they are expelled from the host (Bryant, 1974; Ehrenford, 1954; Robinson et al., 1989).

B. CHARACTERISTICS OF INFECTION

1. Primary infection

Most strains of mice fail to produce a protective immune response on first exposure to *H. polygyrus* and primary infections are usually chronic, lasting from 4 weeks to 10 months depending on mouse strain (Ehrenford, 1954; Robinson et al., 1989; Wahid et al., 1989). There is no significant loss of worms during the tissue phase of a primary infection, despite the inflammatory reaction induced by the developing larvae (Liu, 1965; Sukhdeo et al., 1984). Initial contact with worm antigen stimulates both cellular and humoral responses. There is dense accumulation of neutrophils, eosinophils and macrophages around the encysted larvae (Cypess et al., 1974 and 1988; Liu, 1965), and mastocytosis, which is common in other intestinal helminthiasis is suppressed in *H. polygyrus* primary infections (Behnke et al., 1993; Dehlawi and

Wakelin, 1988). There is increased cellular proliferation within the secondary lymphoid organs (Liu, 1965; Price and Turner, 1983) and increased levels of IgG1, IgE, lymphocytes, neutrophils and particularly eosinophils in the peripheral blood (Behnke et al., 1977; Pritchard et al., 1984a,b; Prowse et al., 1978; Urban et al., 1991b). Although the role of these responses in a primary infection is still controversial, it appears that while they may not be effective at the time of a first infection, they provide the basis for immunity upon re-exposure (Ali and Behnke 1985; Dobson, 1982; Pritchard et al., 1983).

The inability of mice to mount rapid protective responses to primary infections with *H. polygyrus* contrasts with the rapid loss of other helminths in rodents, and has been attributed to the immunodepressive activity of the adult worm (reviewed by Behnke, 1987). There is some evidence that immunmodulatory factors (IMF) are secreted locally by the parasite (Losson et al., 1985; Monroy et al., 1989), and immunodepression may in part result from increased suppressor T cell activity and defective antigen presenting cells (Pritchard et al., 1984a) as well as suppressed mastocytosis (Dehlawi and Wakelin, 1988). Also, it has been suggested that the parasite may be able to resist host effector mechanisms due to its extraordinarily high levels of oxygen radical scavenging enzymes protecting against host granulocyte generated free-radicals (Smith and Bryant, 1986).

2. Challenge infection

Resistance to *H. polygyrus* can be engendered in a number of ways. If primary infections are terminated by anthelmintic drugs, protective responses can be demonstrated after reinfection (Behnke and Robinson, 1985; Behnke and Wakelin, 1977) and, similarly, when mice are exposed to repeated doses of infective larvae, resistance to reinfection may develop (Prowse et al., 1979).

There is considerable variation in the degree of resistance evoked in different strains of mice by identical immunizing procedures (Prowse et. al, 1979).

It is believed that acquired immunity to *H. polygyrus* is elicited by and preferentially directed against the L_4 during its tissue phase (Hagan et al., 1981; Pritchard et al., 1983). As in a primary infection, inflammatory and humoral responses are elicited by the larvae in a challenge infection, but they are more intense than in primary infections (reviewed by Monroy and Enriquez, 1992). The expression of immunity to reinfection is manifested by a reduction in the number of larvae that penetrate the intestinal mucosa, delayed maturation and migration of larvae into the gut lumen, arrested larval development and death of larvae in situ, elimination of young adult worms as they emerge in the lumen and of adult worms over several weeks of infection and, finally, reduction of worm size and fecundity (reviewed by Behnke, 1987, and Monroy and Enriquez, 1992).

C. IMMUNE REGULATION

As with other helminth infections, *H. polygyrus* stimulates an immune response that includes substantial increases in serum IgG1 (Crandall et al., 1974) and IgE (Urban et al., 1991b), and blood eosinophilia (Cypess et al., 1974; Prowse et al., 1978). Intestinal mastocytosis which is suppressed in a primary infection develops upon challenge (Dehlawi and Wakelin, 1988). These responses are characteristic of helminth infections, and they appear to be regulated by T-cells since they do not occur in athymic or T-cell depleted animals (Katona et al., 1988; Mitchell, 1980). Recently, Urban et al. (1991a) showed that CD4+ T cells (T helper cells) but not CD8+ T-cells (cytotoxic T-cells) regulate protective immunity to *H. polygyrus*.

CD4+ T lymphocytes can be divided into two functional groups based on the profile of cytokines produced: Th1 (T helper 1) gives rise to cell-mediated immunity and is largely dominated by IFN-gamma and IL-2, whereas Th2 is responsible for antibody-mediated immunity and is associated with elevated levels of IL-4 and IL-5, and also IL-6, IL-9 and IL-10 (Mosmann and Coffman, 1989). There is considerable evidence that infection of mice with *H. polygyrus* stimulates a T-cell response that has Th2 characteristics (Svetic et al., 1991 and 1993) and the associated eosinophilia, mastocytosis, IgG and IgE responses support this hypothesis. IL-4 induces IgE and IgG1 production by B cells, IL-5 regulates blood and tissue eosinophilia, and cytokine induction of mucosal mastocytosis involves IL-3, IL-4, IL-9 and IL-10 (reviewed by Finkelman et al., 1991; Sher and Coffman, 1992; Urban et al., 1992).

D. IMMUNE EFFECTORS

1. Eosinophils

An increase in the numbers of peripheral and tissue eosinophils is a major hallmark of helminth infections especially during the invasive stage of the parasite (Befus and Bienenstock, 1982). Since large parasites such as helminths cannot be phagocytosed, extracelluar killing by eosinophils appears to have evolved to help cope with this situation (Roitt, 1994). These inflammatory cells infiltrate into mucosal tissue of infected animals and are capable of generating a variety of potent mediators (Moqbel, 1986). Upon activation they produce a particularly strong respiratory burst with concomitant generation of active oxygen metabolites (Roitt, 1994).

The precise mechanisms of killing larvae by eosinophils are still uncertain. In *H. polygyrus* infection, eosinophils increase during a primary infection but are not involved in killing

the larvae until 6-7 days after challenge (Hurley and Vadas, 1983). Eosinophils from immune mice exhibit larvicidal activity in the presence of non-heat inactivated serum, but neither these cells nor the serum alone kills *H. polygyrus* larvae (Pentilla et al., 1983). Monroy and Enriquez (1992) suggested that the lack of larvicidal activity of eosinophils during a primary infection may be related to the lack of protective IgG1 which mediates eosinophil killing in mice (Pritchard et al., 1983). Pentilla et al. (1983) showed that the adherence of eosinophils to the L_4 stage of *H. polygyrus* in vitro can be mediated both by parasite-bound antibody and by parasite-bound complement C3, but that very little adherence to the L3 or adult worm occurs.

Although eosinophils are thought to play a protective role against *H. polygyrus*, recent studies have reported that neutralization of IL-5 by in vivo administration of anti-IL-5 monoclonal antibody (mAb) totally blocked the development of blood eosinophilia but had no effect on adult worm accumulation or fecundity in a challenge infection (Urban et al., 1991b). It was suggested that IL-5 and eosinophils may have no obligatory role in the protective immune response to *H. polygyrus*, although a redundant role is possible.

2. IgG1

Serum IgG1 levels rise within 2 weeks of a primary infection with *H. polygyrus* and stabilize at approximately double the control level (Crandall et al., 1974; Molinari et al., 1978). Although primary infection sera contain parasite-specific IgG1, passive transfer studies showed that immunity cannot be transferred to naive mice using this serum (Williams and Behnke, 1983). In contrast, challenged mice have a more substantial increase of IgG1 in serum with levels exceeding 20-30 mg/ml (Chapman et al., 1979; Crandall et al., 1974; Prowse et al., 1978). Serum

from challenged mice is capable of transferring immunity (Behnke and Parish, 1981) and of acting synergistically with immune mesenteric lymph node cells (Behnke and Parish, 1981). Pritchard et al. (1983) further showed that the anti-parasite activity of this sera could be largely accounted for by purified IgG1 fractions. Purified IgG1 was shown to react with antigenic components common to both adult worms and adult excretory-secretory antigens. In vivo, IgG1 was the only purified immunoglobulin isotype to cause significant reduction in worm numbers in the gut when administered alone, and to have any cooperative effect when administered with immune mesenteric lymph node cells (Pritchard et al., 1983). IgG1 also caused severe stunting of worms, and promoted the adherence of peritoneal exudate cells (eosinophils, neutrophils and macrophages) to the worm surface in vitro (Pritchard et al., 1983).

The role of IgG1 in a primary infection is still uncertain. Wahid and Behnke (1993) hypothesized that if IgG1 is involved in limiting primary infections, an inverse relationship should exist between the intensity and rapidity of specific IgG1 responses and parasite survival. These authors found a significant inverse correlation across mouse strains (from high to low responders) between the intensity of their specific IgG1 response to parasite antigen and worm survival, supporting a protective role for IgG1. However, there was no correlation within mouse strains between serum IgG1 concentration and loss of worms by particular individuals.

3. IgE

It is well known that helminth infections typically induce large increases in serum IgE level (Befus and Bienenstock, 1982). However, it was not until recently that a polyclonal IgE response was reported in *H. polygyrus* infections (Urban et al., 1991b). It has been shown that

in vivo administration of anti IL-4 or anti IL-4 receptor antibody blocked the polyclonal IgE response to *H. polygyrus* and abrogated protective immunity to infection (Urban et al., 1991b). The complexing of IgE receptors on mucosal mast cells with IgE antibody specific for parasite derived antigens is postulated to be important in protective immunity to intestinal helminth parasites because of the subsequent release of mediators of inflammation by mast cells that could adversely affect parasite survival (Castro, 1989). The complexing of parasite specific IgE on mast cells also triggers the release of chemotactic factors for granulocytes, leading to an influx of plasma IgG, complement, polymorphs and eosinophils (Roitt, 1994). In such a context, the ability of eosinophils to damage IgG-coated helminths and the elevated IgE response would constitute an effective defense against helminth parasites (Roitt, 1994).

II. EXPERIMENTAL DESIGN

The study was divided into two experiments, consisting of two different infection protocols using the *H. polygyrus* mouse model of intestinal nematodes infection. A preliminary study was first conducted with a primary infection protocol during which the host immune response against the parasite does not usually provide protection. Experiment 1 included both a primary infection protocol and a challenge infection protocol which stimulates protective host immunity to the parasite. The experimental design was a 3 x 2 factorial design that combined three levels of dietary protein (24%, 7% and 3%) with two levels of dietary zinc (60 mg zinc/kg and 3 mg zinc/kg) (Table 2.1). The control group was fed a diet containing 24% protein and 60

mg zinc/kg. Two levels of protein deficiencies were included and consisted of a 7% protein diet to produce a marginal deficiency and a 3% protein diet to produce a moderate deficiency. One level of zinc deficiency was included and consisted of a diet containing 3 mg zinc/kg to produce a marginal deficiency. The sample size for each experiment is shown in Table 2.1.

III. EXPERIMENTAL PROTOCOL

A. PRELIMINARY STUDY (PRIMARY INFECTION)

The protocol for the preliminary study is shown in Figure 2.2. Female BALB/c mice were received at 21 days of age and were acclimatized for three days on the control diet. On day zero, which was defined as the first day of the experiment, all mice were weighed and randomly divided among the six dietary treatments. Body weights and food intakes were measured every other day and food measurements were adjusted for spillage.

On day 15 of the dietary treatment, 5 mice per group were subjected to a delayed type hypersensitivity test (DTH) as described in Section VI. The DTH test was included as a general measure of acquired immune competence and nutritional status prior to infection.

Baseline blood samples were obtained from the tail vein for the measurements of eosinophils on day 21. On day 22, all mice were infected with 100 infective third stage larvae (L_3) of *H. polygyrus*; this constituted day 0 post-infection. Blood samples were obtained from the tail vein on days 7, 14, and 21 post infection for the enumeration of eosinophils. Twenty-four hour stool collections were obtained on days 14, 21 and 28 post infection for the determination of daily parasite egg output.

Blood was collected just prior to necropsy on days 9 and 29 post-infection by retro-orbital plexus puncture for determination of plasma albumin and zinc concentrations, and alkaline phosphatase activity. Mice were then necropsied as described in Section VI.

B. EXPERIMENT 1 (PRIMARY AND CHALLENGE INFECTIONS)

The protocol for experiment 1 was identical to that of the preliminary study for the first 21 days, with the exception of the DTH test and blood eosinophil counts which were not done prior to infection (Figure 2.3) On day 22 of the dietary treatment, the challenge infection protocol was started. Eleven mice per dietary group were given a first infection with 100 L_3 *H. polygyrus* (day zero post-primary infection). Of these animals, 3 per group served as controls to verify the establishment of the parasite and were killed on day 9 post-primary infection (establishment controls). The other eight animals per diet group were treated with 175 mg pyrantel pamoate (Combantrin) (Pfizer, Montreal, Quebec) per kg of body weight on day 9 and again on day 14 post-primary infection, to remove established *H. polygyrus*. On day 21 post-primary infection (day 0 post-challenge), three of these animals per diet group were killed and served as drug controls to verify the efficacy of the drug, and the remaining five animals per group were challenged with 100 L_3 *H. polygyrus* (challenge infection group). At the same time, five naive mice per dietary group were infected for the first time with 100 L_3 *H. polygyrus* (primary infection group).This group was included for comparison with the challenge infection protocol, which elicits a stronger host immune response. Beginning on day 0 post-challenge infection, animals from the two infection protocols were treated alike and all experimental measurements were obtained at the same time for the two groups.

Blood was obtained from the tail vein on day 0 post-challenge infection for baseline immunoglobulin measurement, and on days 7, 14 and 21 post-challenge infection for the determination of blood eosinophils and serum immunoglobulins. Twenty-four hour stool collections were made on days 16, 22 and 28 post-challenge infection for the determination of parasite daily egg output. All mice were necropsied on day 29 post-challenge as described in Section VI.

IV. ANIMALS AND HOUSING

Weanling, inbred BALB/c female mice were obtained from Charles River (St. Constant, Quebec) at 21 days of age. Mice were individually housed in Nalgene cages (27 x 21 x 14 cm) having stainless steel covers. Stainless steel grids were present on the floor of the cages to prevent coprophagy (Fisher Scientific, Montreal, Quebec). The experimental diets were dispensed *ad libitum* in plastic Mouse Powder Feeders (Lab Products Inc., Montreal, Quebec) specifically designed to minimize food spillage. Deionized water was available *ad libitum* from Nalgene bottles with neoprene stoppers (Fisher Scientific, Montreal, Quebec). Cages, stainless steel covers and grids, bottles and feeders were all acid washed in 10% HCl prior to use and plastic filter tops were placed over the cages to prevent environmental zinc contamination. Room temperature was maintained between 22 to 25^{0}C and lighting was on a 14 h light and 10 hr dark cycle.

V. DIETS

A. CONTROL DIET

All animals were fed a semi-purified, biotin-fortified egg white based diet. The control diet was formulated as described by Minkus (1990) (Table 2.2). Spray-dried egg-white solids (ICN Biochemicals, St. Laurent, Quebec) were chosen as the sole source of protein because they are a complete protein with very low zinc residues. A level of 24% protein and 30%, each, of glucose and cornstarch were chosen based on levels reported in the literature that resulted in adequate growth and reproduction in mice (Beach et al., 1981; Filteau and Woodward, 1982; Flanagan et al., 1983; Luecke and Fraker, 1979; NRC, 1978). The NRC (1978) gives no recommendation for the level of fat in the diet of the mouse. In the recent literature, when the level of dietary protein was 24%, the diet most often contained 8% corn oil as a source of essential fatty acids and a minor source of energy.

A biotin-fortified vitamin mix was formulated as described by Minkus (1990) to meet NRC (1978) recommendations for all vitamins (Tables 2.3 and 2.4). Since zinc deficiency and protein deficiency are known to cause anorexia, the mix was formulated to provide approximately 2 to 6.5 times the NRC (1978) requirements for the mouse to ensure that requirements were met should reduced food intake occur. Excess biotin was added at 18 times requirement to control for the biotin-chelating component, avidin, in the egg-white solids that were used as the protein source (Williams and Mills, 1970).

The control mineral mix was formulated to provide twice the NRC requirements for all minerals including zinc in anticipation of decreased food intake known to accompany zinc and

protein deficiencies (Minkus, 1990) (Table 2.5). Zinc was added as zinc carbonate (Fisher Scientific, Montreal, Quebec) to provide 60 mg/kg diet.

B. DEFICIENT DIETS

1. Zinc deficient diets

The zinc-restricted mineral mix was identical to the control mineral mix except for the zinc content. Zinc was added as zinc carbonate (Fisher Scientific, Montreal, PQ) to provide 3 mg/kg to the zinc-restricted diets (Table 2.6). The level of 3 mg/kg was chosen based on previous experiments conducted in our laboratory showing that a diet containing 5 mg zinc/kg diet resulted in mild zinc deficiency in outbred CD1 female mice and had no effect on *H. polygyrus* infection (Minkus et al., 1992), whereas a diet containing 0.75 mg zinc/kg resulted in severe zinc deficiency and led to higher parasite burdens in zinc deficient BALB/c female mice (Shi et al., 1994a). Since the goal of this study was to examine the effects of marginal zinc deficiency, an intermediate value of 3 mg/kg zinc was chosen.

2. Protein deficient diets

Two levels of protein deficiency were chosen for these experiments. A level of 7% protein was chosen to produce a mild deficiency based on NRC (1978) nutrient requirements of the mouse, which from several early studies indicated that levels of 11.3% egg protein produced maximal growth in CF1 mice (Korsrud, 1966), whereas 13.6% casein was the minimal level required for acceptable growth, reproduction, and lactation in Swiss STM mice (Goettsch, 1960). Also, albino mice fed diets containing 9.8% casein grew at a slightly subnormal rate compared

to mice fed diets containing higher casein levels (Bing et al., 1932). Therefore, a level of 7% was expected to be below the minimal requirement for maximal growth as well as to produce some degree of nutritional stress. A level of 3% protein was chosen to produce a more severe deficiency based on a preliminary experiment in our laboratory that showed a 2% egg white protein diet to produce significant weight loss and mortalities in weanling BALB/c female mice. Also, several published experiments reporting the effects of protein deficiency on *H. polygyrus* infection in mice have shown that levels of 2% and 3% protein produce significant effects on worm burdens and egg production during challenge or repeated infections (Keymer and Tarlton, 1991; Slater and Keymer, 1986; Slater and Keymer, 1988). The protein deficient diets were made isocaloric to the control diet on a weight basis by replacing the amount of protein in the deficient diets with an equal amount of starch; all other dietary constituents of the diets were identical.

VI. EXPERIMENTAL TECHNIQUES

A. BLOOD COLLECTION

1. Tail vein

Blood was collected from the tail vein of mice at specified time intervals during the preliminary study and experiment 1 as described in the experimental protocols (Figures 2.2 and 2.3). Mice were restrained lightly by holding the tail which was warmed with a heat lamp for approximately one minute. The lateral tail vein was swiftly incised with a scalpel blade, in a site within the distal one third of the tail. Blood drops were collected in a non-heparinized

microhematocrit capillary tube. Blood was subsequently transferred into serum separator tubes (Microtainer Brand Serum Separator Tubes, Becton Dickinson) which were centrifuged (Microspin 12S, Sorvall Instruments, Biomedical Products, Mississauga, ON) at 6000 x g for 10 minutes to obtain serum. Serum tubes were stored at -20°C for later determination of immunoglobulins. Whole fresh blood (25 µl) was also immediately transferred to UNOPETTE capillary pipettes (Fisher Scientific, Montreal, PQ) for eosinophil counts.

2. Orbital plexus

Blood was obtained by retro-orbital plexus puncture, just prior to necropsy in both experiments. Mice were first anaesthetized with Metofane and blood was obtained by introducing the end of a heparanized microhematocrit tube with axial rotation at the medial canthus of the orbit. Whole blood was immediately placed in acid-washed (10% HCL) 1.5 ml polyethylene eppendorf tubes which were then centrifuged at 6000 x g for 15 minutes to obtain plasma. Plasma was transferred to acid-washed polyethylene eppendorf tubes, using acid-washed pasteur pipettes, and was immediately frozen on dry ice and stored at -80°C.

3. Cardiac puncture

Blood was also collected by cardiac puncture on half of the animals in experiment 1. Mice were first anaesthetized with Metofane and blood was obtained by introducing the end of a heparinized 1-ml syringe with a 23-G needle at the base of the sternum at an angle of 30 degrees into the heart. Whole blood was aspirated and immediately placed in acid-washed (10% HCL) 1.5 ml polyethylene eppendorf tubes which were then centrifuged at 6000 x g for 15 minutes to

obtain plasma. Plasma was transferred to acid-washed polyethylene eppendorf tubes, using acid-washed pasteur pipettes, and was immediately frozen on dry ice and stored at -80°C.

B. DELAYED TYPE HYPERSENSITIVITY RESPONSE (DTH)

The allergen used was 2,4-dinitrofluorobenzene (DNFB) (Sigma Diagnostics, St-Louis, MO). On experimental day 15 of the preliminary study (i.e. day 15 of the dietary treatment, prior to infection), the abdomen of 5 mice per dietary group was shaved using a small animal clipper. Using a micropipettor with disposable tips, the mice were sensitized by the epicutaneous application of 20 ul of 0.5% DNFB in 4:1 acetone/olive oil solution onto the shaved abdomens on days 15 and 16. Mice were restrained 3 to 5 seconds to allow some of the solvent to evaporate on the skin and to minimize contamination of the cages. These steps are the afferent (sensitization) phase, while the following steps are the efferent (elicitation) phase. Five days later, the baseline ear thickness of the animal's right ear was measured using a thickness gauge. Immediately following ear measurement, mice were treated on each side of the right ear by the epicutaneous application of 10 ul of 0.2% DNFB in 4:1 acetone/olive oil solution (20 µl total). The ear thickness was measured again 24 hours later. The change in ear thickness was calculated by subtracting the baseline ear thickness from the ear thickness 24 hr after elicitation. Three naive mice served as negative controls for non-specific ear swelling induced by the irritating effects of DNFB.

C. LARVAL CULTURE

The *Heligmosomoides polygyrus* strain has been maintained in CD1 outbred mice at the

Institute of Parasitology of McGill University for several years. Infected stock mice were placed in cages with stainless steel grids and faecal pellets were collected over a 24 hour period in deionized water. The faecal sample was then rinsed through two sieves (150 µm and 45 µm) with distilled water to obtain the parasite eggs in the sediment remaining on the 45 µm sieve. The sediment was flushed into plastic centrifuge tubes and was allowed to settle at the bottom of the tubes with the excess water being discarded. Several petri dishes were set up with a 5.5 cm filter paper onto which faecal sediments were spread. The petri dishes were covered and kept in dark boxes containing water dishes to prevent drying, and were stored in a cool dark place (a cupboard) for 7 days, during which time the eggs hatched and developed into infective third stage larvae.

To obtain the larvae, distilled water was added to each petri plate at the level of the filter paper. The water was gently run along the edge of the filter paper to gather the larvae which were transferred into plastic centrifuge tubes with approximately 2 ml of deionized water and kept in a refrigerator at $4^{o}C$ until needed for infection.

D. INFECTION

The appropriate volume of larval suspension to achieve 100 infective L_3 was obtained by counting the number of live larvae in several 10 ul aliquots. The volume of the larval suspension was then adjusted to contain 100 larvae; the number of larvae was counted in several sham doses to ensure that an average of 100 was achieved. The larval suspension was administered orally to each mouse, using a 200 ul automatic pipetman with soft plastic tips.

E. ANTHELMINTIC TREATMENT

The anthelmintic treatment is a modification of that developed by Tanguay and Scott (1987). This protocol is used to stimulate host immunity to infection (Behnke and Wakelin, 1977). On days 9 and 14 post primary infection of experiment 1, mice were given 175 mg pyrental pamoate (0.1 ml of 30% Combantrin/25 g mouse)/kg body weight orally using a 200 ul automatic pipetman with soft plastic tips. The efficacy of the drug treatment was confirmed by opening and examining the intestine of 3 mice per dietary group, designated as drug controls, 7 days after the last drug administration and just prior to the challenge infection. It was assumed that the drug was effective if the drug controls were free of worms.

F. TWENTY-FOUR HOUR EGG COUNTS

Water was placed at the bottom of each cage to collect faecal pellets over a 24 hour period. Faeces of mice passed through the stainless steel grid floor into the water. After exactly 24 hours, the water was collected from each cage, individually, into a plastic beaker. The faecal samples were rinsed through 2 sieves (150 µm and 45 µm) with distilled water to collect the eggs. The sediment remaining on the 45 µm sieve (containing the eggs) was flushed into a 25 ml graduated cylinder to obtain a total volume of 14 ml. This solution was then transferred to a 30 ml plastic tube and resuspended with 14 ml of a saturated aqueous NaCl to obtain a total volume of 28 ml. The tubes were capped and gently inverted a uniform number of times, and an aliquot taken with a pasteur pipette and transferred to the two half of a McMaster Egg Counting chamber (Hawkesley and Sons, Ltd., Sussex, UK). The number of eggs within each half of the chamber was counted using a compound microscope, and the average egg number per chamber

determined. Calculations were done to determine the total number of eggs per mouse per day. Egg counts for each mouse were expressed as total eggs/day on days 14, 21 and 28 post infection and also as eggs/female worm on day 28 post-infection.

G. NECROPSY OF MICE

Mice were killed by cervical dislocation under Metofane anaesthesia, immediately after completion of last blood collection (see protocol, Figures 2.2 and 2.3). The liver, spleen and thymus were cleaned of adhering tissue, weighed and then placed in polyethylene eppendorf tubes. The entire left posterior leg was removed and placed in a polyethylene eppendorf tube for later extraction of tibia bone. All tubes containing the tissues were immediately put on dry ice and stored at -80^{0}C. The small intestine of each mouse was removed and placed in a plastic twirlbag and stored at -20^{0}C for later determination of worm burden.

VII. ANALYTICAL PROCEDURES

A. DIETS

A sample from each diet was analyzed for zinc concentration by flame atomic absorption spectrophotometry (Perkin-Elmer model 3100, Perkin-Elmer, Canada Ltd., Montreal, Quebec) after wet ashing in nitric acid according to the method of Clegg et al. (1981). Zinc analysis was performed following the specifications of the Perkin-Elmer model 3100 operation manual.

B. PARASITOLOGICAL PARAMETERS

1. Worm burden

The entire small intestine from each mouse was thawed and slit open onto a glass plate. The mucosal surface was scanned with a dissecting microscope, and the gender and number of adult *H. polygyrus* were determined. The number of L_4 in the wall of the intestine was counted when this stage of parasite was present.

C. NUTRITIONAL PARAMETERS

1. Protein status

a. Plasma albumin

Albumin concentration was determined by dye-binding of bromcresol green to the protein at pH 4.2 (Albumin procedure No. 631, Sigma Diagnostics, St-Louis, MO). Analyses were conducted on the Abbott VP Super System Discrete Analyzer (Abbott Diagnostic, Mississauga, ON). The method is based on the procedure of Doumas (1971).

b. Blood urea nitrogen

BUN concentration was determined by coupled enzyme reactions involving urease and glutamate dehydrogenase (BUN rate Procedure No. 67-uv, Sigma Diagnostics, St-Louis, MO). Analyses were performed on the Abbott VP Super System Discrete Analyzer. This method is based on the procedure of Talks and Schubert (1965).

2. Zinc status

a. Plasma alkaline phosphatase

Alkaline phosphatase activity was determined by the hydrolysis of p-nitrophenyl phosphate (Alkaline phosphatase (ALP) Procedure No. 245, Sigma Diagnostics, St-Louis, MO.). Analyses were performed, immediately after thawing of plasma, on the Abbott VP Super System Discrete Analyzer. The method is based on the procedure of Bowers and McComb (1966).

b. Plasma zinc

Plasma zinc concentration was determined by flame atomic absorption spectrophotometry. Plasma samples were diluted 1:5 with deionized water. Standards and blank solutions were prepared with 5% glycerol in deionized water to approximate the viscosity of plasma.

c. Tissue zinc

Tibia and livers from all mice were analyzed for zinc concentration by flame atomic absorption spectrophotometry. Tissues were freeze-dried for at least 24 h prior to wet ashing with nitric acid. Liver samples were fat extracted with petroleum ether on a Soxtec System HT2. A blank containing nitric acid only as well as National Bureau of Standards Reference Material (Bovine Liver 1577a) were digested and analyzed simultaneously with the samples.

D. IMMUNOLOGICAL PARAMETERS

1. Eosinophils

Eosinophils were counted with the UNOPETTE Test 5877 (Fisher Scientific, Montreal,

Quebec). Whole blood was obtained from each mouse by the tail vein on the days indicated in the experimental protocol (Figures 2.2 and 2.3). Fresh whole blood was added to the diluent which preserves all leucocytes but stains only eosinophils. The diluted specimen was added to a Neubauer hemacytometer, and the eosinophils which appear bright orange-red were counted under 100X magnification, using a compound microscope. The average from two chambers was calculated for each blood sample.

2. IgG1

The level of total IgG1 in serum was determined by enzyme-linked immunosorbent assay (ELISA). Immunon II plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with serial dilutions of purified mouse IgG1 Kappa (MOPC-31c) purified immunoglobulin (Sigma Immuno Chemicals, St. Louis, Mo), together with diluted serum samples, and incubated overnight at 4°C. Peroxidase conjugated rabbit anti-mouse IgG1 mAb (Serotec, England) was diluted as a detecting antibody (1:1000). The reaction was visualized with the BIO-RAD Peroxidase substrate kit ABTS (BIO-RAD, Hercules, CA) at 415 nm on a ELISA reader.

VIII. STATISTICAL ANALYSIS

A. PRELIMINARY STUDY

In the preliminary study, the infection protocol consisted of a primary infection that included infected animals, only, and the dietary treatment was a 3 X 2 factorial design that combined 3 levels of dietary protein (24%, 7% and 3%) and 2 levels of dietary zinc (60 mg/kg

and 3 mg/kg). The data for the nutritional parameters were analyzed with a two-way analysis of variance that included the effects of protein, zinc, and their interaction. When the main effects of protein, zinc or protein and zinc interaction were significant, post-hoc multiple pairwise comparisons were done with Tukey's test to detect significant differences among pairs of means. The data for the nutritional parameters were analyzed with the Multivariate General Linear Hypothesis (MGLH) procedure of the SYSTAT (Systat Inc., 1990) statistical program.

The parasitological and immunological data were analyzed with a one-way analysis of variance that included the effect of dietary protein, only. The data were not analyzed for the zinc-restricted animals because of problems encountered with the infection protocol in these groups, as described in the result section. Because parasite data are often aggregated and not normally distributed, all parasite parameters (worm burden, eggs/day and eggs/female) and also blood eosinophils were first tested for normality. The residual (difference between observed and predicted) was tested for normality, rather than the specific variable itself, in order to minimize treatment effects on the distribution. The PROC UNIVARIATE procedure of the SAS program (SAS Institute Inc., 1985) produced a test statistic for the null hypothesis that the input data values were from a normal distribution. In this case, the Shapiro-Wilk statistic, W, was computed, with small values of W leading to a rejection of the null hypothesis ($p<0.05$). The test indicated that all the data met the assumptions of normality and, therefore, data transformation was not required. The parasitological and immunological data were analyzed with a one-way analysis of variance to test the main effect of dietary protein. When the effect of protein was significant, post-hoc multiple pairwise comparisons were done with Tukey's test to detect significant differences among the three protein levels. The parasitological and immunological data were

analyzed with the PROC GLM procedure of SAS.

B. EXPERIMENT 1

Experiment 1 included two infection protocols with infected animals only. The diet treatment design was identical to that of the preliminary study. Since two different infection protocols were administered in experiment 1, the data for the nutritional parameters were first analyzed with a three-way analysis of variance including infection (primary or challenge), protein, zinc and their interactions in the model. When infection exerted no significant effect, this factor was dropped from the model and the data were pooled and re-analyzed with a two-way analysis of variance that included only the effects of protein, zinc and their interaction; the means of the pooled data are reported in the results section. When infection was significant, the data were not pooled and were re-analyzed separately for the primary and challenge infection protocols, in a two-way analysis of variance that included the effects of protein, zinc and their interaction. When the main effects of protein, zinc or protein and zinc interaction were significant, post-hoc multiple pairwise comparisons were done to detect statistical differences within treatment means using Tukey's test. Statistical analysis for the nutritional parameters were done with the MGLH procedure of SYSTAT.

The immunological data were also analyzed using the above procedures, but data were always analyzed separately for the primary and challenge infection protocols. Immunological data were analyzed with the SAS statistical program using the PROC GLM procedure.

Parasitological data were first tested for normality, as described for the preliminary study. When the data were not normally distributed, the data were transformed with a square root

(SQRT) transformation which resulted in all the data meeting the assumptions of normality. Data which required SQRT transformation are indicated in the appropriate tables in the Results section. The parasitological data were analyzed separately for primary and challenge infection protocols in a two-way analysis of variance, and the model described above was used with the SAS PROC GLM procedure.

Repeated measure analyses were also performed on eggs/day, eosinophils and IgG1 measurements using the PROC GLM procedure of SAS in order to determine whether dietary treatment affected the pattern of these parameters over time.

In all statistical analyses, the level of significance was set at alpha = 0.05.

Table 2.1 Experimental design and sample size for both preliminary study (primary infection protocol) and experiment 1 (primary and challenge infection protocols)

Parameter	Zinc Level (mg/kg)	Protein Level (%) 24	7	3
		Preliminary study		
Total N	**60**	10	10	10
	3	10	10	10
Necropsy day 9 post-infection	**60**	5	5	5
	3	5	5	5
Necropsy day 29 post-infection	**60**	5	5	5
	3	5	5	5
		Experiment 1		
Total N	**60**	16	16	16
	3	16	16	16
Establishment controls	**60**	3	3	3
	3	3	3	3
Drug controls	**60**	3	3	3
	3	3	3	3
Primary infected animals	**60**	5	5	5
	3	5	5	5
Challenge infected animals	**60**	5	5	5
	3	5	5	5

Figure 2.2. Protocol for the preliminary study

EXPERIMENTAL DAY	PRIMARY INFECTION DAY	PROCEDURE
0		- RECEIVED MICE - EXPERIMENTAL DIETS
15		- SENSITIZATION WITH DNFB FOR DTH TEST
16		- SENSITIZATION WITH DNFB FOR DTH TEST
20		- ELICITATION WITH DNFB FOR DTH TEST
21		- MEASURED EAR THICKNESS FOR DTH PESPONSE TO DNFB - BASELINE BLOOD SAMPLES FOR EOSINOPHILS
22	0	- INFECTION WITH 100 LARVAE
29	7	- EOSINOPHIL COUNTS
31	9	- KILLED 1/2 OF MICE IN EACH DIETARY TREATMENT - WORM BURDEN (ESTABLISHMENT) - BLOOD SAMPLES: PLASMA ALBUMIN, ALKALINE PHOSPHATASE & ZINC - TISSUE SAMPLES: LIVER & TIBIA FOR ZINC ANALYSIS
36	14	- EOSINOPHIL COUNTS - 24 HR EGG COUNTS
43	21	- EOSINOPHIL COUNTS - 24 HR EGG COUNTS
50	28	- EOSINOPHIL COUNTS - 24 HR EGG COUNTS
51	29	- KILLED 1/2 OF MICE IN EACH DIETARY TREATMENT - WORM BURDEN (SURVIVAL) - BLOOD & TISSUE SAMPLES AS FOR DAY 9 POST INF.

Figure 2.3. Protocol for experiment 1.

EXPERIMENTAL DAY	PRIMARY INFECTION DAY	CHALLENGE INFECTION DAY	PROCEDURE
			- RECEIVED MICE
0			- EXPERIMENTAL DIETS
22	0		- INFECTED 11 MICE PER DIET GROUP WITH 100 LARVAE
31	9		- KILLED 3 MICE PER DIET GROUP (ESTABLISHMENT CONTROLS) - DRUG TREATMENT OF 8 MICE PER DIET GROUP (CHALLENGE GROUP & DRUG CONTROLS)
36	14		- DRUG TREATMENT OF CHALLENGE GROUP AND DRUG CONTROLS
44	22	0	- KILLED DRUG CONTROLS - BASELINE IgG1 - RE-INFECTED CHALLENGE GROUP WITH 100 LARVAE - INFECTED 5 NAIVE MICE PER DIET GROUP (PRIMARY GROUP)
51		7	- EOSINOPHILS & IgG1
58		14	- EOSINOPHILS & IgG1
60		16	- 24 HR EGG COUNTS
65		21	- EOSINOPHILS & IgG1
66		22	- 24 HR EGG COUNTS
72		28	- EOSINOPHILS & IgG1 - 24 HR EGG COUNTS
73		29	- KILLED ALL MICE - WORM BURDEN (SURVIVAL) - BLOOD SAMPLES: PLASMA ALBUMIN, BUN, ALKALINE PHOSPHATASE & ZINC SERUM IgG1 - TISSUE SAMPLES: LIVER & TIBIA FOR ZINC ANALYSIS

TABLE 2.2 Composition of basal diet

Ingredient	Amount	
	(g/kg)	(%)
Egg white solids (spray-dried)[1]	240	24.0
Glucose[2]	296	29.6
Cornstarch[3]	296	29.6
Corn oil[4]	80	8.0
Cellulose[5]	30	3.0
Vitamin mix[6]	12	1.2
Mineral mix[7]	46	4.6

[1] Ovalbumin, ICN Biochemicals Canada Ltd., Montreal, QC.
[2] Dextrose (anhydrous), ICN Biochemicals, Canada Ltd., Montreal, QC.
[3] ICN Biochemicals, Canada Ltd., Montreal, QC.
[4] ICN Biochemicals, Canada Ltd., Montreal, QC.
[5] Alphacel, ICN Biochemicals, Canada Ltd., Montreal, QC.
[6] see Tables 2.3 - 2.4
[7] see Tables 2.5 - 2.9

TABLE 2.3 Composition of vitamin mixture

Ingredient	mg/kg diet	Mouse NRC Req't mg/kg diet[1]	Supplier
Niacin	50	10	ICN
d-Calcium pantothenate	32	10	Anachemia
Riboflavin	24	7	ICN
Pyridoxine hydrochloride	4	1	ICN
Thiamine hydrochloride	16	5	ICN
Folic acid	2	0.5	ICN
d-Biotin	3.6	0.2	ICN
Cyanocobalamin (mw 1355.2)	0.05	0.01	ICN
Alpha-tocopheryl acetate[2]	60 IU	20 IU	ICN
Menaquinone	9.0	3.0	ICN
Cholecalciferol[3]	1000 IU	150 IU	ICN
Vitamin A acetate[4]	2000 IU	500 IU	ICN
Choline chloride	4000	600	ICN

[1] Nutritional Requirements of Laboratory animals, Third Revised Edition, NRC. National Academy of Sciences, Washington, D.C., 1978.
[2] Supplied as 1.0 IU/mg dl-alpha-tocopheryl acetate.
[3] Supplied as 400 000 IU/g of vitamin D_3.
[4] Supplied as 500 000 IU/g vitamin A acetate.

TABLE 2.4 Formulation of Vitamin Mixture

Ingredient	Grams	g/kg diet
	(A) Fat Soluble	
Vitamin A acetate	0.8	
Cholecalciferol	0.5	
Alpha-tocopheryl acetate	12.0	
Menaquinone	1.8	
Cellulose	784.9	
Total	800	4
	(B) Water Soluble	
Niacin	10.0	
d-Calcium pantothenate	6.4	
Riboflavin	4.8	
Pyridoxine hydrochloride	0.8	
Thiamin Hydrochloride	3.2	
Folic acid	0.4	
d-Biotin	0.7	
Cyanocobalamin	10.0 mg	
Butylated Hydroxytoluene	20.0	
Cellulose[1]	753.7	4
TOTAL	800.0	
	(c) Choline chloride	4

[1] Alphacel, ICN Biochemicals Canada Ltd., Montreal, QC

TABLE 2.5 Composition of mineral mix[1,2] for zinc-adequate diets

Ingredient	g/kg salt	g/kg diet @ 4.6%	Supplier
$CaHPO_4$	590.43	27.16	Sigma
$KHCO_3$	222.83	10.25	Anachemia
NaCl	55.21	2.54	Anachemia
$MgSO_4$	107.61	4.95	Anachemia
$CrK(SO_4)_2.12H_2O$	0.8348	0.0384	Anachemia
$CuCO_3.Cu(OH).H_2O$	0.3413	0.0157	Anachemia
KIO_3	0.0217	0.001	Anachemia
$FeSO_4.7H_2O$	5.41	0.2489	Anachemia
$MnCO_3$	4.09	0.1883	Anachemia
$ZnCO_3$	2.50	0.115	Fisher
Na_2SeO_3	0.0087	0.0004	ICN
$NaMoO_4.H_2O$	0.0043	0.0002	Anachemia
$KF.2H_2O$	0.2152	0.0099	Anachemia
SUBTOTAL	989.51	45.52	
(Citric acid)	10.49	0.48	ICN
TOTAL	1000.00	46.00=4.6%	

[1] See Table 2.8 for elemental composition of salt mixture.
[2] See Table 2.7 for elemental composition of macroelements.

TABLE 2.6 Composition of mineral mix[1,2] for zinc-restricted diets

Ingredient	g/kg salt	g/kg diet @ 4.6%	Supplier
$CaHPO_4$	590.43	27.16	Sigma
$KHCO_3$	222.83	10.25	Anachemia
NaCl	55.21	2.54	Anachemia
$MgSO_4$	107.61	4.95	Anachemia
$CrK(SO_4)_2.12H_2O$	0.8348	0.0384	Anachemia
$CuCO_3.Cu(OH).H_2O$	0.3413	0.0157	Anachemia
KIO_3	0.0217	0.001	Anachemia
$FeSO_4.7H_2O$	5.41	0.2489	Anachemia
$MnCO_3$	4.09	0.1883	Anachemia
$ZnCO_3$	0.125	0.006	Fisher
Na_2SeO_3	0.0087	0.0004	ICN
$NaMoO_4.H_2O$	0.0043	0.0002	Anachemia
$KF.2H_2O$	0.2152	0.0099	Anachemia
SUBTOTAL	987.131	45.4088	
(Citric acid)	12.869	0.5912	ICN
TOTAL	1000.00	46.00 = 4.6%	

[1] See Table 2.9 for elemental composition of salt mixture.
[2] See Table 2.7 for elemental composition of macroelements.

TABLE 2.7 Elemental composition of macroelements for diets

Ingredient (mw)	mg/100g diet	Ca	Mg	Na	K	P	Cl	S
		mg/100g diet						
$CaHPO_4$ (136.06)	2716	800				618		
$KHCO_3$ (100.11)	1025				400			
NaCl (58.45)	254			100			154	
$MgSO_4$ (120.38)	495		100					132
sub-total	4490							
microelements	100							
a. TOTAL	4590	800	100	100	400	618	154	132
b. NRC 1978[1]		400	50		200	400		
a:b		2.0	2.0		2.0	1.5		

[1] Nutrient Requirements of Laboratory Animals, Third revised Edition, National Academy of Sciences, Washington, D.C., 1978.

TABLE 2.8 Elemental composition of salt mixture for zinc-adequate diets

Ingredient (mw)	mg/kg diet	Cr	Cu	Fe	I	Mn	Mo	Se	Zn	F
		mg/kg diet								
$CrK(SO_4)_2.12H_2O$ (499.40)	38.41	4								
$CuCO_3.Cu(OH).H_2O$ (222.13)	15.73		9							
KIO_3 (214.00)	1.01				.6					
$FeSO_4.7H_2O$ (278.01)	248.94			50						
$MnCO_3$ (114.94)	188.29					90				
$ZnCO_3$ (125.38)	115.06								60	
Na_2SeO_3 (172.96)	0.438							.2		
$NaMoO_4.2H_2O$ (218.97)	0.228						.1			
$KF.2H_2O$ (94.12)	9.91									2
Citric acid	381.98									
a. TOTAL	1000	4	9	50	.6	90	.1	.2	60	2
b. NRC 1978[1]		2	4.5	25	.3	45			30	
a:b		2	2	2	2	2			2	

[1] Nutrient Requirements of Laboratory Animals, Third revised Edition, National Academy of Sciences, Washington, D.C., 1978.

TABLE 2.9 Elemental composition of salt mixture for zinc-restricted diets

Ingredient (mw)	mg/kg diet	Cr	Cu	Fe	I	Mn	Mo	Se	Zn	F
		mg/kg diet								
$CrK(SO_4)_2.12H_2O$ (499.40)	38.41	4								
$CuCO_3.Cu(OH).H_2O$ (222.13)	15.73		9							
KIO_3 (214.00)	1.01				.6					
$FeSO_4.7H_2O$ (278.01)	248.94			50						
$MnCO_3$ (114.94)	188.29					90				
$ZnCO_3$ (125.38)	6.0								3	
Na_2SeO_3 (172.96)	0.438							.2		
$NaMoO_4.2H_2O$ (218.97)	0.228						.1			
$KF.2H_2O$ (94.12)	9.91									2
Citric acid	491.04									
a. TOTAL	1000	4	9	50	.6	90	.1	.2	3	2
b. NRC 1978[1]		2	4.5	25	.3	45			30	
a:b		2	2	2	2	2			0.1	

[1] Nutrient Requirements of Laboratory Animals, Third revised Edition, National Academy of Sciences, Washington, D.C., 1978.

CHAPTER 3. RESULTS

1. PRELIMINARY STUDY

In this preliminary study, only the effects of protein restriction are reported for the parasite parameters, whereas both the effects of zinc restriction and the effects of protein restriction are reported for the nutritional parameters. This was necessary since problems were encountered with L_3 viability during the infection procedure in the zinc deficient animals. During the procedure, the infection dose was first administered randomly among the mice receiving diets containing 3 mg zinc/kg because these animals were housed in a separate cage rack from mice receiving diets containing 60 mg zinc/kg. Prior to beginning infection of the second rack of cages (the 60 mg zinc/kg diet group), the inoculum was reconfirmed. The number of live larvae per aliquot was unacceptably low (30 compared to an intended 100). For this reason a new batch of larvae was used to infect the 60 mg zinc/kg diet groups. Parasite establishment in the 60 mg zinc/kg diet fed animals was normal and much higher than that in the 3 mg zinc/kg diet fed animals. Since the L_3 used for the two groups (3 mg/kg vs. 60 mg/kg zinc) were from different batches and because the establishment differed greatly in the two groups, the effect of zinc restriction on parasite parameters was not examined.

A. PARASITE PARAMETERS

1. Worm burdens

The data are summarized in Table 3.1.1. Parasite establishment measured on day 9 post-

infection was not affected by the level of dietary protein. There was no significant difference in the total number of worms, the number of male and female worms or the male/female ratio among the three dietary groups. The number of immature L_4 that remained in the intestinal wall on day 9 post-infection, however, was significantly lower in the 3% protein group compared to the 7% and 24% protein groups (AOV: p=0.0001). By day 29 post-infection, a greater number of worms survived in the 3% protein group than in the 7% and 24% protein groups (AOV: p=0.004). At this time, there was a significantly greater number of male worms in the 3% protein group than in the 24% protein group (AOV: p=0.007); the number of worms in the 7% protein group was intermediate and not significantly different from either the 3% or 24% protein groups. There was also a greater number of female worms in the 3% protein group than in the 7% and 24% protein groups (AOV: p=0.005), which were not significantly different from one another. The male/female ratio was not statistically different among the three dietary groups.

2. Worm egg production

Data on worm egg production were expressed as eggs/day for days 14, 21 and 28 post-infection (Table 3.1.2), and as eggs/female worm for day 28 when data on numbers of females were available. A significant effect of dietary protein on egg production was detected at day 21 post-infection only, where egg output was significantly greater in the 3% and 7% protein groups than in the 24% protein group (AOV: p=0.008). The average eggs/female worm produced on day 28 post-infection were 770.6 ± 183.6, 387.1 ± 96.6 and 331.2 ± 92.1 for the 24%, 7% and 3% diet groups respectively (Figure 3.1.1), but these differences were not statistically significant.

3. Eosinophils

The numbers of eosinophils in whole blood measured on days 0, 7, 14 and 21 post-infection are shown in Table 3.1.3. There was no effect of the level dietary protein on eosinophil numbers at any measured points.

B. NUTRITIONAL PARAMETERS

1. DTH test

The results from the DTH response prior to infection are shown in Figure 3.1.2. There was no significant effect of the level of dietary protein or dietary zinc on the response of mice to DNFB after 21 days on the experimental diets.

2. Food intake

The food intake of the animals necropsied on day 9 post-infection is shown in Table 3.1.4. The cumulative food intake was significantly greater in mice fed 7% protein diets than in those fed the 24% and 3% protein diets (AOV: $p=0.04$); there was no significant difference between the 3% and the 24% protein groups. The relative food intake calculated as the total food consumed divided by the final body weight was significantly greater in animals fed 3% protein diets than in the other groups (AOV: $p<0.0001$).

In contrast, on day 29 post-infection (Table 3.1.5) cumulative food intake was significantly lower in the 3% protein groups than in the 7% and 24% protein groups (AOV: $p<0.0001$). which were not significantly different from one another. The relative food intake, as on day 9, was significantly greater in the 3% protein groups compared to the other groups (AOV:

p<0.0001).

The cumulative and relative food intake were not affected by the level of dietary zinc on day 9 and 29 post-infection, and there was no interaction between the levels of dietary protein and zinc.

3. Body and organ weights

The data are shown in Table 3.1.4 for the groups killed on day 9 post-infection and in Table 3.1.5 for the groups killed on day 29 post-infection. The initial body weight was not significantly different among the dietary groups for the animals necropsied on either day 9 post-infection or on day 29 post-infection.

For the animals killed on day 9 post-infection (Table 3.1.4), final body weight was significantly lower in the 3% protein groups than in the other groups (AOV: p<0.0001). In contrast, the body weight gain was significantly greater in the 7% protein groups compared with the 24% and 3% protein groups, while the body weight gain was significantly lower in the 3% protein groups than in the 7% and 24% protein groups (AOV: p<0.0001). The absolute liver weight but not the relative liver weight (calculated as the % of the final body weight) was significantly different among the dietary groups. Mice fed the 3% protein diets had smaller livers than mice fed the 7% and 24% protein diets, which were not different from one another (AOV: p<0.0001). There was no effect of dietary zinc or interaction between dietary protein and zinc levels on final body weight or liver weights. Both protein and zinc had significant effects on absolute (AOV: protein p<0.0001; zinc p=0.02) and relative spleen weights (AOV: protein p=0.0004; zinc p=0.004) which were lower in the 3% protein groups compared to the 7% and

24% protein groups, and in the 3 mg/kg zinc groups compared to the 60 mg/kg zinc groups. The thymus weight was significantly lower in the 3% protein groups than in the 7% protein groups but there was no difference between the 3% and 24% protein groups (AOV: p=0.04); the relative thymus weight did not differ among the diet groups. Again, there was no significant effect of dietary zinc and no significant interaction between levels of dietary protein and zinc on thymus weights.

On day 29 post-infection (Table 3.1.5), final body weight and body weight gain were significantly lower in mice fed the 3% protein diets than in the other groups (AOV: p<0.0001). Both the absolute and relative liver weights were affected by the level of dietary protein (AOV: p<0.0001). Mice fed 3% protein had significantly lower absolute liver weight but significantly higher relative liver weight than the other groups. The absolute spleen weight was significantly lower in the 3% protein groups compared to the other groups (AOV: p<0.0001), but the relative spleen weight was not affected by diet. Finally, the absolute thymus weight was not affected by diet but the relative thymus weight was significantly greater in the 3% protein groups compared to the 7% protein groups (AOV: p=0.007); there was no difference between the 7% and the 24% protein groups or between the 3% and the 24% protein groups. There was no significant effect of dietary zinc level and no interaction between dietary protein and zinc levels on any of the body and organ weights, on day 29 post-infection.

4. Plasma analysis and tibia zinc concentration

The data for day 9 post-infection are shown in Table 3.1.6. Plasma albumin concentration was affected by both the level of dietary protein (AOV: p=0.0002) and the level of dietary zinc

(AOV: p=0.01). Albumin was significantly lower in the 3% protein groups compared to the higher protein levels and in the 60 mg/kg zinc groups compared to the 3 mg/kg zinc groups. Plasma alkaline phosphatase and plasma zinc concentrations were not affected by either the level of dietary protein or zinc. Tibia zinc concentration was affected by both protein (AOV: p<0.0001) and zinc (AOV: p=0.03), and there was a significant interaction effect between protein and zinc (AOV: p=0.03). The interaction was such that animals fed 3 mg/kg zinc had lower tibia zinc than animals fed 60 mg/kg zinc when the protein level was 24% but not 7% or 3%. This is due to the fact that the zinc content in the tibia of mice fed 60 mg/kg zinc also decreased significantly with decreasing dietary protein level.

On day 29 post-infection (Table 3.1.7) the plasma albumin concentration was significantly higher in the 7% protein groups than in the other groups, and there was no difference between the 3% and 24% protein groups (AOV: p=0.002). Plasma alkaline phosphatase activity was significantly higher in mice fed 3% protein compared to the other groups (AOV: p<0.0001). There was no significant effect of level of dietary zinc or interaction between dietary protein and zinc levels on either plasma albumin concentration or plasma alkaline phosphatase activity. Plasma zinc concentration was significantly affected by protein (AOV: p=0.0008) and there was also an interaction between protein and zinc (AOV: p=0.03). It can be seen from Table 3.1.7 that plasma zinc was signif cantly lower in animals fed 60 mg/kg zinc with 3% protein than in animals fed 60 mg/kg zinc with 24% protein or 3 mg/kg zinc with 7% protein. Plasma zinc concentration of animals fed 3 mg/kg zinc with 3% protein was lower than that of animals fed 60 mg/kg zinc and 24% protein, but there was no difference in plasma zinc level among the other groups. Tibia zinc concentration was affected by both protein (AOV: p<0.0001) and zinc

($p<0.0001$) and there were also protein and zinc interactions (AOV: $p=0.03$). Tibia zinc was lower in animals fed 3 mg/kg zinc than in animals fed 60 mg/kg zinc when comparisons were made at the 24% and 7% protein levels but not at the 3% protein level, where there was no difference between the 3 mg/kg and 60 mg/kg zinc fed animals. Again, this is because tibia zinc also decreased with protein levels. In contrast to day 9 post-infection, however, the decrease was observed for the 3% protein fed animals, only. The 7% protein fed animals were no longer different from the 24% protein fed animals at either zinc levels.

II. EXPERIMENT 1

A. PARASITE PARAMETERS

1. Worm burdens

Data for the establishment controls are shown in Table 3.2.1. The total number of worms and the number of male worms on day 9 post-infection were significantly affected by the level of dietary protein (AOV: $p=0.02$ and 0.03 respectively). The total number of worms and the number of male worms were significantly greater in the 3% protein groups than in the 7% protein groups, but neither of these groups differed from the controls (24% protein). There was no significant effect of dietary zinc level on parasite establishment or on the number of male worms. Neither the number of female worms, the male/female ratio, nor the number of L_4 that remained in the intestinal wall were affected by the level of dietary protein or zinc.

The drug controls in each dietary treatment, which received the same drug treatment as the challenge infection group and were killed on the day of the challenge infection, were all free

of worms. Therefore, the drug treatment was not affected by dietary treatment and was judged to be equally effective in all dietary groups.

The data for parasite survival on day 29 post-infection are summarized in Table 3.2.2. In the primary infection group, mice fed 3% and 7% protein had significantly more worms than the mice fed 24% protein (AOV: p=0.0006), and mice fed 3 mg/kg zinc had significantly more worms than the mice fed 60 mg/kg zinc (AOV: p=0.03). The number of male worms was also significantly greater in the 3% and 7% protein groups than in the 24% protein groups (AOV: p=0.0006), and in the 3 mg/kg zinc groups compared to the 60 mg/kg zinc groups (AOV: p=0.03). The number of female worms was significantly higher in the 3% protein groups than in the 24% protein groups (AOV: p=0.008); the 7% protein groups were intermediate and not significantly different from either the 3% or 24% protein groups. There were also significantly more female worms in the 3 mg/kg zinc groups than in the 60 mg/kg zinc groups (AOV: p=0.02). Finally, there was no effect of the level of dietary protein or zinc on the male/female ratio. Mice which had either zero male or zero female worms were not included in the calculating ratio.

In the challenge infection group, mice fed 3% protein had significantly higher worm burdens than mice fed 7% and 24% protein, which were not significantly different from one another (AOV: p<0.0001). The number of male worms and female worms were also significantly higher in the 3% protein groups compared to the 7% and 24% protein groups which, again, were not significantly different from one another (AOV: p<0.0001 for both male and female worms). The male/female ratio was not affected by diet and mice with either zero male or zero female were not included in the ratio. In contrast to the primary infection group, the level of zinc in the

diet had no effect on final worm burdens.

There was no significant interaction between the levels of dietary protein and zinc on any of the worm burden parameters for either the primary or the challenge infection groups.

2. Worm egg production

The data for egg production were expressed as total eggs/day for days 16, 22 and 28 post-infection (Table 3.2.3) and as eggs/female worm on day 28 post-infection (Table 3.2.4).

For the primary infection group (Table 3.2.3), egg production per day was comparable among the 6 dietary groups on day 16 post-infection, but by day 22 post-primary infection, there was a significant effect of protein (AOV: p=0.0001) and a significant zinc and protein interaction (AOV: p=0.006) on total egg production per mouse. The interaction is shown in Table 3.2.4, and in general it can be seen that egg output was greater in mice fed 3 mg/kg zinc than in mice fed 60 mg/kg zinc when the protein level of the diet was 3%, but there was no difference due to zinc when the protein levels were 7% or 24%. On day 28 post-primary infection, mice fed 3% protein had significantly greater egg output than mice fed 7% and 24% protein, which were not significantly different from one another (AOV: p=0.004). There was also a significant effect of dietary zinc, such that mice fed diets containing 3 mg zinc/kg had higher egg output than mice fed diets containing 60 mg zinc/kg (AOV: p=0.02).

Repeated measures analyses were performed on egg production from day 14 to 28. There was a significant effect of time on total egg output (Wilks Lambda p=0.04), and also an interaction of time with protein level (Wilks Lambda p=0.001). Egg output decreased with time in animals fed 24% and 7% protein, was stable in animals fed 3% protein and 60 mg/kg zinc,

and increased with time in animals fed 3% protein with 3 mg/kg zinc. The test of hypothesis for among diets effects resulted in a significant effect of protein (repeated measures p=0.01) on overall egg production.

The number of eggs produced per female worm on day 28 post-primary infection (Table 3.2.4) was affected by protein (AOV: p=0.001) and there was also an interaction between protein and zinc (AOV: p=0.0008). Mice fed 24% protein and 60 mg/kg zinc had fewer eggs produced per female worm than all the other groups except the 7% protein and 3 mg/kg zinc group. There was no significant difference in eggs per female worms among the other groups (mice with zero female worms were not included).

Total egg production per day in the challenge infection group (Table 3.2.3) was affected by the level of protein on all three days (AOV: $p<0.0001$ for day 16, 22 and 28). Mice fed 3% protein had significantly greater egg output than mice fed other protein levels. In contrast to the primary infected group, there was no significant effect of dietary zinc and no interaction between dietary protein and zinc levels on total eggs produced per day.

Repeated measures analysis yielded no statistically significant effects of time and no interaction between time and diet. Therefore, the pattern of egg output through time did not change in each dietary group. The test of hypothesis for among diets effects, however, indicated a significant effect of protein level (repeated measures p=0.0001). Mice fed 3% protein produced more eggs throughout time than mice fed other protein levels.

The number of eggs produced per female worm on day 28 post-challenge infection (Table 3.2.4) was affected by the protein level in the diet (AOV: p=0.007). Mice fed 7% protein had fewer eggs produced per female worm than mice fed either 24% protein or 3% protein, whereas

there was no difference between the 24% and 3% protein fed animals. There was no significant effect of dietary zinc and no interaction between dietary protein and zinc levels on eggs/female worm in the challenge infection.

B. Immunological parameters

1. Eosinophils

The number of eosinophils found in blood samples from the mice is shown in Table 3.2.5 for both the primary and challenge infection groups.

In the course of the primary infection, eosinophils were affected by dietary zinc on two occasions. Mice fed diets containing 3 mg zinc/kg had lower eosinophilia than mice fed diets containing 60 mg zinc/kg on day 7 (AOV: p=0.005) and on day 21 (AOV: p=0.002) post-primary infection. There was no effect due to zinc on day 14 post-primary infection. Eosinophil numbers were also affected by the level of dietary protein on day 14 (AOV: p=0.002) and day 21 post-primary infection (AOV: p=0.02). On day 14, mice fed 7% protein had higher eosinophil counts than mice fed 24% and 3% protein, but on day 21, mice fed 3% protein had lower eosinophilia than mice fed 24% and 7% protein. Repeated measures analysis indicated a significant effect of time on eosinophilia (Wilks Lambda p=0.0001), a significant interaction of protein level with time (Wilks Lambda p=0.01) and a significant interaction of time with protein and zinc (Wilks Lambda p=0.01). The test of hypothesis for the among diets effects indicated a significant effect of zinc (repeated measures p=0.009), and it can be seen from Table 3.2.5 that mice fed diets containing 3 mg zinc/kg had overall lower eosinophil numbers than mice fed diets containing 60 mg zinc/kg.

In the course of the challenge infection, eosinophilia was affected by dietary zinc on one occasion only. On day 7 post-challenge infection, mice fed diets containing 3 mg zinc/kg had lower eosinophilia than mice fed 60 mg/kg zinc (AOV: p=0.006). The number of eosinophils was affected by dietary protein level on all three measurement days. On day 7 post-challenge, mice fed 3% protein had lower eosinophilia than mice fed 7% and 24% protein, which did not differ from one another (AOV: p=0.002). On day 14 post-challenge infection, mice fed 3% protein had lower eosinophilia than mice fed 24% protein, and mice fed 7% protein were intermediate and not significantly different from the 24% or 3% protein groups (AOV: p=0.008). Finally, on day 21 post-challenge infection, mice fed 3% protein had lower eosinophilia than mice fed 7% and 24% protein, which were not significantly different from one another (AOV: p=0.003). Repeated measures analysis indicated a significant effect of time on eosinophil pattern (Wilks Lambda p=0.0001) but there was no interaction of time with diet. The test of hypothesis for the among diets effect indicated a significant effect of protein level on eosinophilia through time. From Table 3.2.5 it can be seen that mice fed 24% and 7% protein had higher eosinophilia than mice fed 3% protein throughout the experimental period.

2. Serum IgG1 profile

The serum IgG1 profile is shown in Table 3.2.6. In the primary infection protocol, baseline levels of IgG1 (prior to infection) were not statistically different among the dietary groups. By day 7 post-primary infection, there was a significant interaction between dietary protein and zinc levels such that the animals fed 3% protein with 3 mg/kg zinc had a significantly higher serum IgG1 level than all the other groups (AOV: p=0.02). Therefore, these

animals had an earlier rise in IgG1 concentration following a primary infection. For the remainder of the primary infection, serum IgG1 concentration was affected by dietary protein level, only. On day 14 and 21, mice fed 3% protein had significantly lower serum IgG1 than mice fed 7% and 24% protein, which were not different from one another (day 14, AOV: p=0.01 and day 21, AOV: p=0.03). On day 29, mice fed 3% protein had significantly lower serum IgG1 than mice fed 24% protein, but mice fed 7% protein were intermediate and not significantly different from mice fed either 24% or 3% protein (AOV: p=0.01).

Repeated measures analysis indicated a significant effect of time on serum IgG1 profile (Wilks Lambda p<0.0001) and a significant interaction between time and dietary protein level (Wilks Lambda p=0.05) in a primary infection. It can be seen from Table 3.2.6 that serum IgG1 levels rose between day 0 and day 21 in all the groups but that, by day 29, serum IgG1 levels continued to rise in animals fed 24% protein whereas it either plateaued or decreased slightly in animals fed 7% and 3% protein. Thus, animals fed 24% protein had a continued rise in serum IgG1 over the entire experimental period, whereas serum IgG1 peaked on day 21 in animals fed 7% and 3% protein and subsequently declined in the 7% protein fed animals and plateaued in the 3% protein fed animals. Also, the test of hypothesis for the among diets effects indicated an overall effect of dietary protein level (repeated measure p=0.01).

In the challenge infection protocol, baseline IgG1 levels were higher than for the primary infection protocol since the challenge infected animals had been previously exposed to the parasite when they received the anthelmintic-abbreviated primary infection. In this situation, baseline serum IgG1 concentration (prior to the challenge infection) was affected by both dietary protein and zinc levels: mice fed diets containing 3 mg zinc/kg had significantly lower serum

IgG1 concentration than mice fed diets containing 60 mg zinc/kg (AOV: $p<0.0001$), and mice fed 3% protein had significantly lower serum IgG1 levels than mice fed 24% protein. Mice fed 7% protein were intermediate and not significantly different from mice fed either 24% or 3% protein (AOV: $p=0.01$). On day 7, serum IgG1 was affected by protein level, only, such that mice fed 7% and 3% protein had significantly lower serum IgG1 levels than mice fed 24% protein (AOV: $p=0.01$). Serum IgG1 concentrations were not different among the dietary groups on day 14 post-challenge infection, but on day 21, IgG1 level was significantly lower in mice fed 3% protein than in mice fed 24% protein, whereas mice fed 7% protein were intermediate and not significantly different from mice fed either 24% or 3% protein (AOV: $p=0.02$). On day 29, IgG1 concentration was significantly lower in mice fed 3% protein than in mice fed either 7% or 24% protein, which were not different from one another (AOV: $p<0.0001$).

Repeated measures analysis of IgG1 profile in the challenge infection protocol indicated a significant effect of time (Wilks Lambda $p<0.0001$) and also a significant interaction between time and dietary protein level (Wilks Lambda $p=0.03$). It can be seen in Table 3.2.6 that serum IgG1 levels rose more rapidly in animals fed 24% protein, peaked on day 21 and decreased by day 29, whereas IgG1 levels rose less rapidly in animals fed 7% protein but also peaked on day 21 and decreased by day 29. In contrast, IgG1 level peaked on day 14 in animals fed 3% protein, plateaued until day 21 and decreased by day 29. The test of hypothesis for the among diets effects revealed a significant effect of protein level on the overall IgG1 concentration (repeated measures $p=0.04$). There was no effect of zinc restriction on the serum IgG1 level in either the primary or the challenge infection protocol.

C. Nutritional parameters

The nutritional parameters of mice necropsied on day 29 post-infection are summarized in Tables 3.2.7 and 3.2.8. The data was first tested with infection in the statistical model to determine whether infection type (primary vs challenge) had an effect on nutritional parameters. When infection type (primary vs challenge) was not significant, the data for the primary and challenge infection groups were pooled and reanalysed for the main effects of zinc and protein The results for the pooled data are shown in Table 3.2.7. When infection type (primary vs challenge) was significant the data were not pooled but were re-analyzed separately for the main effects of zinc and protein. In this case the data for the primary and challenge infection groups are shown separately in Table 3.2.8.

1. Food intake

The cumulative food intake throughout the experiment was not affected by infection and the data are shown in Table 3.2.7. There was a significant effect of dietary protein level on total food intake (AOV: $p<0.0001$). Mice fed 7% protein ate more food than mice fed 24% and 3% protein, which did not differ from one another. The relative food intake, calculated as the total food eaten divided by the body weight gain, was also significantly affected by dietary protein level (AOV: $p<0.0001$), such that the relative food intake was significantly increased in mice fed 3% protein. There was no significant effect of dietary zinc and no significant interaction between dietary protein and zinc levels on food intake.

2. Body and organ weights

Body weight and organ weights were not affected by infection type (Table 3.2.7) with the exception of liver weight which is described in Table 3.2.8. The initial body weight of mice was not statistically different among the dietary groups. The final body weight and the body weight gain in both the primary and challenge infection groups, however, were affected by the level of protein in the diet (AOV: $p<0.0001$). Mice fed 3% protein had lower final body weight and lower body weight gain than mice fed 7% and 24% protein, which were not significantly different from one another.

The spleen weight was affected by dietary protein level (Table 3.2.7) (AOV: $p<0.0001$). Mice fed 7% protein had greater spleen weights than mice fed 24% and 3% protein, and mice fed 3% protein had lower spleen weights than mice fed either 24% or 7% protein. The relative spleen weights, calculated as the percent of body weight. did not follow the same pattern. Mice fed 7% protein had higher relative spleen weights than mice fed 24% and 3% protein, which were not significantly different from one another. The thymus weight was significantly smaller in mice fed 3% protein compared to the higher protein levels (AOV: $p=0.0001$), but the relative thymus weight was not affected by dietary treatment. There was no effect of dietary zinc or significant interaction between dietary protein and zinc levels on body or organ weights.

The liver weight and relative liver weight were affected by the infection type (AOV: $p=0.025$ and 0.006 respectively) (Table 3.2.8), such that both were significantly greater in the challenge infected animals than in the primary infected animals. In the primary infection group, liver weight was affected by the level of protein (AOV: $p<0.0001$), but not by the level of dietary zinc. The 3% protein groups had smaller livers than the 7% and 24% protein groups, which did

not differ from one another. In contrast, relative liver weight was affected by dietary zinc (AOV: p=0.008), but not by dietary protein level. Mice fed diets containing 3 mg zinc/kg had higher relative liver weight than mice fed diets containing 60 mg zinc/kg. In the challenge infection group, there was a significant protein effect (AOV: p<0.0001) and also a significant interaction between protein and zinc levels on liver weight. Mice fed diets containing 3 mg zinc/kg and 3% protein had significantly smaller livers than all the other groups, whereas mice fed diets containing 60 mg zinc/kg and 3% protein had smaller livers than mice fed 60 mg/kg zinc and 7% protein only. The relative liver weight was affected by zinc (AOV: p=0.03) and by protein (AOV: p=0.001), and there was also an interaction between dietary protein and zinc levels (AOV: p=0.008). In this case, mice fed diets containing 60 mg zinc/kg and 3% protein had higher relative liver weights than all the other groups.

3. Plasma analysis

Plasma albumin and alkaline phosphatase activity were affected by infection type (AOV: p=0.002 and p=0.01 respectively) and, therefore, the data are shown separately in Table 3.2.8. The pooled data for BUN is shown in Table 3.2.7 as this parameter was not affected by infection type.

Plasma albumin and alkaline phosphatase activity exhibited a different pattern in the primary versus challenge infected animals. In the primary infected group, albumin was affected by dietary zinc (AOV: p<0.0001) but not by dietary protein. Animals fed diets containing 3 mg zinc/kg had higher plasma albumin than animals fed diets containing 60 mg zinc/kg. Alkaline phosphatase activity was affected by dietary zinc (AOV: p=0.001) and by dietary protein

($p<0.0001$), and there was also a significant interaction between dietary protein and zinc levels (AOV: $p=0.03$). The interaction was such that alkaline phosphatase activity was greater in mice fed 3% protein than mice fed higher levels of protein, and the increase was significantly greater in mice fed 3% protein with 3 mg zinc/kg diet than in mice fed 3% protein with 60 mg zinc/kg diet. In the challenge infected group, plasma albumin was affected by the level of dietary protein (AOV: $p=0.05$) and the level of dietary zinc (AOV: $p=0.02$). Mice fed 7% protein had higher albumin concentration than mice fed 24% protein but did not differ from mice fed 3% protein. Mice fed 3% and 24% protein were not significantly different from one another. Also, mice fed diets containing 3 mg zinc/kg had lower albumin concentrations than mice fed diets containing 60 mg zinc/kg. Alkaline phosphatase activity was affected by the level of dietary protein (AOV: $p<0.0001$) but not by the level of zinc. Alkaline phosphatase activity was significantly higher in mice fed 3% protein than mice fed higher levels of dietary protein.

BUN was not affected by infection type (primary vs. challenge). In both infection groups, BUN was significantly affected by protein level (AOV: $p<0.0001$), such that this index decreased significantly with decreasing protein levels. There was no effect of dietary zinc on BUN concentration.

4. Tissue trace minerals analysis

Tibia and liver zinc concentrations were significantly affected by infection type (AOV: $p=0.01$ and $p=0.001$ respectively), and therefore, the results are shown separately for each infection group in Table 3.2.8. Liver copper and the ratio of liver zinc to liver copper were not affected by infection type and the pooled results are shown in Table 3.2.7.

The concentration of zinc in the tibia was higher in the challenge infected group than in

the primary infected group (AOV: p=0.001). In both the primary and challenge infection groups, tibia zinc concentration was affected by protein (AOV: p<0.0001) and zinc (AOV: p<0.0001). Tibia zinc concentration was significantly lower in mice fed 3% protein compared to higher protein levels; there was no difference between the 7% and 24% protein groups. Tibia zinc was further decreased by zinc restriction such that mice fed diets containing 3 mg zinc/kg had lower tibia zinc than mice fed diets containing 60 mg zinc/kg for each level of dietary protein intake.

Liver zinc concentration was also different between the primary and challenge infected animals. Liver zinc was higher in the primary infected group than in the challenge infected group (AOV: p=0.01). In the primary infection group, liver zinc concentration was affected by dietary zinc (AOV: p=0.001) and by dietary protein (AOV: p=0.009). Mice fed diets containing 3 mg zinc/kg had lower liver zinc than mice fed diets containing 60 mg zinc/kg, and mice fed 3% protein had lower liver zinc than mice fed 24% protein; the 7% protein group was intermediate and not significantly different from either the 24% or 3% protein groups. In the challenge infection group, liver zinc concentration was affected by dietary protein (AOV: p<0.0001) and by an interaction between zinc and protein (AOV: p=0.009). The interaction was such that the concentration of zinc was lower in the 3 mg/kg and 3% protein group compared to all other groups, whereas there was no effect of a 3% protein diet when the dietary level of zinc was 60 mg/kg.

Liver copper concentration was not affected by the different infection protocols (Table 3.2.7), but it was affected by the level of protein in the diet (AOV: p=0.04). The liver copper concentration was significantly higher in mice fed 3% protein than in the animals fed 7% and 24% protein, which were not significantly different from one another. The ratio of zinc to copper

in the liver was also not affected by the different infection protocols but was affected by the protein level in the diet (AOV: $p<0.0001$). Mice fed a 3% protein diet had a lower ratio than mice fed a 7% or a 24% protein diet, which did not differ from one another.

TABLE 3.1.1 The effect of dietary protein restriction on *H.polygyrus* establishment on day 9 and survival on day 29 post-primary infection (preliminary)[1,2].

Parameter (Absolute Number)	Protein Level (%) 24	7	3
		DAY 9	
Total worms[3]	60.8 ± 4.3	61.6 ± 5.7	60.8 ± 7.2
L_4[4]	7.6 ± 0.5[a]	7.4 ± 1.2[a]	1.4 ± 0.5[b]
Male worms[5]	21.8 ± 2.1	27.6 ± 1.7	27.6 ± 3.3
Female worms[6]	31.0 ± 3.6	29.4 ± 3.2	31.8 ± 4.9
Male/Female ratio[7]	0.74 ± 0.1	0.99 ± 0.1	0.93 ± 0.1
		DAY 29	
Total worms[8]	5.4 ± 1.4[b]	25.4 ± 13.9[b]	54.0 ± 2.8[a]
Male worms[9]	1.8 ± 0.6[b]	10.8 ± 5.9[ab]	22.4 ± 2.6[a]
Female worms[10]	3.6 ± 0.9[b]	14.6 ± 8.1[b]	31.6 ± 1.6[a]
Male/Female ratio[11]	0.55 ± 0.2	0.90 ± 0.2	0.72 ± 0.1

[1] Values are means ± SEM; the number of animals in each group was 5.
[2] Values within each parameter with different letter superscripts are statistically different ($p \leq 0.05$) based on Tukey's multiple pairwise comparisons.
[3-11] Significance of main effects from AOV:
[3,5-7,11] protein (NS)
[4] protein ($p=0.0001$)
[8] protein ($p=0.004$)
[9] protein ($p=0.007$)
[10] protein ($p=0.005$)

TABLE 3.1.2 The effect of dietary protein restriction on *H. polygyrus* egg output in mice during a primary infection (preliminary)[1,2]

Day Post-Infection	Protein Level (%)		
	24	7	3
14[3]	8898 ± 1105	12851 ± 2539	8090 ± 732
21[4]	5939 ± 1082[b]	12698 ± 1882[a]	11802 ± 853[a]
28[5]	3059 ± 1345	6886 ± 3977	12784 ± 1974

[1] Values are means ± SEM; the number of animals in each group was 5.
[2] Values within each infection day with different letter superscripts are statistically different ($p \leq 0.05$) based on Tukey's multiple pairwise comparisons.
[3-5] Significance of main effects from AOV:
[3,5] protein (NS)
[4] protein ($p=0.008$)

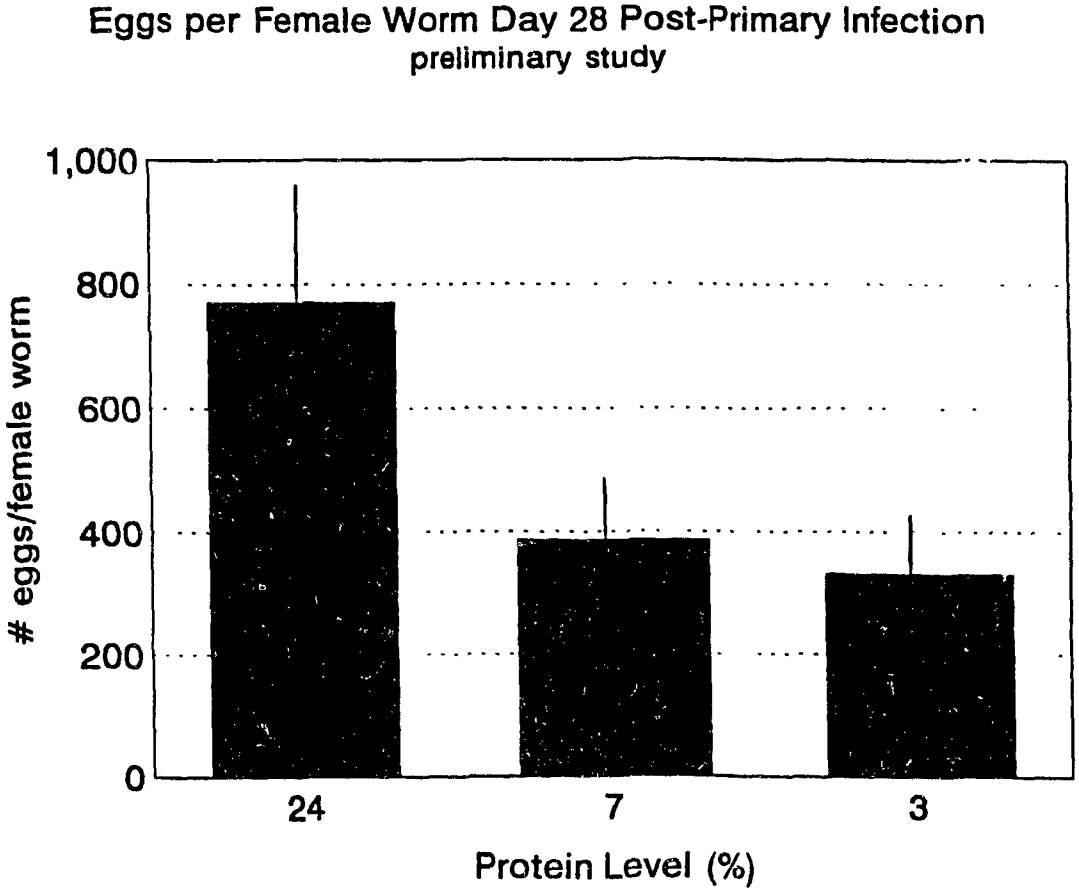

Figure 3.1.1 The effect of dietary protein restriction on H. Polygyrus female worm egg production on day 29 post-primary infection.

TABLE 3.1.3 The effect of dietary protein restriction on the number of eosinophils/mm^3 in whole blood of mice infected with *H. polygyrus* (preliminary)[1]

Day Post-Infection	Protein Level (%)		
	24	7	3
0[2]	220.0 ± 47.3	148.0 ± 17.7	144.0 ± 0.0
7[2]	352.0 ± 46.1	419.2 ± 50.4	668.8 ± 201.7
14[2]	172.8 ± 38.7	467.2 ± 207.7	832.0 ± 547.4
21[2]	100.0 ± 64.8	88.0 ± 26.5	80.0 ± 0.8

[1] Values are means ± SEM; the number of animals in each group was 5.
[2] Significance of main effects from AOV: protein (NS)

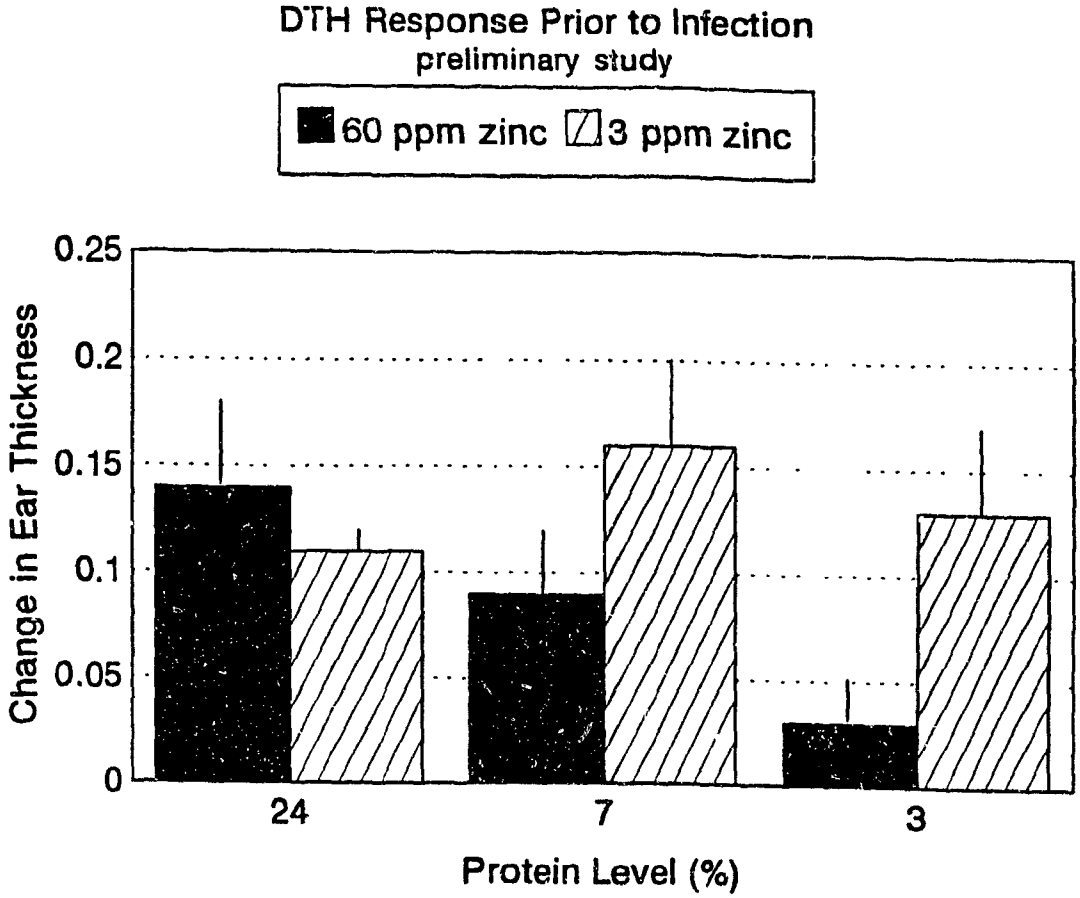

Figure 3.1.2 The effect of dietary protein and zinc restrictions on the DTH response to DNFB on experimental day 21.

TABLE 3.1.4 The effect of dietary protein and zinc restrictions on nutritional parameters of mice on day 9 post-infection (preliminary, experimental day 32)[1,2]

Parameter	Zinc Level (mg/kg)	Protein Level (%) 24	7	3	pooled
Total food intake (g)[3]	**60**	85.1 ± 2.7	95.5 ± 2.8	88.7 ± 3.9	89.7 ± 2.1
	3	90.6 ± 2.9	100.0 ± 2.0	87.8 ± 3.1	92.9 ± 2.1
	pooled	87.5 ± 2.1[b]	97.7 ± 1.8[a]	88.2 ± 2.4[b]	
Food intake/ body wt gain[4]	**60**	15.1 ± 1.3	13.8 ± 0.6	67.0 ± 17.1	31.9 ± 8.5
	3	14.7 ± 1.2	14.9 ± 0.2	51.7 ± 6.1	28.0 ± 5.3
	pooled	14.9 ± 0.8[b]	14.4 ± 0.4[b]	59.4 ± 8.9[a]	
Initial body wt (g)[5]	**60**	10.3 ± 0.6	9.8 ± 0.5	11.0 ± 0.4	10.4 ± 0.3
	3	10.3 ± 0.6	11.3 ± 0.3	10.4 ± 0.1	10.7 ± 0.2
	pooled	10.3 ± 0.4	10.5 ± 0.4	10.7 ± 0.2	
Final body wt (g)[6]	**60**	16.0 ± 0.6	16.7 ± 0.6	12.7 ± 0.6	15.1 ± 0.6
	3	16.6 ± 0.7	18.0 ± 0.2	12.3 ± 0.3	15.5 ± 0.7
	pooled	16.3 ± 0.4[a]	17.4 ± 0.4[a]	12.5 ± 0.3[b]	
Body wt gain (g)[7]	**60**	5.8 ± 0.4	6.9 ± 0.3	1.6 ± 0.3	4.8 ± 0.6
	3	6.2 ± 0.3	6.7 ± 0.1	1.8 ± 0.3	4.8 ± 0.6
	pooled	6.0 ± 0.3[b]	6.8 ± 0.2[a]	1.7 ± 0.2[c]	
Absolute Liver wt (g)[8]	**60**	0.68 ± 0.02	0.70 ± 0.03	0.50 ± 0.04	0.64 ± 0.02
	3	0.70 ± 0.02	0.77 ± 0.02	0.56 ± 0.02	0.67 ± 0.03
	pooled	0.69 ± 0.01[a]	0.73 ± 0.02[a]	0.55 ± 0.02[b]	
Relative Liver wt (% bwt)[9]	**60**	4.25 ± 0.12	4.17 ± 0.06	4.21 ± 0.13	4.21 ± 0.58
	3	4.23 ± 0.11	4.25 ± 0.11	4.57 ± 0.14	4.36 ± 0.08
	pooled	4.24 ± 0.07	4.22 ± 0.06	4.38 ± 0.11	
Absolute Spleen wt (g)[10]	**60**	0.12 ± 0.004	0.12 ± 0.01	0.06 ± 0.01	0.10 ± 0.01[a]
	3	0.09 ± 0.005	0.10 ± 0.01	0.05 ± 0.01	0.08 ± 0.01[b]
	pooled	0.11 ± 0.005[a]	0.11 ± 0.01[a]	0.06 ± 0.01[b]	

TABLE 3.1.4 continued

Parameter	Zinc Level (mg/kg)	Protein Level (%) 24	7	3	pooled
Relative Spleen wt (% bwt)[11]	**60**	0.73 ± 0.03	0.69 ± 0.06	0.49 ± 0.03	0.63 ± 0.04[a]
	3	0.56 ± 0.02	0.58 ± 0.06	0.41 ± 0.05	0.51 ± 0.03[b]
	pooled	0.65 ± 0.03[a]	0.63 ± 0.04[a]	0.45 ± 0.03[b]	
Absolute Thymus wt (g)[12]	**60**	0.05 ± 0.01	0.04 ± 0.004	0.03 ± 0.005	0.04 ± 0.004
	3	0.03 ± 0.01	0.05 ± 0.005	0.04 ± 0.002	0.04 ± 0.004
	pooled	0.04 ± 0.01[ab]	0.05 ± 0.004[a]	0.03 ± 0.003[b]	
Relative Thymus wt (% bwt)[13]	**60**	0.29 ± 0.05	0.23 ± 0.02	0.20 ± 0.03	0.24 ± 0.02
	3	0.17 ± 0.04	0.29 ± 0.03	0.30 ± 0.02	0.26 ± 0.02
	pooled	0.23 ± 0.04	0.26 ± 0.02	0.25 ± 0.02	

[1] Values are means ± SEM; the number of animals in each group was 5, except 4 in the 24% protein with 3 mg/kg zinc group.
[2] Values within each parameter with different letter superscripts are statistically different ($p \leq 0.05$) based on Tukey's multiple pairwise comparisons; main effects of protein and zinc are shown with the pooled means and zinc*protein interactions are shown with the individual means
[3-13] Significance of main effects from AOV:
[3,9,13] protein (NS); zinc (NS); zinc*protein (NS)
[4,5,7-8] protein ($p<0.0001$); zinc (NS); zinc*protein (NS)
[6] protein ($p=0.004$); zinc (NS); zinc*protein (NS)
[10] protein ($p<0.0001$);zinc ($p=0.02$); zinc*protein (NS)
[11] protein ($p=0.0004$); zinc ($p=0.004$); zinc*protein (NS)
[12] protein ($p=0.04$); zinc (NS); zinc*protein (NS)

TABLE 3.1.5 The effect of dietary protein and zinc restrictions on nutritional parameters of mice on day 29 post-infection (preliminary, experimental day 51)[1,2]

Parameter	Zinc Level (mg/kg)	Protein Level (%) 24	7	3	pooled
Total food intake (g)[3]	**60**	160.5 ± 2.7	167.6 ± 3.9	138.1 ± 7.0	155.4 ± 4.2
	3	158.6 ± 2.8	171.1 ± 4.0	136.4 ± 6.7	155.4 ± 4.6
	pooled	159.5 ± 1.9[a]	169.4 ± 2.7[a]	137.3 ± 4.6[b]	
Food intake/ body wt gain[4]	**60**	18.3 ± 1.1	19.6 ± 1.1	66.8 ± 18.5	32.6 ± 7.7
	3	17.7 ± 0.7	17.7 ± 0.8	57.8 ± 10.5	31.1 ± 6.0
	pooled	18.0 ± 0.6[b]	18.7 ± 0.7[b]	61.8 ± 9.5[a]	
Initial body wt (g)[5]	**60**	10.6 ± 0.5	10.8 ± 0.4	9.9 ± 0.3	10.4 ± 0.3
	3	10.2 ± 0.6	9.4 ± 0.4	10.1 ± 0.6	9.9 ± 0.3
	pooled	10.4 ± 0.4	10.1 ± 0.3	10.0 ± 0.3	
Final body wt (g)[6]	**60**	19.4 ± 0.4	19.4 ± 0.3	12.3 ± 0.7	17.0 ± 0.9
	3	19.3 ± 0.8	19.2 ± 0.5	12.9 ± 0.6	17.1 ± 0.9
	pooled	19.4 ± 0.4[a]	19.3 ± 0.3[a]	12.6 ± 0.4[b]	
Body wt gain (g)[7]	**60**	8.9 ± 0.4	8.6 ± 0.4	2.3 ± 1.0	6.6 ± 0.9
	3	9.0 ± 0.4	9.7 ± 0.6	2.8 ± 0.6	7.2 ± 0.9
	pooled	8.9 ± 0.3[a]	9.2 ± 0.4[a]	2.5 ± 0.5[b]	
Absolute Liver wt (g)[8]	**60**	0.78 ± 0.02	0.83 ± 0.01	0.56 ± 0.03	0.72 ± 0.03
	3	0.80 ± 0.04	0.81 ± 0.04	0.59 ± 0.04	0.73 ± 0.03
	pooled	0.79 ± 0.02[a]	0.82 ± 0.02[a]	0.57 ± 0.02[b]	
Relative Liver wt (% bwt)[9]	**60**	4.03 ± 0.04	4.27 ± 0.06	4.55 ± 0.08	4.29 ± 0.06
	3	4.17 ± 0.10	4.20 ± 0.12	4.56 ± 0.10	4.31 ± 0.07
	pooled	4.10 ± 0.06[b]	4.24 ± 0.06[b]	4.56 ± 0.06[a]	
Absolute Spleen wt (g)[10]	**60**	0.09 ± 0.006	0.10 ± 0.004	0.06 ± 0.01	0.08 ± 0.01
	3	0.09 ± 0.006	0.09 ± 0.004	0.05 ± 0.01	0.07 ± 0.01
	pooled	0.09 ± 0.004[a]	0.10 ± 0.003[a]	0.05 ± 0.01[b]	

TABLE 3.1.5 continued

Parameter	Zinc Level (mg/kg)	Protein Level (%) 24	7	3	pooled
Relative Spleen wt (% bwt)[11]	**60**	0.47 ± 0.03	0.51 ± 0.02	0.50 ± 0.11	0.49 ± 0.03
	3	0.45 ± 0.03	0.48 ± 0.02	0.37 ± 0.05	0.43 ± 0.02
	pooled	0.46 ± 0.02	0.50 ± 0.01	0.43 ± 0.06	
Absolute Thymus wt (g)[12]	**60**	0.04 ± 0.004	0.04 ± 0.004	0.04 ± 0.004	0.04 ± 0.002
	3	0.05 ± 0.010	0.04 ± 0.010	0.04 ± 0.003	0.04 ± 0.004
	pooled	0.05 ± 0.004	0.04 ± 0.004	0.04 ± 0.002	
Relative Thymus wt (% bwt)[13]	**60**	0.23 ± 0.02	0.19 ± 0.02	0.29 ± 0.02	0.24 ± 0.01
	3	0.26 ± 0.04	0.19 ± 0.04	0.32 ± 0.04	0.25 ± 0.03
	pooled	0.24 ± 0.02^{ab}	0.19 ± 0.02^{b}	0.30 ± 0.02^{a}	

[1] Values are means ± SEM; the number of animals in each group was 5.
[2] Values within each parameter with different letter superscripts are statistically different ($p \leq 0.05$) based on Tukey's multiple pairwise comparisons; main effects of protein and zinc are shown with the pooled means and zinc*protein interactions are shown with the individual means.
[3-13] Significance of main effects from AOV:
[3,4] protein ($p<0.0001$); zinc (NS); zinc*protein (NS)
[5,11-12] protein (NS); zinc (NS); zinc*protein (NS)
[13] protein ($p=0.007$); zinc (NS); zinc*protein (NS)

TABLE 3.1.6 The effect of dietary protein and zinc restrictions on the nutritional status of mice on day 9 post-infection (preliminary, experimental day 32)[1,2]

Parameter	Zinc Level (mg/kg)	Protein Level (%) 24	7	3	pooled
Albumin (g/dl)[3]	**60**	2.96 ± 0.05	3.04 ± 0.07	2.67 ± 0.10	2.89 ± 0.06[b]
	3	3.24 ± 0.18	3.37 ± 0.12	2.77 ± 0.09	3.12 ± 0.10[a]
	pooled	3.09 ± 0.09[a]	3.20 ± 0.09[a]	2.72 ± 0.06[b]	
Alkaline phosphatase (IU)[4]	**60**	134.8 ± 4.8	133.9 ± 15.1	142.3 ± 7.7	137.0 ± 5.5
	3	146.5 ± 6.1	127.1 ± 7.1	130.9 ± 8.7	134.0 ± 4.6
	pooled	140.0 ± 4.1	130.5 ± 7.9	136.6 ± 5.8	
Plasma zinc (µg/ml)[5]	**60**	0.76 ± 0.06	0.82 ± 0.08	0.62 ± 0.09	0.73 ± 0.05
	3	0.71 ± 0.11	0.68 ± 0.03	0.64 ± 0.04	0.67 ± 0.03
	pooled	0.74 ± 0.05	0.75 ± 0.05	0.63 ± 0.05	
Tibia zinc (µg/g)[6]	**60**	222 ± 4[a]	191 ± 13[b]	146 ± 5[cd]	186 ± 9
	3	164 ± 8[bc]	167 ± 5[bc]	127 ± 4[d]	152 ± 6
	pooled	196 ± 11	178 ± 8	137 ± 4	

[1] Values are means ± SEM; the number of animals in each group was 5.
[2] Values within each parameter with different letter superscripts are statistically different ($p \leq 0.05$) based on Tukey's multiple pairwise comparisons; main effects of protein and zinc are shown with the pooled means and zinc*protein interactions are shown with the individual means.
[3-6] Significance of main effects from AOV:
[3] protein (p=0.0002); zinc (p=0.01); zinc*protein (NS)
[4-5] protein (NS); zinc (NS); zinc*protein (NS)
[6] protein (p<0.0001); zinc (p<0.0001); zinc*protein (p=0.03)

TABLE 3.1.7 The effect of dietary protein and zinc restrictions on the nutritional status of mice on day 29 post-infection (prelirninary, experimental day 51)[1,2]

Parameter	Zinc Level (mg/kg)	Protein Level (%) 24	7	3	pooled
Albumin (g/dl)[3]	**60**	2.64 ± 0.10	3.07 ± 0.07	2.71 ± 0.17	2.81 ± 0.08
	3	2.96 ± 0.08	3.10 ± 0.11	2.60 ± 0.12	2.89 ± 0.08
	pooled	2.80 ± 0.08[b]	3.09 ± 0.06[a]	2.65 ± 0.10[b]	
Alkaline phosphatase (IU)[4]	**60**	108.6 ± 4.7	108.4 ± 5.8	164.6 ± 9.4	127.2 ± 8.0
	3	115.0 ± 2.6	105.9 ± 3.0	169.0 ± 7.4	130.0 ± 7.9
	pooled	111.8 ± 2.8[b]	107.2 ± 3.1[b]	166.8 ± 5.7[a]	
Plasma zinc (µg/ml)[5]	**60**	1.32 ± 0.20[a]	0.76 ±0.04[abc]	0.47 ± 0.07[c]	0.81 ± 0.12
	3	0.92 ± 0.08[abc]	1.10 ± 0.21[ab]	0.64 ± 0.03[bc]	0.88 ± 0.09
	pooled	1.10 ± 0.13	0.93 ± 0.11	0.55 ± 0.05	
Tibia zinc (µg/g)[6]	**60**		212 ± 13[a]	146 ± 6[bc]	197 ± 11
	3	234 ± 5[a]	150 ± 6[b]	121 ± 9[c]	146 ± 6
	pooled	165 ± 9[b]	181 ± 12	134 ± 6	
		200 ± 12			

[1] Values are means ± SEM; the number of animals in each group was 5.
[2] Values within each parameter with different letter superscripts are statistically different ($p \leq 0.05$) based on Tukey's multiple pairwise comparisons; main effects of protein and zinc are shown with the pooled means and zinc*protein interactions are shown with the individual means.
[3-6] Significance of main effects from AOV:
[3] protein (p=0.002); zinc (NS); zinc*protein (NS)
[4] protein (p<0.0001); zinc (NS); zinc*protein (NS)
[5] protein (p=0.0008); zinc (NS); zinc*protein (p=0.03)
[6] protein (p<0.0001); zinc (p<0.0001); zinc*protein (p=0.03)

TABLE 3.2.1 The effect of dietary protein and zinc restrictions on *H. polygyrus* establishment day 9 post-primary infection in establishment control mice (experiment 1, experimental day 31).[1,2]

Parameter (Absolute Number)	Zinc (mg/kg)	Protein Level (%) 24	7	3	pooled
Total worms[3]	**60**	62.3 ± 13.0	59.0 ± 2.9	68.0 ± 4.7	63.1 ± 4.3
	3	54.0 ± 2.6	53.0 ± 7.5	83.3 ± 2.7	63.4 ± 5.5
	pooled	58.2 ± 6.2^{ab}	56.0 ± 3.8^{b}	75.7 ± 4.2^{a}	
L_4[4]	**60**	1.3 ± 0.7	2.3 ± 0.9	1.3 ± 0.3	1.7 ± 0.4
	3	2.0 ± 1.0	2.0 ± 0.6	1.0 ± 0.6	1.7 ± 0.4
	pooled	1.7 ± 0.6	2.2 ± 0.5	1.2 ± 0.3	
Male worms[5]	**60**	31.0 ± 8.6	28.0 ± 4.0	36.3 ± 2.3	31.8 ± 3.1
	3	27.7 ± 0.9	23.3 ± 3.3	42.7 ± 4.7	31.2 ± 3.4
	pooled	29.3 ± 3.9^{ab}	25.7 ± 2.5^{b}	39.5 ± 2.7^{a}	
Female worms[6]	**60**	30.0 ± 5.7	28.7 ± 1.4	30.3 ± 2.3	29.7 ± 1.8
	3	24.3 ± 0.9	27.7 ± 7.0	39.7 ± 4.0	30.6 ± 3.3
	pooled	27.2 ± 2.9	28.2 ± 3.2	35.0 ± 2.9	
Male/Female ratio[7]	**60**	1.0 ± 0.1	1.0 ± 0.2	1.2 ± 0.1	1.1 ± 0.1
	3	1.1 ± 0.1	1.0 ± 0.2	1.1 ± 0.6	1.1 ± 0.1
	pooled	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	

[1] Values are means ± SEM; the number of animals in each group was 3.
[2] Values within each parameter with different letter superscripts are statistically different ($p \leq 0.05$) based on Tukey's multiple pairwise comparisons; main effects of protein and zinc are shown with the pooled means and zinc*protein interactions are shown with the individual means.
[3] Protein ($p=0.02$); zinc (NS); zinc*protein (NS)
[5] Protein ($p=0.03$); zinc (NS); zinc*protein (NS)
[4,6-7] protein (NS); zinc (NS); zinc*protein (NS)

TABLE 3.2.2 The effect of dietary protein and zinc restrictions on *H.polygyrus* survival on day 29 post-primary and challenge infections in mice (experiment 1)[1,2]

Parameter (Absolute number)	Zinc (mg/kg)	Protein Level (%) 24	7	3	pooled
		PRIMARY INFECTION			
Total worms[3]	**60**	5.6 ± 3.1	39.4 ± 16.2	65.0 ± 4.9	36.7 ± 8.4[b]
	3	34.8 ± 14.9	62.4 ± 11.7	71.0 ± 5.2	56.1 ± 7.3[a]
	pooled	20.2 ± 8.7[b]	50.9 ± 10.2[a]	68.0 ± 3.5[a]	
Male worms[4]	**60**	2.2 ± 1.6	16.4 ± 6.2	28.4 ± 3.0	15.7 ± 3.6[b]
	3	13.0 ± 5.8	31.0 ± 6.4	29.8 ± 4.3	24.6 ± 3.7[a]
	pooled	7.6 ± 3.4[b]	23.7 ± 4.9[a]	29.1 ± 2.5[a]	
Female worms[5*]	**60**	3.4 ± 1.7	23.0 ± 10.6	36.6 ± 2.6	21.0 ± 5.0[b]
	3	21.8 ± 9.3	31.4 ± 5.9	41.2 ± 3.3	31.5 ± 4.1[a]
	pooled	12.6 ± 5.4[b]	27.2 ± 5.9[ab]	38.9 ± 2.1[a]	
Male/Female ratio[6*]	**60**	0.8 ± 0.4 (2)	1.6 ± 0.6	0.8 ± 0.1	1.1 ± 0.3
	3	0.6 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	0.8 ± 0.1
	pooled	0.6 ± 0.1	1.3 ± 0.3	0.8 ± 0.1	
		CHALLENGE INFECTION			
Total worms[7*]	**60**	2.4 ± 0.7	3.0 ± 1.2	65.8 ± 6.6	23.7 ± 8.2
	3	4.4 ± 0.7	3.0 ± 1.3	60.0 ± 7.4	22.5 ± 7.5
	pooled	3.4 ± 0.6[b]	3.0 ± 0.8[b]	62.9 ± 4.8[a]	
Male worms[8*]	**60**	1.2 ± 0.4	1.2 ± 0.4	29.6 ± 4.5	10.7 ± 3.8
	3	0.8 ± 0.4	0.6 ± 0.6	28.0 ± 2.8	9.8 ± 3.5
	pooled	1.0 ± 0.3[b]	0.9 ± 0.3[b]	28.8 ± 2.5[a]	
Female worms[9*]	**60**	1.2 ± 0.5	1.8 ± 1.1	36.2 ± 2.2	13.1 ± 4.4
	3	3.6 ± 0.7	2.4 ± 0.7	32.0 ± 5.5	12.7 ± 4.0
	pooled	2.4 ± 0.6[b]	2.1 ± 0.6[b]	34.1 ± 2.9[a]	
Male/Female ratio[10*]	**60**	0.7 ± 0.2 (3)	0.8 ± 0.5 (4)	0.8 ± 0.1	0.8 ± 0.1
	3	0.4 ± 0.1 (3)	0.6 ± 0.0 (1)	1.0 ± 0.2	0.7 ± 0.1
	pooled	0.5 ± 0.1	0.7 ± 0.4	0.9 ± 0.1	

TABLE 3.2.2 continued

[1] Values are means ± SEM; the number of animals in each group was 5, unless indicated otherwise in parenthesis.
[2] Values within each parameter with different letter superscripts are statistically different ($p \leq 0.05$) based on Tukey's multiple pairwise comparisons; main effects of protein and zinc are shown with the pooled means and zinc*protein interactions are shown with he individual means.
[*] The data were not normally distributed and were transformed to square root.
[3-11] Significance of main effects from AOV:
[3-4] protein ($p=0.0006$); zinc ($p=0.03$); zinc*protein (NS)
[5] protein ($p=0.008$); zinc ($p=0.02$); zinc*protein (NS)
[6,10] protein (NS); zinc (NS); zinc*protein (NS)
[7-9] Protein ($p<0.0001$); zinc (NS); zinc*protein (NS)

TABLE 3.2.3 The effect of dietary protein and zinc restrictions on *H. polygyrus* daily egg output during primary and challenge infections in mice (experiment 1)[1,2]

Day Post-Infection	Zinc (mg/kg)	Protein Level (%) 24	7	3	pooled
		PRIMARY INFECTION			
16[3]	**60**	9643 ± 3447	16531 ± 3892	11273 ± 1611	12482 ± 1854
	3	12886 ± 4620	13658 ± 2543	11962 ± 514	12835 ± 1645
	Pooled	11264 ± 2770	15095 ± 2243	11617 ± 806	
	60	1932 ± 1277[a]	15406 ± 3937[bc]	9526 ± 1379[ab]	8954 ± 1996
22[4]	**3**	5914 ± 1867[ab]	10450 ± 1718[ab]	21168 ± 2644[c]	12510 ± 2049
	pooled	3923 ± 1256	12928 ± 2187	15347 ± 2396	
28[5*]	**60**	370 ± 214	9475 ± 3986	11071 ± 1135	6972 ± 1796[b]
	3	8854 ± 4989	8820 ± 3070	19488 ± 2083	12387 ± 2341[a]
	pooled	4612 ± 2746[b]	9148 ± 2374[b]	15280 ± 1794[a]	
		CHALLENGE INFECTION			
16[6]	**60**	554 ± 205	739 ± 469	9190 ± 1091	3494 ± 1139
	3	1579 ± 587	538 ± 250	7602 ± 506 (4)	2928 ± 865
	pooled	1067 ± 339[b]	638 ± 253[b]	8484 ± 672[a]	
22[7*]	**60**	286 ± 147	437 ± 175	8534 ± 479	3086 ± 1043
	3	823 ± 333	386 ± 305	8350 ± 1649	3186 ± 1110
	pooled	554 ± 194[b]	412 ± 166[b]	8442 ± 809[a]	
28[8*]	**60**	168 ± 127	0 ± 0	9962 ± 2006	3377 ± 1391
	3	1025 ± 437	84 ± 65	8127 ±482 (4)	2718 ± 1572
	pooled	596 ± 258[b]	42 ± 34[b]	9146 ± 2260[a]	

TABLE 3.2.3 continued

[1] Values are means ± SEM; the number of animals in each group was 5, unless indicated otherwise in parenthesis.
[2] Values within each post-infection day and infection type with different letter superscripts are statistically different ($p \leq 0.05$) based on Tukey's multiple pairwise comparisons; main effects of protein and zinc are shown with the pooled means and zinc*protein interactions are shown with the individual means.
[*] The data were not normally distributed and were transformed to square root.
[3-8] Significance of main effects from AOV:
[3] protein (NS); zinc (NS); zinc*protein (NS)
[4] protein ($p=0.0001$); zinc (NS); zinc*protein ($p=0.006$)
[5] protein ($p=0.0004$); zinc ($p=0.02$); zinc*protein (NS)
[6-8] protein ($p<0.0001$); zinc (NS); zinc*protein (NS)

TABLE 3.2.4 The effect of dietary protein and zinc restrictions on the number of eggs produced by *H. polygyrus* female worms on day 28 post-primary and challenge infections in mice (experiment 1)[1,2]

		Protein Level (%)			
Infection	**Zinc (mg/kg)**	**24**	**7**	**3**	**pooled**
Primary[3*]	**60**	59 ± 34[a] (4)	742 ± 212[c]	417 ± 80[b]	430 ± 107
	3	322 ± 78[b]	259 ± 55[ab]	482 ± 62[b]	354 ± 43
	pooled	205 ± 64	501 ± 131	449 ± 49	
Challenge[4*]	**60**	140 ± 98 (3)	0 ± 0 (3)	294 ± 77	172 ± 56
	3	321 ± 144	37 ± 33	239 ± 127 (4)	196 ± 68
	pooled	253 ± 98[a]	23 ± 21[b]	269 ± 67[a]	

[1] Values are means ± SEM; the number of animals in each group was 5 unless indicated otherwise in parenthesis.
[2] Values within infection type with different letter superscripts are significantly different ($p \leq 0.05$) based on Tukey's multiple pairwise comparisons; main effects of protein and zinc are shown with the pooled means and zinc*protein interactions are shown with the individual means.
[*] The data were not normally distributed and were transformed to square root.
[3-4] Significance of main effects from AOV:
[3] protein (p=0.001); zinc (NS); zinc*protein (p=0.0008)
[5] protein (p=0.007); zinc (NS); zinc*protein (NS)

TABLE 3.2.5 The effect of dietary protein and zinc restrictions on the number of eosinophils/mm^3 in whole blood of mice during primary and challenge infections with *H. polygyrus* (experiment 1)[1,2]

Day Post-Infection	Zinc (mg/kg)	Protein Level (%) 24	7	3	pooled
		PRIMARY INFECTION			
7[4*]	60	214.4 ± 36.0	428.0 ± 123.5(4)	392.0 ± 189.6(4)	334.8 ±69.8[a]
	3	150.4 ± 14.8	192.0 ± 18.2	112.0 ± 25.3(4)	154.3 ±13.5[b]
	pooled	182.4 ± 21.2	296.9 ± 66.0	252.0 ± 103.1	
14[5]	60	345.6 ± 64.9	672.0 ± 82.1	275.2 ± 62.9	430.9 ± 59.6
	3	403.2 ± 66.0	416.0 ± 54.5	278.4 ± 69.5	365.9 ± 37.9
	pooled	374.4 ± 44.7[b]	544.0 ± 63.1[a]	276.8 ± 44.2[b]	
21[6*]	60	297.6 ± 108.3	195.2 ± 44.8(4)	60.0 ± 26.4(4)	193.1 ±47.4[a]
	3	86.4 ± 18.0	140.0 ± 12.0(4)	22.4 ± 8.2	78.8 ± 14.9[b]
	pooled	192.0 ± 62.6[a]	170.7 ± 26.0[a]	39.1 ± 13.4[b]	
		CHALLENGE INFECTION			
7[7]	60	720.0 ± 133.6	726.4 ± 66.1	342.4 ± 58.5	596.3 ±68.9[a]
	3	646.4 ± 138.8	380.8 ± 115.6	180.0 ± 38.3(4)	418.3 ±80.0[b]
	pooled	683.2 ± 91.6[a]	553.6 ± 85.2[a]	270.2 ± 44.8[b]	
14[7]	60	563.2 ± 159.2	502.4 ± 153.5	220.8 ± 24.5	428.8 ± 79.4
	3	908.8 ± 279.5	665.6 ± 95.1	211.2 ± 89.9	595.2±122.6
	pooled	736.0 ± 162.2[a]	584.0 ± 89.4[ab]	216.0 ± 43.9[b]	
21[8]	60	236.8 ± 55.1	275.2 ± 42.2	72.0 ± 13.9(4)	203.4 ± 33.1
	3	147.2 ± 33.3	243.2 ± 80.9	64.0 ± 20.9	151.5 ± 33.9
	pooled	192.0 ± 33.8[a]	259.2 ± 43.3[a]	67.5 ± 12.4[b]	

TABLE 3.2.5 continued

[1] Values are means ± SEM; the number of animals in each dietary and infection groups was 5 unless indicated otherwise in parenthesis.
[2] Values within one infection type and one post-infection day with different letter superscripts are significantly different based on Tukey's multiple pairwise comparisons; main effets of protein and zinc are shown with the pooled means and zinc*protein interactions are shown with the individual means.
[*] The data were not normally distributed and were transformed to square root.
[3-8] Significance of main effects from AOV:
[3] protein (NS); zinc (p=0.005); zinc*protein (NS)
[4] protein (p=0.002); zinc (NS); zinc*protein (NS)
[5] protein (p=0.02); zinc (p=0.006); zinc*protein (NS)
[6] protein (p=0.002); zinc (p=0.03; zinc*protein (NS)
[7] protein (p=0.008); zinc (NS); zinc*protein (NS)
[8] protein (p=0.003); zinc (NS); zinc*protein (NS)

TABLE 3.2.6. The effect of dietary protein and zinc restrictions on total serum IgG1 concentration during primary and challenge infections with *H. polygyrus* (experiment 1)[1,2].

Day Post-Infection	Zinc (mg/kg)	Protein Level (%) 24	7	3	pooled
		IgG1 (µg/ml) PRIMARY INFECTION			
0[2]	**60**	2868 ± 453	2850 ± 247	2356 ± 230	2691 ± 185
	3	2649 ± 260	2268 ± 251	1944 ± 166	2311 ± 149
	pooled	2759 ± 249	2559 ± 192	2173 ± 157	
7[3]	**60**	6450 ± 765b[b]	5149 ± 369[b]	4590 ± 175[b]	5220 ± 377
	3	5226 ± 238[b]	5347 ± 255[b]	7142 ± 873[a]	5915 ± 388
	pooled	5991 ± 515	5248 ± 214	6048 ± 698	
14[4]	**60**	13254 ± 1800	12250 ± 1175	8486 ± 845	11330 ± 899
	3	11078 ± 1856	11640 ± 1171	7445 ± 558	10241 ± 892
	pooled	12166 ± 1272[a]	11945 ± 789[a]	8024 ± 532[b]	
21[5]	**60**	15239 ± 829	15714 ± 1044	11448 ± 616	14449 ± 705
	3	13142 ± 669	14230 ± 942	12369 ± 1556	13247 ± 628
	pooled	14307 ± 633[a]	14971 ± 709[a]	11974 ± 884[b]	
29[6]	**60**	16218 ± 1422	12232 ± 285	11423 ± 1022	13424 ± 1233
	3	16555 ± 1911	12681 ± 528	10556 ± 1406	13264 ± 1002
	pooled	16387 ± 1125[a]	12457 ± 1365[ab]	10941 ± 864[b]	

TABLE 3.2.6 continued

Day Post-Infection	Zinc (mg/kg)	Protein Level (%) 24	7	3	pooled
		IgG1 (µg/ml) CHALLENGE INFECTION			
0[7]	60	8234 ± 1014	5788 ± 625	5292 ± 487	6438 ± 525[a]
	3	4343 ± 1058	3535 ± 721	2383 ± 281	3420 ± 458[b]
	pooled	6289 ± 948[a]	4661 ± 586[ab]	3838 ± 553[b]	
7[8]	60	11238 ± 1615	7734 ± 893	8842 ± 816	9381 ± 758
	3	11355 ± 648	9326 ± 297	7702 ± 1405	9586 ± 593
	pooled	11296 ± 821[a]	8618 ± 485[b]	8335 ± 744[b]	
14[9]	60	15524 ± 3631	15763 ± 270	13429 ± 938	14953 ± 1554
	3	16005 ± 1125	14877 ± 1747	16434 ± 705	15772 ± 690
	pooled	15738 ± 1971	15320 ± 1506	14932 ± 786	
21[10]	60	20554 ± 1624	17657 ± 1708	14173 ± 1354	17461 ± 1136
	3	18818 ± 698	18930 ± 1100	16908 ± 1431	18176 ± 677
	pooled	19686 ± 881[a]	18364 ± 934[ab]	15693 ± 1051[b]	
29[11]	60	18939 ± 1528	16216 ± 2026	10596 ± 666	15184 ± 1218
	3	15413 ± 575	15811 ± 1979	10611 ± 839	13840 ± 989
	pooled	17260 ± 1022[a]	16013 ± 1337[a]	10604 ± 505[b]	

TABLE 3.2.6 continued

[1] Values are means ± SEM; the number of animals in each dietary and infection groups was 5.
[2] Values within one infection type and one post-infection day with different letter superscripts are significantly different based on Tukey's multiple pairwise comparisons; main effets of protein and zinc are shown with the pooled means and zinc*protein interactions are shown with the individual means.
[3-11] Significance of main effects from AOV:
[3] Protein (NS); zinc (NS); zinc*pprotein (NS)
[4] Protein (NS); zinc (NS); zinc*protein (p=0.020
[5] prorein (p=0.01); zinc (NS); zinc*protein (NS)
[6] protein (p=0.03); zinc (NS); zinc*protein (NS)
[7] protein (p=0.01); zinc (NS); zinc*protein (NS)
[8] protein (p=0.01); zinc (p<0.0001); zinc*protein (NS)
[9] protein (p=0.012); zinc (NS); zinc*protein (NS)
[10] protein (ns); zinc (NS); zinc*protein (NS)
[11] protein (p=0.02); zinc (NS); zinc*protein (NS)

TABLE 3.2.7 The effect of dietary protein and zinc restrictions on nutritional parameters of mice on day 28 post-infection (experiment 1, experimental day 73)[1,2]

Parameter	Zinc (mg/kg)	Protein Level (%) 24	7	3	pooled
Total food intake (g)[3]	**60**	213.6 ± 1.7	245.9 ± 2.4	205.8 ± 7.0	221.7 ± 4.0
	3	217.9 ± 4.4	239.8 ± 4.4	205.8 ± 5.9	221.2 ± 3.8
	pooled	215.7 ± 2.4[b]	242.8 ± 2.5[a]	205.8 ± 4.5[b]	
Food intake/ body wt gain[4]	**60**	21.0 ± 0.6	22.2 ± 0.5	62.0 ± 12.5	35.1 ± 5.4
	3	22.0 ± 1.4	25.9 ± 0.9	50.9 ± 4.3	32.7 ± 2.8
	pooled	21.5 ± 0.8[b]	23.6 ± 0.6[b]	56.5 ± 6.5[a]	
Initial body wt (g)[5]	**60**	10.5 ± 0.2	10.7 ± 0.3	10.4 ± 0.2	10.5 ± 0.1
	3	10.3 ± 0.3	10.5 ± 0.3	10.2 ± 0.2	10.3 ± 0.1
	pooled	10.4 ± 0.2	10.6 ± 0.2	10.3 ± 0.1	
Final body wt (g)[6]	**60**	20.7 ± 0.3	21.8 ± 0.2	14.6 ± 0.6	19.1 ± 0.6
	3	20.4 ± 0.5	20.2 ± 0.4	14.5 ± 0.4	18.4 ± 0.6
	pooled	20.6 ± 0.3[a]	21.0 ± 0.3[a]	14.6 ± 0.4[b]	
Body wt gain (g)[7]	**60**	10.3 ± 0.3	11.1 ± 0.3	4.2 ± 0.6	8.5 ± 0.6
	3	10.2 ± 0.5	9.7 ± 0.4	4.3 ± 0.3	8.1 ± 0.5
	pooled	10.2 ± 0.3[a]	10.4 ± 0.3[a]	4.2 ± 0.3[b]	
Absolute Spleen wt (g)[8]	**60**	0.09 ± 0.004	0.11 ± 0.007	0.05 ± 0.002	0.08 ± 0.005
	3	0.09 ± 0.005	0.11 ± 0.005	0.06 ± 0.006	0.08 ± 0.004
	pooled	0.09 ± 0.003[b]	0.11 ± 0.004[a]	0.06 ± 0.003[c]	
Relative Spleen wt (% bwt)[9]	**60**	0.45 ± 0.01	0.49 ± 0.03	0.36 ± 0.01	0.43 ± 0.016
	3	0.42 ± 0.02	0.52 ± 0.02	0.41 ± 0.03	0.45 ± 0.016
	pooled	0.43 ± 0.01[b]	0.50 ± 0.02[a]	0.39 ± 0.02[b]	
Absolute Thymus wt (g)[10]	**60**	0.05 ± 0.003	0.04 ± 0.003	0.03 ± 0.003	0.04 ± 0.002
	3	0.04 ± 0.004	0.04 ± 0.004	0.03 ± 0.002	0.04 ± 0.002
	pooled	0.05 ± 0.003[a]	0.04 ± 0.003[a]	0.03 ± 0.002[b]	
Relative Thymus wt (% bwt)[11]	**60**	0.24 ± 0.02	0.19 ± 0.01	0.19 ± 0.02	0.21 ± 0.01
	3	0.19 ± 0.02	0.20 ± 0.02	0.21 ± 0.01	0.20 ± 0.01
	pooled	0.22 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	

TABLE 3.2.7 continued

Parameter	Zinc (mg/Kg)	Protein Level (%) 24	7	3	pooled
BUN (mg/dl)[12]	**60**	22.8 ± 1.2	16.7 ± 0.9	10.0 ± 0.9 (9)	16.7 ± 1.1
	3	22.0 ± 1.5	16.4 ± 0.6	12.7 ± 0.6 (9)	17.2 ± 0.9
	pooled	22.4 ± 0.9[a]	16.5 ± 0.5[b]	11.3 ± 0.6[c]	
Liver copper (µg/g)[13]	**60**	19.2 ± 0.7	18.6 ± 0.5	22.4 ± 1.5	20.1 ± 0.9
	3	18.3 ± 0.6	18.9 ± 0.8	19.1 ± 0.5	18.7 ± 0.4
	pooled	18.8 ± 0.5[b]	18.7 ± 0.5[b]	20.7 ± 0.9[a]	
Liver zinc/copper (µg/g)[14]	**60**	6.1 ± 0.2	6.2 ± 0.1	5.3 ± 0.2 (9)	5.9 ± 0.1
	3	6.4 ± 0.1 (9)	6.2 ± 0.2	4.9 ± 0.2	5.8 ± 0.2
	pooled	6.2 ± 0.1[a]	6.2 ± 0.1[a]	5.1 ± 0.2[b]	

[1] Values are means ± SEM; the data for the primary and challenge infections are pooled; the number of animals in each group was 10 unless indicated otherwise in parenthesis.
[2] Values within each parameter with different letter superscripts are statistically different ($p \leq 0.05$) based on Tukey's multiple pairwise comparisons; main effects of protein and zinc are shown with the pooled means and zinc*protein interactions are shown with the individual means.
[3-14] Significance of main effects from AOV:
[3-4,6-10,12,14] protein ($p<0.0001$); zinc (NS); zinc*protein (NS)
[5,11] protein (NS); zinc(NS); zinc*protein (NS)
[13] protein ($p=0.04$); zinc (NS); zinc*protein (NS)

TABLE 3.2.8 The effect of dietary protein and zinc restrictions on nutritional parameters of mice on day 28 post-infection (experiment 1, experimental day 73)[1,2]

Parameter	Zinc (mg/kg)	Protein Level (%) 24	7	3	pooled
		PRIMARY INFECTION			
Liver wt (g)[3]	**60**	0.83 ± 0.05	0.88 ± 0.04	0.59 ± 0.04	0.77 ± 0.04
	3	0.85 ± 0.06	0.87 ± 0.02	0.64 ± 0.05	0.79 ± 0.04
	pooled	0.84 ± 0.03[a]	0.88 ± 0.02[a]	0.62 ± 0.03[b]	
Relative Liver wt (% bwt)[4]	**60**	4.02 ± 0.18	4.06 ± 0.13	4.10 ± 0.11	4.06 ± 0.08[b]
	3	4.26 ± 0.14	4.39 ± 0.09	4.43 ± 0.15	4.38 ± 0.07[a]
	pooled	4.14 ± 0.12	4.20 ± 0.09	4.29 ± 0.10	
Albumin (g/dl)[5]	**60**	2.71 ± 0.1	2.79 ± 0.2	3.19 ± 0.1	2.90 ± 0.1[b]
	3	3.23 ± 0.1	3.40 ± 0.1	3.30 ± 0.1	3.33 ± 0.1[a]
	pooled	2.97 ± 0.1	3.11 ± 0.1	3.25 ± 0.1	
Alkaline Phosphatase (IU)[6]	**60**	90.3 ± 6.5[a]	92.5 ± 5.8[a]	161.9 ± 5.6[b]	116.5 ± 9.9
	3	108.9 ± 6.1[a]	107.6 ± 4.5[a]	206.4 ± 4.9[c]	153.4 ± 12.7
	pooled	99.6 ± 5.2	100.9 ± 4.3	184.1 ± 8.2	
Tibia zinc (µg/g)[7]	**60**	225 ± 8	226 ± 12	164 ± 5	205 ± 9[a]
	3	170 ± 3	159 ± 5	132 ± 6	153 ± 5[b]
	pooled	198 ± 10 [a]	196 ± 14[a]	148 ± 6[b]	
Liver zinc (µg/g)[8]	**60**	122 ± 5	116 ± 4	115 ± 1	118 ± 2[a]
	3	113 ± 3 (4)	112 ± 3	99 ± 2	108 ± 2[b]
	pooled	118 ± 3[a]	114 ± 2[ab]	107 ± 3[b]	

TABLE 3.2.8 continued

Parameter	Zinc (mg/kg)	Protein Level (%) 24	7	3	pooled
		CHALLENGE INFECTION			
Liver wt (g)[9]	**60**	0.85 ± 0.01^{ab}	0.98 ± 0.03^{a}	0.76 ± 0.05^{b}	0.86 ± 0.03
	3	0.91 ± 0.06^{ab}	0.84 ± 0.02^{ab}	0.65 ± 0.03^{c}	0.80 ± 0.04
	pooled	0.88 ± 0.03	0.91 ± 0.03	0.71 ± 0.03	
Relative Liver wt (% bwt)[10]	**60**	4.07 ± 0.09^{a}	4.51 ± 0.17^{a}	5.17 ± 0.15^{b}	4.58 ± 0.14
	3	4.35 ± 0.20^{a}	4.14 ± 0.07^{a}	4.46 ± 0.15^{a}	4.32 ± 0.09
	pooled	4.20 ± 0.11	4.32 ± 0.11	4.81 ± 0.15	
Albumin (g/dl)[11]	**60**	2.85 ± 0.1	3.19 ± 0.1	3.01 ± 0.2	3.00 ± 0.1^{a}
	3	2.59 ± 0.2	2.90 ± 0.03	2.78 ± 0.1	2.75 ± 0.1^{b}
	pooled	2.72 ± 0.1^{b}	3.03 ± 0.07^{a}	2.90 ± 0.11^{ab}	
Alkaline Phosphatase (IU)[12]	**60**	105.7 ± 4.4	101.5 ± 10.4	159.4 ± 23.6	119.5 ±10.0
	3	93.4 ± 4.8	87.7 ± 2.6	137.9 ± 16.9	106.4 ± 8.1
	pooled	99.5 ± 3.7^{b}	94.6 ± 5.6^{b}	147.5 ± 13.6^{a}	
Tibia zinc (µg/g)[13]	**60**	222 ± 3	241 ± 8	179 ± 6	214 ± 8^{a}
	3	180 ± 4	193 ± 16	163 ± 6	179 ± 6^{b}
	pooled	201 ± 7^{a}	217 ± 12^{a}	171 ± 5^{b}	
Liver zinc (µg/g)[14]	**60**	110 ± 1^{a}	114 ± 6^{a}	104 ± 3^{a}	110 ± 2
	3	113 ± 3^{a}	119 ± 3^{a}	87 ± 3^{b}	106 ± 4
	pooled	112 ± 1	116 ± 3	96 ± 3	

[1] Values are means ± SEM.
[2] The number of animals in each dietary and infection groups was 5.
[3] Values within one parameter and infection type with different letter superscripts are significantly different ($p \leq 0.05$) based on Tukey's multiple pairwise comparisons; main effects of zinc and protein are shown with the pooled means and zinc*protein interactions are shown with the individual means.
[3-14] Significance of main effects from AOV:

TABLE 3.2.8 continued

[3] protein (p<0.0001); zinc (NS); zinc*protein (NS)
[4] protein (NS); zinc (p=0.008); zinc*protein (NS)
[5] protein (NS); zinc (p<0.0001); zinc*protein (NS)
[6] protein (p<0.0001); zinc (p=0.001); zinc*protein (p=0.03)
[7] protein (p<0.0001); zinc (p<0.0001); zinc*protein (NS)
[8] protein (p=0.009); zinc (p<0.0001); zinc*protein (NS)
[9] protein (p<0.0001); zinc (NS); zinc*protein (p=0.03)
[10] protein (p=0.001); zinc (p=0.03); zinc*protein (p=0.008)
[11] protein (p=0.05); zinc (p=0.02); zinc*protein (NS)
[12] protein (p<0.0001); zinc (NS); zinc*protein (NS)
[13] protein (p<0.0001); zinc (p<0.0001); zinc*protein (NS)
[14] protein (p<0.0001); zinc (NS); zinc*protein (p=0.006)

CHAPTER 4. DISCUSSION

The effects of single and combined dietary protein and zinc restrictions on *H. polygyrus* infection in both immune and non-immune mice were investigated. Worm burdens and worm egg production, and the associated IgG1 levels and eosinophili counts were compared in groups of mice fed 24, 7 or 3% protein combined with 60 or 3 mg zinc/kg diet. The effects of protein and zinc restrictions on host nutritional status were also investigated. This represents the first comprehensive study of the effects of combined dietary protein and zinc restrictions on a chronic intestinal parasitic infection. The model used in this study is also unique as it allows the study of the effects of dietary restrictions in both immune (challenge infection) and non-immune (primary infection) hosts. The complexity of the protein and zinc-parasite interaction is illustrated in this study by the array of significant findings which are subsequently discussed.

I. HOST NUTRITIONAL STATUS

A. ZINC STATUS

The effect of marginal zinc deficiency rather than of severe zinc deficiency on the outcome of an intestinal helminth infection was studied in the present experiment because of the growing awareness that marginal zinc deficiency may be more important and widespread than severe zinc deficiency in human populations (Casey and Hambidge, 1980; Keen et al., 1988; Kimberlly et al., 1991; Lindsay, 1993; Prasad, 1983). Many studies concerning the effects of zinc deficiency in laboratory animals have used severely deficient levels of zinc which produce

marked failure to grow, anorexia, alopecia, diarrhoea, skin lesions and sometimes death (Aggett, 1989 and 1991; Burch et al., 1975; Williams and Mills, 1970) and results from these studies may not be relevant to human populations. In humans, impaired linear growth has been the most significant feature observed in zinc deficient children in several countries (Ferguson et al., 1993; Halstead et al., 1972; King, 1990; Walravens et al., 1983 and 1989; Walravens and Hambidge, 1976), and mildly zinc deficient children responded to zinc supplementation with changes in indexes of body composition rather than growth (Cavan et al., 1993a,b).

The level of zinc restriction chosen for this study was based on previous experiments in this laboratory using similar diet formulations and showing that diets containing 5 mg zinc/kg resulted in a 40% reduction in serum zinc but had no effects on other clinical or biochemical indicators of zinc status such as food intake, body weight gain, tissue zinc stores and alkaline phosphatase activity (Minkus, 1990). A more severe zinc restriction with diets containing 0.75 mg zinc/kg resulted in reduced food intake, weight loss, lower liver and plasma zinc concentrations, reduced relative spleen and thymus weights, hair loss and skin lesions (Shi et al., 1994a). Diets containing an intermediate value of 3 mg zinc/kg were expected to produce a marginal zinc deficiency.

Luecke and Fraker (1979) attempted to determine the zinc requirement of young growing female mice, and found maximum weight gain and normal antibody-mediated responses at a level of 5.9 mg zinc/kg in the diet (these results indicated that the zinc requirement of the mice is lower than that of the rat which is 12 mg/kg). A level of 3.3 mg zinc/kg diet resulted in lower food intake and lower body weight gain in outbred Swiss mice, but had no significant effect on these parameters in inbred A/J mice although a trend towards lower values were observed. In

addition, the effects of a 3.3 mg/kg level were much milder than the effects observed with a level of 0.7 mg/kg. It may be suggested that the different effect of the 3.3 mg/kg zinc restriction in the Swiss mice and the A/J mice may have been due to their differing growth rate. The Swiss and A/J mice weighed 11.1 and 10.5 grams respectively at the start of the experiment at three weeks of age. The control Swiss mice which weighed 21 grams at age 5 weeks grew faster than the control A/J mice which weighed only 16 grams. The BALB/c mice used in the present experiment have small body size and weighed only 16 grams at age 7 weeks when fed the control diet. This strain may have zinc requirements similar to those of the A/J mouse as determined in the experiment of Luecke and Fraker (1979).

The level of zinc in the diets used in this study ranged from 2.9 mg/kg in the 3% protein diet to 4.0 mg/kg in the 24% protein diet when measured by atomic absorption spectrophotometry. These levels may have been borderline and not sufficiently low to impair body weight gain and food intake, as these parameters were not altered. Furthermore, reducing the protein content of zinc deficient diets has been shown to increase food intake and body weight gain (Chesters and Will, 1973; Hunt and Johnson, 1992) and, therefore, the protein restricted diets in this study may have required even lower levels of zinc to produce clear signs of deficiency. There was also no effect of this level of restriction on the relative spleen and thymus weights which are commonly reduced in moderate to severe zinc deficiency (Beach et al., 1980a and 1982; Fraker, 1977; Fraker et al., 1982b and 1984; Luecke, 1978). In addition, there was no effect on alkaline phosphatase activity which is a zinc dependent enzyme often used as an indicator of zinc status (Aggett, 1991; Roth and Kirchgessner, 1980; Thompson, 1991). Parameters of zinc status which were altered by the level of restriction used in this investigation

included tibia zinc concentration in both primary and challenge infected animals and liver zinc concentration in the primary infection; liver zinc concentration was only decreased in mice fed the lowest protein level in the challenge infection group.

The response of specific tissues to zinc deficiency is not uniform (King, 1990). The concentration of zinc in bone has been reported to drop by as much as 65%, that of plasma by approximately 45% and that of liver within the range of 7 20%, in moderate to severe zinc restrictions (Giugliano and Millward, 1984; Jackson et al., 1982; King, 1990). Zinc is thought to be mobilized from those tissues to support high priority tissues such as muscle and skin (Giugliano and Millward, 1984), and is thought to be derived from bone to a larger extent than from liver or plasma (Giugliano and Millward, 1984; King, 1990).

Although bone is very sensitive to dietary zinc restriction, it must be pointed out that its role as a potential zinc store has been controversial. Brown et al. (1978) suggested that the 30% reduction in total femur zinc observed in young zinc deficient rats could be redistributed to other tissues to support minimal growth during periods of dietary zinc restriction. Giugliano and Millward (1984) estimated that the sum of zinc loss from bone together with that obtained from the diet was equal to the amount of zinc deposited in muscle in zinc deficient rats, and suggested that storage zinc in the skeleton serves as a zinc reservoir. Hurley and Swererton (1971), Hurley and Tao (1972), and Tao and Hurley (1975), however, rejected the possibility that bone zinc could be redistributed when needed elsewhere because zinc deficient pregnant rats (which had high zinc requirements) could not mobilize bone zinc in an amount above that mobilized by non-pregnant zinc deficient rats. These authors argued that, in the mature skeleton, zinc is firmly bound in bone mineral and is essentially unavailable in times of need, and that any release of

zinc from bone is secondary to normal bone turnover rather than being the result of active zinc-releasing mechanisms. Murray and Messer (1981) also argued that bone zinc could not serve as a store, since zinc deficiency did not affect the resorption rate of bone in either weanling or adult rats; zinc was lost from bone in a passive manner that was dependent on, but did not influence, bone turnover. This study however, only measured bone zinc turnover at one time point, at 4 weeks after dietary zinc restriction. More recently, Zhou et al. (1993) have provided evidence to show the existence of two zinc pools in the skeleton, by following the redistribution of ^{65}Zn among rat tissues throughout a period of 5 weeks of marginal zinc restriction. These authors suggested that one pool of bone zinc is a rapidly turning over pool from which zinc can be released within the first week of marginal zinc restriction (6 mg zinc/kg) to maintain zinc homeostasis and growth. The other pool is a slowly turning over pool, or sink, from which zinc cannot be utilized without bone resorption. Based on these observations, it is possible that the reduction in bone zinc observed in the zinc restricted animals in the present study may have been used to maintain body weight gain and to prevent the development of further signs of zinc deficiency. Also, the fact that the zinc restricted animals were first acclimatized on the high zinc diet (60 mg/kg) may have helped in maintaining zinc homeostasis following marginal zinc restriction. Harland et al. (1975) demonstrated that zinc supplementation prior to zinc restriction resulted in improved growth of Japanese quails. These investigators suggested that bone may store zinc consumed in excess of the requirement and that this zinc may be available for utilization during a subsequent period of zinc deprivation. Acclimatization on a commercial chow diet which contains lower zinc (12-20 mg/kg) or placing the weaning animals directly on the low zinc diet may have been better alternatives to produce further signs of deficiency.

Bone and liver zinc concentrations were also found to be affected differently in a primary vs a challenge infection protocol. Bone zinc concentration was significantly higher in challenged animals than in primary infected animals; liver zinc was significantly lower at all protein levels in the primary infection but only significantly lower in the 3% protein fed animals in the challenge infection. Since animals from both infection protocols were fed identically, these effects may be due to the infection protocol itself, although the fact that uninfected animals were not included in the study makes it difficult to speculate on the effects of infection on tissue zinc concentration. The challenge infection protocol stimulates a more intense immune response than a primary infection protocol (Bartlett and Ball, 1974; Benhke and Parish, 1979; Cypess et al., 1988). The acute phase protein response which occurs during inflammation and certain infections and which is regulated by IL-1, IL-6 and TNF can result in disturbances in tissue mineral concentrations and alteration in liver protein synthesis profile. Hepatic uptake of amino acids, zinc and iron from the plasma, as well as an increased hepatic synthesis of acute phase proteins and reduction in albumin synthesis are observed (Grimble, 1989 and 1990). Cousins and Leinart (1988) examined changes in tissue zinc dynamics in mice treated with recombinant IL-1 to simulate an acute phase response and found that uptake of labelled zinc into bone, skin and intestine was decreased, whereas that of liver, bone marrow and thymus was enhanced. These authors suggested that when host defences are challenged, the redistribution of zinc from skin and bone to liver, thymus and bone marrow may occur to supply these high priority tissues for nucleic acid and protein synthesis. Whether this kind of interaction between infection and tissue zinc stores could explain the difference observed in bone and liver zinc concentrations between the primary and challenge infection groups is not clear. It has not been previously established that

H. polygyrus stimulates an acute phase protein response in a challenge infection, but it has been demonstrated that the intestinal helminths *Trichinela spiralis* and *Nippostrongylus brasiliensis* do not elicit synthesis of acute phase proteins (Stadnyk et al., 1990). Furthermore, Minkus et al. (1992) observed no effect of *H. polygyrus* primary or challenge infection on plasma or liver iron or on zinc concentrations and plasma albumin levels compared with uninfected animals. These authors concluded that the acute phase protein response was not likely to have been stimulated, although liver and splenic copper were increased and splenic iron level was decreased, by unknown mechanisms, in challenge infected animals compared to uninfected controls.

B. PROTEIN STATUS

Three dietary protein levels were chosen for this study to represent a broad range of protein intake. A level of 24% was chosen for the control diet, as described by Minkus (1990). This level is higher than the NRC (1978) recommendation of 18% protein, which is stated to be adequate for most strains of mice and not excessive for any. The level for the present experiment was formulated at 24% in the event that reduced food intake should have occurred in the zinc restricted animals fed the control level of protein. The level of 7% protein was expected to represent a marginal protein intake based on reports from Korsrud (1966) stating that levels of 11.3% from egg protein produced maximum growth in CF1 mice, and reports from Goettsh (1960) stating that 13.6% protein as casein was the minimal concentration that supported acceptable growth, reproduction and lactation in Swiss STM mice. Finally the level of 3% protein was chosen as a low protein diet and was not expected to produce severe signs of deficiency. Studies using diets containing less than 2% protein, have been shown to result in excessive body

weight loss and edema in weanling mice, and to produce marked effects on immune function (e.g. Filteau and Woodward, 1987; Watson et al., 1983; Woods and Woodward, 1991; Woodward et al., 1992). Although some have reported a 2% protein diet to maintain weaning weight in mice (Filteau and Woodward, 1984) others have reported body weight loss at this level of protein (Slater and Keymer, 1988). Previous experiments in this laboratory have shown that female BALB/c mice cannot maintain weaning weight on a 2% protein diet which induces a loss of about 3 g of body weight from weaning and approximately 20% mortality. Severe protein deficiency was not chosen for this study because it may have caused a high mortality rate, considering the length of this experiment (10 weeks), and because two nutritional deficiencies were combined. Also, it was desirable to examine the effects of protein deficiency at a level that would not induce excessive morbidity and mortality, which may be more realistic to the human situation of stunting malnutrition.

Unexpectedly, the level of 7% protein did not produce any signs of deficiency and appeared to be an adequate level for growth. Mice fed 7% protein gained as much weight and ate more food than mice fed 24% protein. The feed utilization ratios, were calculated as the amount of food eaten divided by the body weight gain of mice, and represent the extent to which mice converted dietary protein into body tissue. This index showed that mice fed 7% protein had a food utilization ratio that was not significantly different from that of mice fed 24% protein. Therefore, mice fed 24% protein or 7% protein ate the same amount of their respective diet to gain 1 g of body weight. The 7% protein diet also resulted in higher albumin values on day 51 of the preliminary study and on day 73 of experiment 1 in the challenge infection group. BUN in this group, as expected was intermediate between the values for animals fed 24% and 3%

protein. Thus, none of the nutritional parameters indicated that the 7% protein fed animals were receiving sub-optimal protein intake, although this level of dietary protein increased parasite survival in a primary infection and produced slight differences in the plasma IgG1 concentration in a challenge infection.

The 3% protein diet was clearly inadequate for growth, as mice fed this level of protein had significantly lower weight gain than mice fed 7% and 24% protein. This level of protein permitted a slow rate of body weight gain. Petro et al. (1982) found similar growth in female BALB/c mice fed a 4% casein diet supplemented with methionine starting at 6 weeks of age for a period of 5 weeks and qualified this level of restriction as a moderate protein deficiency. Although the 3% protein diet impaired body weight gain in mice, plasma albumin concentration was not consistently reduced. Significantly lower albumin values were observed on day 32 of the preliminary study, but no difference between the 3% and 24% protein fed animals was observed on day 51 of the preliminary study or on day 73 of experiment 1. Food intake is sometimes reduced when animals are fed very low protein diets, but did not differ between mice fed 3% and 24% protein, although mice fed 3% protein had lower food intake than mice fed 7% protein.

Lunn and Austin (1983a and 1983b) demonstrated that hypoalbuminemia, and perhaps other features of protein deficiency seen in animals fed very low protein diets, may occur as an undesirable consequence of the metabolic response required to deal with the excess energy consumption in relation to reduced body weight and maintenance need. Data from balance studies showed that for hypoalbuminemia to occur, not only does dietary protein need to be inadequate, but it is also necessary for energy consumption to be in excess of requirement. It was suggested that in protein deficient animals, energy consumed in excess of the amount required for

maintenance and the protein-limited growth rate, initiates a metabolic response which results in inappropriate use of the meagre protein intake. This "dysadapted" state in the rat was associated with elevated plasma T_3 values. The voluntary reduction of food intake of animals fed very low protein diets is also thought to be related in part to the inability of protein deficient animals to deal with the excess energy intake in relation to protein (Coward et al., 1977) or to the limited ability to store or dissipate the excess energy (Beaton et al., 1965 and 1966). Protein deficient rats which have reduced their food intake can increase intake when exposed to factors, such as cold and exercise, which increase energy dissipation (Beaton et al., 1965). Insulin treatment also resulted in increased food intake and fat deposition in these rats, presumably through an increased ability to convert excess carbohydrate to fat for storage (Beaton et al., 1966). The observation that animals fed the 3% protein diet in the present study did not have decreased plasma albumin and did not decrease their food intake may indicate that the ratio of protein:energy was sufficiently high to permit efficient utilization of energy and protein to maintain body weight gain and plasma albumin levels on a limited protein intake.

Tibia zinc concentration in a primary and challenge infection and liver zinc concentration in a primary infection were also significantly reduced in animals fed 3% protein. Previous observations have indicated that the quantity of protein in the diet can affect zinc deposition into bone when dietary protein and zinc intakes are adequate (Hunt and Johnson, 1992; Johnson and Evans, 1984; Snedeken and Greger, 1985). High intake of protein (30-45% protein) results in greater bone zinc deposition, and it has been observed that protein enhances zinc absorption and bioavailability. It has also been noted that the differences in bone may not have been caused entirely by enhanced zinc absorption, but by unidentified interactions between protein and zinc

in bone (Johnson and Evans, 1984; Snedeken and Greger, 1985). The effects of protein deficient diets on bone zinc concentration have apparently not been fully investigated. Campen and House (1974) observed that rats fed protein deficient diets had reduced absorption of orally administered ^{65}Zn and increased endogenous excretion of zinc. Although bone zinc was not measured the low protein fed animals had decreased plasma, hepatic and small intestinal zinc concentrations. Therefore, an important consequence of protein depletion may be a secondary zinc deficiency. This may raise the possibility that the reduced zinc status in protein deficient animals may have contributed in part to their impaired immune response to *H. polygyrus* infection. Filteau and Woodward (1984), however, demonstrated that the depressed antibody response and delayed hypersensitivity in severely protein deficient mice were not corrected by subcutaneous zinc injections which re-established serum zinc levels to near normal. These authors concluded that secondary zinc deficiency is not likely to be an underlying cause of the immunodepression which occurs in severe protein deficiency.

Finally, protein deficient mice fed the 3% protein diet also had elevated alkaline phosphatase activity compared to mice fed higher protein levels. Alkaline phosphatase is an enzyme found in the liver, bone, placenta, and intestine and is useful in detecting diseases in these organs (Lee and Nieman, 1993). Activity is high in children, during skeletal growth in adolescents, and during pregnancy (Lee and Nieman, 1993). Values are also elevated in patients with hepatic disease such as hepatitis and cirrhosis, and with bone pathology such as rickets in children and osteomalacia in adults (Tilkian et al., 1987). It is possible that protein deficiency may have stressed these tissues in the mice, resulting in elevated alkaline phosphatase values. For example, it is known that protein deficiency with adequate caloric intake causes a fatty liver in

rats and humans; in rats the process progresses spontaneously to cirrhosis (Rudman and Feller, 1990). Also, protein deficient rats show retarded bone growth leading to bones which are shorter and lighter than those of well-fed animals (Le Roith and Pimstone, 1973; Orwoll et al., 1992). Le Roith and Pimstone (1973) observed lower calcium accretion but unchanged calcium resorption in bone of protein deficient rats.

II. EFFECT OF HOST NUTRITION ON WORM BURDEN AND IMMUNE FUNCTION

In the present study, worm burden 29 days post-primary infection was significantly greater in mice fed 7% protein and 3% protein than in mice fed 24% protein, and significantly greater in mice fed diets containing 3 mg zinc/kg than in mice fed diets containing 60 mg zinc/kg. The effects of dietary restriction on the number of female and male parasites followed the same pattern as that on the total number of worms and parasite sex ratio was not affected by dietary restrictions, indicating that female and male worms were equally affected by dietary treatment. Also, there were no interactive effects of combined dietary protein and zinc restrictions on any of the worm burden parameters.

Previously, neither Brailsford and Mapes (1987) nor Slater and Keymer (1988) found dietary protein level to have any effect on *H. polygyrus* burden in a primary infection. Also, Minkus et al. (1992) found no effect of marginal zinc deficiency (5 mg zinc/kg diet) on *H. polygyrus* burden in a primary infection. However, in contrast to the present study, these studies have found that the control and deficient animals remained infected with equally large worm burdens after a period of 3 to 4 weeks post-primary infection. Therefore, the reduction in parasite

number in the control animals in a primary infection (and not a greater parasite numbers in the dietary restricted groups) is the factor from this study which differs from others (Brailsford and Mapes, 1987; Slater and keymer, 1988; Minkus et al., 1992).

In the present experiment, control mice (mice fed 24% protein with 60 mg zinc/kg) were able to reduce their worm burden to 6 ± 3 worms 29 days after an inoculation with 100 larvae. The establishment control mice killed on day 9 post-primary infection, prior to the challenge infection protocol, indicated that parasite establishment in the control mice did not differ from the other dietary groups. Parasite establishment in the control mice on day 9 post-primary infection was 62 ± 13 worms as compared to 6 ± 3 that survived 29 days post-primary infection. Therefore, although similar numbers of worms established in the control mice, the subsequent survival of the parasite was reduced as compared to the other dietary groups. The similar number of eggs produced by all dietary groups on day 16 post-primary infection also suggests that all mice harboured similar numbers of worms at the beginning of the experiment. However, egg production subsequently declined rapidly until the end of the experiment in the control group, which indicates that progressive loss of adult worms was taking place in these mice. In agreement with the present results, Shi et al., (1994a) found severe zinc restriction (0.75 mg zinc/kg diet) to result in higher worm burdens as compared to pair-fed and *ad-libitum*-fed controls, 5 weeks post-primary infection. In the latter study, control animals were also able to reduce their worm burden to approximately 20 - 25 worms, or to 50% of the numbers detected in deficient animals.

The effect of dietary protein and zinc restriction on worm burden in a primary infection observed in this study, and the effects of severe zinc deficiency observed in the study of Shi et

al. (1994a) may differ from other studies which have found no effect of protein or zinc restriction in a primary infection (Brailsford and Mapes, 1987; Minkus et al., 1992; Slater and Keymer, 1988) due to a combination of strain and sex of mice used in the experiments. The present study and the study of Shi et al. (1994) used BALB/c female mice, while others have used either outbred male or female CD1 mice.

The chronicity of primary infections with *H. polygyrus* can vary widely from 4 weeks to 10 months, depending on mouse strain (Ehrenford, 1954; Monroy and Enriquez, 1992; Robinson et al., 1989; Wahid et al., 1989), and low responsiveness and chronic infection appear to reflect genetically determined host susceptibility to parasite immunomodulation. Behnke et Wahid (1991) demonstrated that H-2 linked genes in the mouse determine worm survival. Also, female mice are more resistant to *H. polygyrus* than are male mice (Dobson and Owen, 1978). There is now growing evidence that the BALB/c mouse is a resistant strain which can develop an immune response in a primary infection that may be effective in limiting worm survival (Robinson et al., 1989; Shi et al., 1994a). However, Behnke and Robinson (1985) pointed out that, in their laboratory, different mice of the BALB/c strain have ranged in performance from weak (< 20% protection) to strong responders (almost 100% protection) in response to *H. polygyrus*. These authors have therefore designated this strain as an intermediate responder since it is not as consistent as the other strong responder strains. The findings from other studies that dietary protein or zinc restrictions had no effect on worm burden in a primary infection (Brailsford and Mapes, 1987; Slater and Keymer, 1988; Minkus et al., 1992) could be due to the fact that the outbred mice used in the experiments were more susceptible to parasite-induced immunosuppression and, therefore, any effect caused by dietary restriction would have been

masked. In the present study and in the study of Shi et al., (1994a), control mice did not appear to be susceptible to parasite-induced immunodepression in a primary infection and, therefore, in resistant BALB/c mice, interference of dietary restriction with parasite survival could be detected.

The mechanism by which dietary deficiency alters the worm survival in a primary infection is not evident from the present study. Behnke et al. (1992) suggested that some hosts may be susceptible to infection, fail to mount rapid host-protective immune response and support chronic infections, not because they lack the genes necessary for the expression of an appropriate response, but because they are susceptible to the immunomodulatory products of the worms themselves. This aspect of resistance and susceptibility has not received sufficient attention, but Behnke and Wahid (1991) have shown that it exists and that it is an important determinant of the outcome of infection. Similarly, two possibilities exist for an effect of dietary protein and zinc deficiencies on *H. polygyrus* in a primary infection. One possibility is that protein or zinc restricted mice may fail to produce an effective protective response in a primary infection due to direct effects of host nutrition on immune function, and the other possibility is that protein or zinc restricted mice may be more susceptible to parasite-induced immunomodulation.

Two parameters of immune function were measured in the present study which were eosinophilia and serum IgG1 profile. In a primary infection, the eosinophil response was more prominently affected by zinc restriction as it was delayed and less pronounced in zinc restricted animals. The eosinophil response was not affected to the same degree by the two levels of dietary protein restrictions. The magnitude of the response was increased in animals fed the 7% protein diet while it was only by day 21 post-infection that eosinophils were lower in the animals fed 3% protein as compared to higher protein levels. Therefore, decreased eosinophilia in a primary

infection could only contribute to the higher parasite survival in mice fed the low zinc diets and to a lesser extent in mice fed 3% protein, but it does not appear to be related to worm burden in mice fed 7% protein. The absence of a clear relationship between the eosinophil pattern and worm survival does not support a role of eosinophils in limiting worm survival in a primary infection. The effect of protein and zinc restrictions on IgG1 profile also did not readily offer a mechanism for the higher worm survival in deficient animals. Serum IgG1 levels were significantly lower in the 3% protein groups starting on day 14 until the end of the experiment, whereas mice fed 7% protein and mice fed 3 mg zinc/kg diet did not differ from controls throughout the period.

Slater and Keymer (1988) also found no effect of protein deficiency (2% protein) on the eosinophilia in mice during a primary infection with *H. polygyrus*, although uninfected protein deficient mice had lower peripheral eosinophil numbers than uninfected mice fed control levels of protein. These results are similar to ours in which eosinophils in mice fed 3% protein were only lower on day 21 post-primary infection when levels were returning to baseline. In contrast, Shi et al. (1994a) found that the % eosinophils in peripheral blood, measured on day 21 post-primary infection, was significantly decreased by severe zinc deficiency. Although these authors measured eosinophils at only one time point, the results agree with the reduced eosinophilia seen in the zinc restricted animals in the present study. Thus, it appears that eosinophilia in a primary infection with *H. polygyrus* is more susceptible to dietary zinc restriction than to dietary protein restriction. In contrast to our study, Shi et al. (1994a) found severe zinc deficiency to result in significantly lower serum IgG1 2 weeks and 5 weeks post-primary infection. Thus, IgG1 production may not be sensitive to marginal zinc deficiency as we observed no effect of marginal

zinc restriction on serum IgG1. Although the study of Shi et al. (1994a) suggested a possible link between decreased IgG1 response and eosinophilia, and increased worm survival, our study suggests that other mechanism must be involved as increased worm survival occurred in zinc restricted animals, despite the normal IgG1 response and increased worm survival occurred in mice fed 7% protein despite normal IgG1 response and eosinophilia.

The immunological processes accompanying loss of *H. polygyrus* in a primary infection are not understood and the role of eosinophils and IgG1 in limiting a primary infection is still controversial. Some studies have shown that serum from animals exposed to a primary infection contains parasite specific IgG1, but passive transfer studies have revealed that immunity cannot be transferred using this serum (Pritchard et al., 1984; Williams and Behnke, 1983). Recently, Urban et al. (1991a) showed that depletion of CD4+ T cells by anti-CD4+ monoclonal antibody treatment did not affect *H. polygyrus* survival in BALB/c mice during a primary infection, despite the marked reduction in serum IgG1 and IgE caused by the treatment, indicating that serum IgG1 and IgE may have no role in limiting a primary infection. In contrast, Wahid and Behnke (1993) demonstrated that mouse strains which speedily limit primary *H. polygyrus* infections, including BALB/c mice, produce a more intense specific IgG1 antibody response and greater amounts of total IgG1 than strains which tolerate chronic infections. These results support a role for IgG1 in a primary infection. However, the authors also pointed out that other data reported in their study did not support a role of IgG1, as there was no relation between the intensity of IgG1 response within mouse strain and loss of worms by particular individuals. Moreover, there was no difference between sexes of mice in the IgG1 response despite the earlier loss of worms in female BALB/c and NIH mice.

The role of eosinophils in a primary infection is also uncertain. Although eosinophils increase in blood and tissue of mice during a primary infection with *H. polygyrus*, these cells are thought to be involved in the killing of the trapped larvae only 6-7 days after challenge infection (Hurley and Vadas, 1983). Monroy and Enriquez (1992) suggested that the lack of larvicidal activity of eosinophils in a primary infection may be related to the lack of protective IgG1 which mediates eosinophil killing in mice (Pritchard et al., 1983), or may be due to the fact that young adult worms escape to the gut lumen before the granulomata are formed.

The data from the present study also did not support a role for IgG1 and eosinophils in limiting a primary infection since some of the dietary treatments which resulted in increased worm survival did not induce an altered IgG1 and/or eosinophil response. Perhaps dietary protein and zinc restrictions in a primary infection may have interfered with other more diverse components of enteric physiology such as goblet and mast cells, epithelial function and smooth muscle cell responses or other factors which may also contribute to protective immunity to intestinal helminths (Castro, 1989). Alternatively, these mice may have been more susceptible to parasite immunomodulatory factors which are released by and thought to act in close proximity to adult worms (Behnke, 1987; Behnke et al.,1992).

In contrast to a primary infection, marginal zinc restriction and marginal protein restriction (7% protein) had no effect on worm survival 29 days post-challenge infection. These animals were able to expel the worms in a manner similar to the control animals. The lowest level of dietary protein (3% protein), however, clearly impaired worm expulsion mechanisms in a challenge infection. These animals remained infected with an average of 63 ± 5 worms after a challenge with 100 larvae, as compared to only 3 ± 1 worms in the other protein groups. When

this value is compared to the primary infection, in which mice fed 3% protein remained infected with 68 ± 4 worms, the data strongly suggest that the response to a challenge immunizing protocol was totally blocked by this level of protein deficiency, as there was practically no reduction in worm numbers from a primary infection. This lack of response to an immunizing protocol provides strong indirect evidence of nutritionally mediated effects on host immune function, as the involvement of the immune response in limiting challenge infections is well documented (Behnke, 1987; Behnke et al., 1992; Monroy and Enriquez, 1992). Both female and male parasites were equally affected by dietary treatment, as there was no difference in the sex ratio of the worms among the dietary groups. Also, there was clearly no interaction between dietary protein and zinc restrictions on any of the worm burden parameters.

Impaired host resistance to *H. polygyrus* due to protein deficiency has been reported previously in experiments using various infection protocols. Slater and Keymer (1986a) reported that mice fed 2% protein accumulated adult worms in direct proportion to exposure with either 5, 10, 20 or 40 larvae every two weeks, while in contrast, mice fed 8% protein acquired a partly effective immunity to reinfection in a dose dependent manner. As in our study, immunity appeared to act with equal force against male and female parasites as no changes in sex ratio were observed. Keymer and Tarlton (1992) reported similar results on final worm burden in female NIH mice fed 3% protein as compared to control mice fed 16% protein, when exposed repeatedly to either 5 or 40 larvae. The ability to immunize mice with gamma irradiated larvae was also found to be depressed by feeding outbred male mice protein deficient diets containing 2% and 4% protein as compared to 8% protein and by feeding female NIH mice 2% protein as compared to 4% or 16% protein (Slater and Keymer, 1988). In addition the resistance of NIH

female mice to a challenge protocol similar to that employed in the present study was also impaired by feeding diets containing 2% protein as compared to 16% protein (Slater and Keymer, 1988). The effects of protein deficiency on the experimental epidemiology of *H. polygyrus* was also studied by Slater and Keymer (1986b) and by Slater (1988). In this system, colonies of mice were fed diets containing 2% protein compared to diets either containing 8% protein (Keymer and Slater (1986) or 16% protein (Slater, 1988). These experiments showed that dietary protein deficiency could influence the rate of parasite transmission in a closed population by increasing the survival of adult worms and by increasing female worm fecundity. From the present experiment, and all of the previous experiments studying the effects of protein deficiency on the resistance of mice to *H. polygyrus*, it is evident that protein deficiency caused by feeding diets containing 2-3% protein impairs the host response to *H. polygyrus*. This study was the first to include an intermediate level of 7% protein, and from the results, it appears that even though feeding a marginal 7% protein diet can result in higher worm burden in a primary infection, there is no subsequent effect on the host response to a challenge infection.

Fewer studies have examined the effects of zinc deficiency on *H. polygyrus* challenge infection. Minkus et al. (1992) reported that although plasma zinc concentration was significantly lower in mice fed zinc restricted diets containing 5 mg zinc/kg, there were no significant differences between zinc restricted and control mice in their cell-mediated immune response to SRBC or in final worm burdens. In contrast, Shi et al. (1994b) found that severe zinc deficiency caused by feeding a diet containing 0.75 mg zinc/kg (which resulted in impaired body weight gain, reduced food intake and lower liver and plasma zinc concentration) resulted in impaired worm expulsion after a challenge infection protocol identical to that of the present study. The

worm burden reported for the zinc deficient animals 5 weeks post-challenge infection was similar to that reported for the 3% protein fed mice of the present study. From the results accumulated in this laboratory, it appears that marginal zinc restriction to a dietary level of either 3 mg/kg or 5 mg/kg is not sufficient to impair the host response to challenge infection, but as the deficiency progresses to a more severe state the host response to challenge is impaired.

Few studies have investigated the mechanisms by which protein or zinc deficiency impairs the host response to re-infection with *H. polygyrus*. Slater and Keymer (1988) reported that immunization to *H. polygyrus* primed all mice, whether well fed or protein restricted, for an intense production of antibody against larval antigen after challenge infection. Although a slightly higher titre of total IgG was detected in the plasma of mice fed a 16% protein diet compared to mice fed a 2% protein diet, these authors concluded that this was unlikely to be sufficient to account for the reduced resistance in the protein deficient mice. The development of eosinophilia in the blood of immunized mice was significantly delayed and reduced in protein deficient mice and it was suggested that a reduction in the number of eosinophils attacking larval worms contributed to the low level of resistance. Shi et al. (1994b) found that zinc deficiency resulted in impaired proliferative responses of spleen cells to T cell mitogens and parasite antigen, but that the production of IL-5 (which regulates eosinophilia) and the production IL-4 (which regulates the IgG1 and IgE response to *H. polygyrus*) were equally reduced by both zinc deficiency and pair-feeding. These results indicate that defects at this level may be due to energy restriction associated with zinc deficiency and not to zinc deficiency per se.

In the present study, eosinophil numbers and IgG1 profile in response to a challenge infection were measured. The eosinophilia was found to be significantly lower in the 3% protein

fed animals throughout the infection period, whereas mice fed 7% and 24% protein did not differ from one another throughout the period. Although the zinc restricted animals started out with fewer eosinophils on day 7 post-challenge infection as compared to mice fed zinc adequate diets, the response was no longer different on days 14 and 21 post-challenge infection. These findings for the challenge infection contrast with those of the primary infection in which it was the zinc restricted animals who had a delayed and shorter lived response, whereas animals fed 3% protein had fewer eosinophils on day 21 post-infection only. The absence of a relationship between worm burden and eosinophilia in a primary infection contrasts markedly with the clear pattern in a challenge infection. Mice fed 3% protein had significantly reduced eosinophilia throughout the period, and were the only group to have impaired response to the challenge infection. The different effects of protein and zinc restrictions observed between a primary and challenge infection suggest different mechanism and require further investigation.

The rise in serum IgG1 in a challenge infection was delayed in mice fed either 7% or 3% protein on day 7 post-challenge infection. Serum IgG1 level in mice fed 3% protein peaked earlier and at a lower level (on day 14 post-infection) compared to mice fed the other protein levels, and was lower than the concentration in control mice for the remainder of the experiment. The IgG1 response in animals fed 7% protein did not differ from controls after day 7 post-infection and was not affected by zinc restriction throughout the challenge period. The effect of diet on IgG1 response in a challenge infection did not differ from that in the primary infection in the sense that mice fed 3% protein were more consistently affected in both protocols, having a response that was delayed and which did not reach the magnitude of that exhibited by mice fed the other protein levels. In contrast to the results observed in the primary infection, the

eosinophilia and IgG1 responses in the challenge infection were consistent with a negative effect of protein deficiency on the host immune response to infection and consequent increased parasite survival.

The hypothesis that combined dietary protein and zinc restrictions would have interactive effects on the final outcome of infection, as measured by final worm burden, was not verified by the present results, although isolated statistical interactions occurred on parasite egg output on day 22 post-primary infection and on female fecundity on day 28 post-primary infection. It appears that combining a marginal level of protein or a low level of protein with a marginal level of zinc produces no effects above those of single protein or zinc restrictions in either primary or challenge infection protocols. The maximum effects on worm burden were observed at a level of 3% protein regardless of dietary zinc level. The number of worms surviving in mice fed 3% protein 29 days post primary and challenge infections appears to be close to the maximum number of parasites that were initially measured in mice on day 9 post-primary infection in both the preliminary study and experiment 1. Therefore, the data suggest that the response is totally blocked by a low 3% protein diet and adding a marginal zinc restriction has no additional effects. Although we did not observe an interaction between the levels of protein and zinc chosen for this study it is possible that interactions could occur at levels of protein between the range of 7% and 3%, or possibly by combining these levels of protein with lower levels of dietary zinc that would produce a greater stress on host nutritional status and immune function and/or on the intestinal environment of the parasite.

III. EFFECT OF HOST NUTRITION ON WORM EGG PRODUCTION

Other parasite parameters which were studied in this experiment included daily parasite egg output and female parasite fecundity. The egg production per day measured on days 16, 22 and 28 post-infection, and female parasite fecundity measured on day 28 post-infection were lower than has been previously reported in this laboratory and by others. For example Scott (1991) reported that BALB/c mice fed commercial chow diets, and infected with 100 larvae, produced approximately 30 000 eggs/mouse/day and 1114 eggs/female worm 4 to 5 weeks post-primary infection, and 5000 eggs/mouse/day or 500 eggs/female worm 8 weeks post-challenge infection. The daily egg output and female parasite fecundity reported in Tables 3.1.2, 3.2.3 and 3.2.4 are clearly lower than these reported values. Several authors have suggested that feeding synthetic diets can suppress parasite fecundity (De Witt and Weinstein, 1964; Slater and Keymer, 1986b). The egg data from the present study, however, are still less than half of the values previously reported in this laboratory using similar synthetic diets (Minkus et al., 1992; Shi et al., 1994a).

Several factors have been found to affect parasite fecundity. Keymer and Hiorns (1986) reported a significant positive relationship between host faecal volume and *H. polygyrus* fecundity; also, both faecal output and worm fecundity were depressed by a period of host food deprivation. Kerboeuf and Jacobs (1983) reported that *H. polygyrus* egg output was stimulated by increasing the flow of digesta through the intestine of infected mice. It is not clear whether a combination of synthetic diet and low food intake and/or low faecal output may account for the relatively small fecundity reported in the present study. Another factor that must be addressed

is the very humid conditions (above 75% relative humidity) which prevailed in the animal facility during experiment 1. Also there were time intervals when the ambient room temperature exceeded 25 ^{0}C because of failure in the temperature control unit during the very warm summer weather. It is possible that the mice ate less food under these environmental conditions thereby affecting parasite fecundity. Alternatively, the possibility exists that female *H. polygyrus* are less fecund during periods of elevated ambient temperature and humidity, although this possibility has not previously been studied with *H. polygyrus*. Finally, it is important to note that the control animals in the present study were able to clear a primary infection much faster than previously observed in BALB/c mice fed commercial chow diets (Scott, 1991) and slightly faster than previously observed in BALB/c mice fed a similar synthetic diet (Shi et al., 1994a), suggesting that host immune response or other factors acting to limit a primary infection were particularly strong in the present study.

A. PRIMARY INFECTION

In a primary infection, repeated measures analysis indicated that the pattern of daily parasite egg production through time differed according to the level of protein in the diet, and that the overall egg production was greater in protein restricted animals. Parasite egg production did not differ among the dietary groups on day 16 post-infection, indirectly indicating that mice were harbouring similar number of worms at this time. In general, daily egg production decreased with time in the 24% protein group, whereas it decreased slightly but to a lesser extent in the 7% protein group. In contrast, daily egg production increased slightly with time in the 3% protein group. This difference in the pattern of egg production may in part be attributed to the progressive loss of worms in the 24% protein group compared to the protein restricted groups.

Although not significant, probably due to high variability and the small sample size, the worm burden in the 7% protein group tended to be smaller than that in the 3% protein group, and this difference could also explain some variation in egg output through time between these two groups. There was no effect of the level of zinc in the diet on the pattern of egg production or on the overall egg production, as indicated by repeated measures analysis, even though zinc restricted animals had significantly higher worm burdens at the end of the experiment when compared to animals fed adequate zinc. This may be due to the fact that the difference in worm burden was smaller between the groups fed the 2 levels of zinc than that between the groups fed the different levels of protein. The egg production on day 28 post-primary infection, however, was significantly greater in mice fed diets containing 3 mg zinc/kg than in mice fed diets containing 60 mg zinc/kg. This outcome partly reflects the significantly higher worm burden in the zinc restricted groups.

Other factors affecting egg production can be assessed better by looking at female parasite fecundity, which was expressed as the total egg production on day 28 divided by the number of female worms in each mouse on day 29 post-infection. The number of eggs per female worm was affected by a significant interaction between the levels of protein and zinc in the diets such that mice fed 24% protein with 60 mg zinc/kg diet had fewer eggs produced per female worm than all the other groups except the mice fed 7% protein with 3 mg zinc/kg diet. The basis for this interaction is difficult to explain as it only happened at one level of protein and no other results, parasitological, immunological or nutritional, suggest an explanation. When the data were examined for the individual mice, two mice in the control group harboured only one female worm with no male worm present and had a fecundity of zero. If those mice are removed,

because zero fecundity can be attributed to the absence of male worms to mate rather than to the diet, the value becomes 117 ± 9 eggs/female worm in the control mice instead of 59 ± 34. Statistical analysis given this factor gave a different pattern such that mice fed 24% protein with 60 mg/kg zinc and mice fed 7% protein with 3 mg/kg zinc had significantly lower egg production than mice fed 7% protein with 60 mg/kg zinc, while the other dietary groups did not differ from one another.

The effect of protein restriction on *H. polygyrus* egg production in a primary infection has not been previously examined. Michael and Bundy (1991) found that feeding mice a low (4%) protein diet had no effect on *Trichuris muris* fecundity in a primary infection, although this level of protein restriction resulted in delayed parasite expulsion. Also, neither marginal zinc deficiency (Minkus et al., 1992) nor severe zinc deficiency (Shi et al., 1994a) had an effect on *H. polygyrus* fecundity in a primary infection. In the present study, there was no difference between animals fed 3% protein or 24% protein, and no effect of zinc at these protein levels when the data were adjusted to exclude mice which harboured no male worms in the control group. However, the interaction between protein and zinc levels makes interpretation of the data difficult. Results from previous studies, however, do not support an effect of protein or zinc deficiency on female parasite fecundity in a primary infection.

B. CHALLENGE INFECTION

In contrast to the primary infection, no difference in the pattern of eggs produced through time could be detected by repeated measures analysis in a challenge infection. Between day 16 and day 28, total egg output remained low in animals fed 24% or 7% protein, while egg output

remained consistently high in animals fed 3% protein. On all measurement days, total egg output was significantly greater in mice fed 3% protein regardless of zinc level, than in mice fed 24% and 7% protein. This result reflects the higher worm burden that was recovered from mice fed 3% protein compared to higher protein levels 29 days post-challenge infection. The early drop in egg production in mice fed 24% and 7% protein (on day 16 post-infection) also reflects the host response that occurs to limit worm survival in a challenge infection. In a primary infection, egg production decreased in the control animals after day 16 post-infection suggesting that adult worms were being expelled, whereas the already low egg production on day 16 post-challenge infection in the two higher protein groups, suggests loss of larvae and early loss of adult worms. In a challenge infection, the host response is directed mainly against the L_4 tissue stage, and very few adult worms emerge in the intestinal lumen. Cypess (1988) reported that even though 25.5% of a larval inoculum were able to mature and emerge from the intestinal wall between 9-13 days post-challenge infection, only 2.6% of the infective dose could be found as adults in the intestinal lumen on day 14 post-challenge. In the present experiment, both larval and adult worm expulsion may have been affected by the 3% protein diet since these mice produced large numbers of parasite eggs throughout the period.

Female fecundity on day 28 post-challenge infection was significantly lower in mice fed 7% protein than in mice fed 24% or 3% protein, and there was no effect of zinc level on this parameter. Acquired immunity can act to depress the size and fecundity of *H. polygyrus* female worms (Behnke and Parish, 1979 and 1981; Keymer and Hiorns, 1986; Slater and Keymer, 1988). Urban et al. (1991a) also showed that depleting CD4+ T cells impaired the immune response acting on female parasite fecundity. The lower parasite fecundity in mice fed 7% protein may

indicate a stronger immune response acting to depress female fecundity or an effect of this protein level on other physiological parameters of the female parasite. Another consideration is that live worms cannot be distinguished from dead worms when they are counted in the intestinal lumen post-mortem. It is possible that some of the few worms recovered from the 7% protein group were already dead and not fecund.

Previously, Slater and Keymer (1986a) found that female *H. polygyrus* fecundity was significantly higher in mice fed 2% protein than in control mice fed 8% protein, after a period of repeated infections to stimulate host immune response. Shi et al. (1994a) also found higher female *H. polygyrus* fecundity in zinc deficient animals than in pair-fed and *ad libitum*-fed animals in a challenge infection (Shi et al., 1994b). These authors have attributed higher parasite fecundity to an altered host immune response in deficient animals. In the present study, mice fed 3% protein had an impaired response to challenge infection as evidenced by the increased parasite survival and lower eosinophil and IgG1 responses; female parasite fecundity, however, did not differ from that of parasites in control mice, although it was lower than in animals fed 7% protein. As mentioned earlier, total egg output and female parasite fecundity were found to be much lower in the present study than has been previously reported. It is possible that other factors such as food intake pattern, faecal output, ambient temperature and humidity or other unknown factors which resulted in low parasite egg output in this study may have masked or prevented detection of the effects of dietary treatment.

IV. SUMMARY

The most significant finding in this study was the different effects of dietary protein and zinc restrictions during a primary and challenge infection protocol. While both marginal and low protein diets, and marginal zinc restriction resulted in increased parasite survival in a primary infection, only the lowest level of protein impaired host response to a challenge infection. The fact that the 3% protein-fed animals could not respond to an immunizing challenge infection protocol, as evidenced by similar parasite numbers recovered from these animals in both protocols, is strong indirect evidence that host immune response operating to reduce parasite survival in a challenge infection was impaired by this level of protein deficiency. Several immune function parameters which are stimulated by *H. polygyrus* infection were measured in both primary and challenge infections and included eosinophils in peripheral blood and serum IgG1 concentration. The eosinophilia was also affected differently by dietary protein and zinc restrictions in a primary vs challenge infection protocol. While there was no clear relationship between the dietary effects on eosinophils and worm burden in a primary infection, these two parameters appeared to be related in a challenge infection. Significantly reduced eosinophilia and slightly reduced IgG1 profile in the 3% protein group in a challenge infection, supported the idea that a negative effect of protein deficiency on the host immune response resulted in increased parasite survival. Finally, the hypothesis that combined dietary protein and zinc restrictions would produce interacting effects on final outcome of infection was not verified by the present results. Combined dietary protein and zinc restrictions, at the levels chosen for this study, resulted in statistically independent effects on worm burden and host immune response in both primary and

challenge infection protocols.

The different effects of marginal protein and zinc restrictions observed in a primary vs challenge infection protocol suggest different mechanisms of impact and require further investigation. The eosinophilia and IgG1 response measured in a primary infection did not appear to be related to worm survival since some of the dietary treatments which resulted in normal eosinophil numbers and/or IgG1 level also resulted in increased worm survival. It is suggested that either marginal protein and zinc restrictions may have impaired other immune parameters acting to limit a primary infection or that, alternatively, these levels of protein and zinc restriction may have increased host susceptibility to parasite immunomodulation. In the challenge infection protocol, parasite expulsion mechanisms were clearly impaired by the 3% protein diet. Although eosinophilia and IgG1 level were reduced in these animals (suggesting a negative effect of protein restriction on the host response to infection), further investigation is required to determine the precise mechanism by which protein deficiency interferes with the complex host response to *H. polygyrus*. Decreased eosinophil numbers and IgG1 levels may indicate reduced production of IL-5 and IL-4, respectively, but whether these mechanisms were specifically involved or whether other mechanisms played a role in increasing parasite survival in protein deficient animals remains to be established.

Results from this and previous studies have shown that host dietary protein status is an important determinant of the host response to an intestinal helminth infection. From this study, it is evident that moderate protein deficiency which permits a slow rate of body weight gain but which is not sufficiently severe to produce wasting, edema, hypoalbuminemia and atrophied lymphoid organs, can still have profound effects on the outcome of an intestinal helminth

infection. This finding may be relevant to human populations in which intestinal parasites and protein malnutrition often co-occur in the same individuals and suggest that mild to moderate protein deficiency may predispose individuals to acquire higher levels of infection.

BIBLIOGRAPHY

Aggett, P.J. (1991) Workshop on 'assessment of zinc status'. Proc. Nutr. Soc. 50:9-17.

Aggett, P.J. (1989) Severe zinc deficiency. in: Mills, C.F. (ed.), Zinc in Human Biology, pp. 259-279. London: Springer-Verlag.

Ali, N.M.H. and Behnke, J.M. (1985) Observations on the gross changes in the secondary lymphoid organs of mice infected with *Nematospiroides dubius*. J. Helminth. 59:167-174.

Allen, J.I., Kay, N.E. and McClain, C.J. (1981) Severe zinc deficiency in humans: association with a reversible T-lymphocyte dysfunction. Ann. Intern. Med. 95:154-157.

Bach, J.F., Dardenne, M., Pleau, J.M. and Bach, M. (1975) Isolation, biochemical characteristics and biological activity of a circulation thymic hormone in the mouse and in the human. Ann. N.Y. Acad. Sci. 249:186-210.

Barrett, J. (1981) Biochemistry of parasitic helminths, London: McMillan.

Bartlett, A. and Ball, P.A.J. (1972) *Nematospiroides dubius* in the mouse as a possible model for endemic hookworm infection. Ann. Trop. Med. Parasitol. 66:129-134.

Bawden, R.J. (1969) Some effects of the diet of mice on *Nematospiroides dubius* (Nematoda). Parasitology 59:203-213.

Beach, R.S., Gershwin, M.E. and Hurley, L.S. (1981) Nutritional factors and autoimmunity. I. Immunolpathology of zinc deprivation in New Zealand mice. J. Immunol. 126:1999-2006.

Beach, R.S., Gershwin, M.E. and Hurley, L.S. (1980a) Growth and development of postnatally zinc-deprived mice. J. Nutr. 110:201-211.

Beach, R.S., Gershwin, M.E. and Hurley, L.S. (1980b) T cell function in the lethal milk (Im/Im) mutant mouse. Proc. Int. Cong. Immunol. 4th 1980 p 171.

Beach, R.S., Gershwin, M.E. and Hurley, L.S. (1982) Nutritional factors and autoimmunity II. Prolongation of survival in zinc-deprived NZB/w mice. J. Immunol. 128:308-313.

Beaton, J.R., Feleki, V. and Stevenson, J.A.F. (1965) Insulin hyperfagia in rats fed a low-protein diet. Can. J. Phisiol. Pharmacol. 43:225-233.

Beaton, J.R., Feleki, V. and Stevenson, J.A.F. (1968) Factors in the reduced food intake of rats fed a low-protein diet. Can. J. Physiol. Pharmacol. 46:19-23.

Befus, D. (1986) Immunity to intestinal helminth infections: present concepts, future directions. Trans. Roy. Soc. Trop. Med. Hyg. 80:735-741.

Befus, D. and Bienenstock, J. (1982) Factors involved in symbiosis and host resistance at the mucosa-parasite interface. Prog Allergy 31:76-177.

Behnke, J.M. (1987) Evasion of immunity by nematode parasites causing chronic infections. Adv. Parasitol. 26:1-71.

Behnke, J.M., Barnard, C.J. and Wakelin, D. (1992) Understanding chronic nematode infections: evolutionary considerations, current hypotheses and the way forward. Int. J. Parasitol. 22:861-907.

Behnke, J.M. and Parish, H.A. (1979) *Nematospiroides dubius*: arrested development of larvae in immune mice. Exp. Parasitol. 47:116-127.

Behnke J.M., and Parish, H.A. (1981) Transfer of immunity to *Nematospiroides dubius*: co-operation between lymphoid cells and antibodies in mediating worm expulsion. Parasite Immnunol. 3:249-259.

Behnke, J.M. and Robinson, M. (1985) Genetic control of immunity to *Nematospiroides dubius*: a 9-day anthelmintic abbreviated immunizing regime which seperates weak and strong responder strains of mice. Parasite Immunol. 7:235-253.

Behnke, J.M. and Wahid, C. (1991) Immunological relashionships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): H-2 linked genes determine worm survival. Parasitology 103:157-164.

Behnke, J.M., Wahid, F.N., Grencis, R.K., Else, K.J., Ben-Smith, A.W. and Goyal, P.K. (1993) Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): downregulation of specific cytokine secretion (IL-9 and IL-10) correlates with poor mastocytosis and chronic survival of adult worms. Parasite immunol. 15:415-421.

Behnke, J.M. and Wakelin, D. (1977) *Nematospiroides dubius*: stimulation of acquired immunity in inbred strains of mice. J. Helminth. 51:167-176.

Beisel, W.R. (1982) Synergism and antagonism of parasitic diseases and malnutrition. Rev. Infect. Dis. 4:746-749.

Beisel, W.R. (1983) Single nutrients and immunity. Am. J. Clin. Nutr. 35:417-468.

Beisel, W.R. (1986) The role of zinc in neutrophil function. In: Prasad, A.S. (ed.), Clinical, Biochemical, and nutritional aspects of trace elements, pp. 203-210, Alan R. Liss, Inc., N.Y.

Bell, R.G. and Hazell, L.A. (1975) Influence of dietary protein restriction on immune competance. J. Exp. Med. 141:127-137.

Bell, R.G., Hazell, L.A. and Price, P. (1976) Influence of dietary protein restriction on immune competance II. Effect of lymphoid tissue. Clin. Exp. Immunol. 26:314-326.

Bhuyan, U.N. and Ramalingaswami, V. (1973) Immune responses of the protein-deficient guinea pig to BCG vaccination. Am. J. Pathol. 72:489-500.

Bhuyan, U.N. and Ramalingaswami, V. (1974) Systemic macrophage mobilization and granulomatous response to BCG in the protein-deficient rabbit. Am. J. Pathol. 76:313-319.

Bing, F.C., Lloyd, W.A. and Bowman, R.O. (1932) The protein requirements of the Albino mouse. J. Nutr. 5:571-579.

Bises, G. Mengheri, E. and Gaetani, S. (1987) T-lymphocyte subsets in zinc deficient and in protein malnourished rats. Nutr. Rep. Int. 36:1371-1378.

Bolin, T.D., Davis, A.E., Cummins, A.G., Duncombe, V.M. and Kelly, J.D. (1977) Effect of iron and protein deficiency on the expulsion of *Nippostrongylus brasiliensis* from the small intestine of the rat. Gut 18:182-186.

Bowers, G.N. and McComb, R.B. (1966) A continuous spectrophotometric method for measuring the activity of serum alkaline phosphatase. Clin. Chem. 12:70.

Brailsford, T.J. and Mapes, C.J. (1987) Comparisons of *Heligmosomoides polygyrus* primary infection in protein-deficient and well-nourished mice. Parasitology 95:311-321.

Brown, E.D., Chan, W. and Smith, C.J. (1978) Bone mineralization during developing zinc deficiency. Proc. Soc. Exp. Biol. Med. 157:211-214.

Bryant, V. (1973) The lyfe cycle of *Nematospiroides dubius*, Baylis, 1926 (Nematoda: Heligmosomoidae). J. Helminth. 47:263-268.

Bundy, D.A.P. and Golden, M.H.N. (1987) The impact of host nutrition on gastrointestinal helminth populations. Parasitology 95:623-635.

Burch, R.E., Hahn, H.K.J. and Sillivan, J.F. (1975) Newer aspects of the roles of zinc, manganese and copper in human nutrition. Clin. Chem. 21:501-520.

Campen, V.C. and House, W.A. Effect of a low Protein diet on retention of an oral dose of ^{65}Zn and on tissue concentrations of zinc, iron, and copper in rats. J. Nutr. 104:84-90.

Castro, G.A. (1989) Immunophysiology of enteric parasitism. Parasitol. Today 5:11-19.

Cavan, K.R., Gibson, R.S., Grazioso, C.F., Isalgue, A.M., Ruz, M. and Solomons, N.W. (1993a) Growth and body composition of periurban guatemalan children in relation to zinc status: a cross-sectional study. Am. J. Clin. Nutr. 57:334-343.

Cavan, K.R., Gibson, R.S., Grazioso, C.F., Isalgue, A.M., Ruz, M. and Solomons, N.W. (1993b) Growth and body composition of periurban guatemalan children in relation to zinc status: a longitudinal zinc intervention trial. Am. J. Clin. Nutr. 57:344-352.

Chandra, R.K. (1984) Parasitic infection, nutrition, and immune response. Fed. Proc. 43:251-255.

Chandra, R.K. (1988) Nutrition and immunology. Alan R. Liss, Inc., N.Y.

Chandra, R.K., Newberne, P.M. Nutrition, immunity and infection: mechanisms of interaction. New York: Plenum, 1977.

Chapman, C.B., Knopf, P.M., Anders, R.F. and Mitchell, G.F. (1979) IgG1 hypergammaglobulinaemia in chronic parasitic infections in mice: evidence that the response refflects chronicity of antigen exposure. Aust. J. Exp. Biol. Med. Sci. 57:389-400.

Chappell, L.H. (1982) Physiology In: Cox, F.E.G. (ed.), Modern Parasitology. A textbook of parasitology, pp. 116-147, Blackwell Scientific Publications, London.

Chesters, J.K. and Will, M. (1973) Some factors controlling food intake by zinc-deficient rats. Br. J. Nutr. 30:555-567.

Chvapil, M. (1976) Effect of zinc in cells and biomembranes. Med. Clin. North Am. 60:799-812.

Chvapil, M., Stankova, L., Zukoski, C. (1977) Inhibition of some functions of polymorphonuclear leukocytes by in vitro zinc. J. Lab. Clin. Med. 89:135-146.

Clegg, M.S., Keen, C.L., Lonnerdal, B. and Hurley, L.S. (1981) Influence of ashing techniques on the analysis of trace elements in animal tissues. Biol. Trace Elem. Res. 3:107-115.

Coovadia, H.M., and Soothill, J.F. (1976) The effect of amino acid restricted diets on the clearance of ^{125}I-labelled polyvinyl pyrolidone in mice. Clin. Exp. Immunol. 23:562-568.

Cousins, R.J. and Leinart, A.S. (1988) Tissue specific regulation of zinc metabolism and metallothionein genes by interleukin 1. FASEB J. 2:2884-2898.

Coward, W.A., Whitehead, R.G. and Lunn, P.G. (1977) Reasons why hypoalbuminaemia may or may not appear in protein-energy malnutrition. Br. J. Nutr. 38:115-126.

Crandall, R.B., Crandall, C.A. and Franco, J.A. (1974) *Heligmosomoides polygyrus* (*Nematospiroides dubius*): humoral and intestinal responses to infection in mice. Exp. Parasitol.

35:275-287.

Cromptom, D.W.T. (1986) Nutritional aspects of infection. Trans. Roy. Soc. Trop. Med. Hyg. 80:697-705.

Crompton, D.W.T. (1987) Host diet as a determinant of parasite growth, reproduction and survival Mammal. Rev. 17:117-126.

Cummins, A.G., Duncombe, T.D., Bolin, A.E., Kelly, J. (1978) Suppression of rejection of *Nippostrongylus brasiliensis* in iron and protein deficient rats: the effect of syngeneic lymphocyte transfer. Gut 19:823-832.

Cummins, A.G., Duncombe, V.M., Bolin, T.D., Davis, A.E. and Young, J. (1987a) The response of the small intestine of the protein-deficient rat to infection with *Nippostrongylus brasiliensis*. Int. J. Parasitol. 17: 1445-1450.

Cummins, A.G., Kenny, A.L., Duncombe, V.M., Bolin, T.D. and Davis, A.E. (1987b) The effect of protein deficiency on systemic release of rat mucosal mast cell protease II during *Nippostrongylus brasiliensis* infection and following systemic anaphylaxis. Immunol. Cell Biol. 65:357-363.

Cypess, R.H. (1974) Blood cell count changes associated with the immunity to *Nematospiroides dubius*. J. Parasitol. 58:563-566.

Cypess, R.H., Lucia, H.L., Dunsforf, H.A. and Enriquez, F.J. (1988) The tissue reactions of mice to infection with *Heligmosomoides polygyrus*. J. Helminth. 62:69-76.

Dardenne M., Savino, W., Wade, S., Kaiserlian, D., Lemonier, D. and Back, J.F. (1984) In vivo and in vitro studies of thymulin in marginally zinc-deficient mice. Eur. J. Immunol. 14:454-458.

Dawson, S., Landman, J., Bundy, D.A.P. and Heikens, G.T. (1987) Helminths and nutritional status in Jamaican todlers. WIMJ 36(suppl.):15

De Witt, W.B. and Weinstein, P.P. (1964) Elimination of intestinal helminths of mice by feeding purified diets. J. Parasitol. 50:429-434.

Dehlawi, M.S. and Wakelin, D. (1988) Suppression of mucosal mastocytosis by *Nematospiroides dubius* results from adult worm-mediated effect upon host lymphocytes. Parasite Immunol. 10:85-95.

Dobson, C. (1982) Passive transfer of immunity with serum in mice infected with *Nematospiroides dubius*: influence of quality and quantity of immune serum. Int. J. Parasitol. 12:207-213.

Dobson, C. and Owen, M.E. (1978) Effect of host sex on passive immunity in mice infected with *Nematospiroides dubius*. Int. J. Parasitol. 8:359-364.

Doumas, B. Watson, W, Biggs, H. (1971) Albumin standards and the measurement of serum albumin with bromcresol green. Clin. Chem. Acta. 31:87-96.

Douvres, F.W. and Urban, J.F. (1983) Factors contributing to the in vitro development of *Ascaris seum* from second-stage larvae to mature adults. J. Parasitol. 69:549-558.

Duncombe, V.M., Bolin, T.D., Davies, A.E., Kelly, D.J. (1981) Delayed expulsion of the nematode *Nippostrongylus brasiliensis* from the rats on a low protein diet: the role of a bone marrow derived component. Am. J. Clin. Nutr. 34:400-403.

Duncombe, V.M. Bolin, T.D. and Davis, A. (1979) The effect of iron and protein deficiency on the development of acquired resistance to reinfestation with *Nippostrongylus brasiliensis* in rats. Am. J. Clin. Nutr. 32:553-558.

Ehrenford, F.A. (1954) The lyfe cycle of *Nematospiroides dubius* Baylis (Nematoda: Heligmosomoidae). J. Parasitol. 40:480-481.

El-Hag, H.M., Macdonald, D.C., Fenwick, P., Aggett, P.J. and Wakelin, D. (1989a) Kinetics of *Nippostrongylus brasiliensis* infection in the zinc-deficient rat. J. Nutr. 119:1506-1512.

El-Hag, H.M., Macdonald, D.C., Fenwick P., and Aggett, P.J. (1989b) The lymphocyte response of zinc-deficient rats to sheep red blood cells and *Nippostrongylus brasiliensis* antigens. Nutr. Rep. Int. 40:1217-1227.

Fenwick, P.K., Aggett, P.J., Macdonald, D.C., Huber, C. and Wakelin, D. (1990a) Zinc deficiency and zinc repletion:effect on the response of rats to infection with *Trichinella spiralis*. Am. J. Clin. Nutr. 52:166-172.

Fenwick, P.K., Aggett, P.J., Macdonald, D.C., Huber, C. and Wakelin, D. (1990b) Zinc deprivation and zinc repletion: effect on the response of rats to infection with *Strongyloides ratti*. Am. J. Clin. Nutr. 52:173-177.

Filteau, S.M. and Woodward, B. (1984) Relationship between serum zinc level and immunocompetence in protein-deficient and well-nourished weanling mice. Nutr. Res. 4:853-866.

Finkelman, F.D., Pearce, E.J., Urban, J.F. and Sher, A. (1991) Regulation and biological function of helminth induced cytokine responses. In Ash, C. and Galagher, R. (ed.), Immunoparasitology Today, pp. A62-A66. Cambridge: Elsevier Trends.

Flanagan, P.R., Haist, J. and Valberg, L.S. (1983) Zinc absorption, intraluminal zinc and intestinal metallothionein levels in zinc-deficient and zinc-replete rodents. J. Nutr. 113:962-972.

Fraker, P.J., Haas, S.M. and Leucke, R.W. (1977) Effect of zinc deficiency on the immune response of the young adult A/J mouse. J. Nutr. 107:1889-1895.

Fraker, P.J., de Pasquale-Jardieu, P., Zwickl, C.M. and Luecke, R.W. (1978) Regeneration of T-cell helper function in zinc deficient adult mice. Proc. Natl. Acad. Sci. USA 75:5660-5664.

Fraker, P.J., Zwickl, C.M., and Luecke, R.W. (1982a) Delayed type hypersensitivity in zinc deficient adult mice: impairment and restoration of responsitivity to dinitrofluorobenzene. J. Nutr. 112:309-313.

Fraker, P.J., Caruss, R., Kierszenbaum, F. (1982b) Alteration of immune and nutritional status of mice by synergy between zinc deficiency and infection with Trypanosoma cruzi. J. Nutr. 112:1224-1229.

Fraker, P.J., Hilderbrandt, K. and Luecke, R.W. (1984) Alteration of antibody-mediated responses of suckling mice to T-cell-dependent and independent antigens by maternal marginal zinc deficiency: restoration of responsivity by nutritional repletion. J. Nutr. 114:170-179.

Fraker P.J., Gershwin, M.E., Good R.A. and Prasad A. (1986) Interrelationship between zinc and immune function. Fed. Proc. 45:1474.

Franke, E.D. and Weinstein, P.P. (1983) *Dipetalonema vitae* (Nematoda: Filariodea): culture of third-stage larvae to young adult in vitro. Science 221:161-163.

Franke, E.D. and Weinstein, P.P. (1984) In vitro cultivation of *Dipetalonema vitae* third-stage larvae: evaluation of culture media, serum and other supplements. J. Parasitol. 70:618-628.

Furguson, E.L., Gibson, R.S., Opare-Obisau, G., Ounpuu, S., Thompson, L.U. and Lehrfeld, J. (1993) The zinc nutriture of preschool children living in two African countries. J. Nutr. 123:1487-1496.

Gershwin, M.E., Beach, R.S. and Hurley, L.S. (1985) Nutrition and immunity. Academic Press, Inc., N.Y.

Giugliano, R. and Millward, D.J. (1984) Growth and zinc homeostasis in the severly zn-deficient rat. Br. J. Nutr. 52:545-560.

Goettsch, M.A. (1960) Comparative protein requirement of the rat and mouse for growth, reproduction and lactation using casein diets. J. Nutr. 70:307-315.

Grazioso, C.F., Isalgue, M., de Ramirez, I., Ruz, M. and Solomons, N.W. (1993) The effect of zinc supplementation on parasitic reinfestation of Guatemalan schoolchildren. Am. J. Clin. Nutr. 57:673-678.

Grimble, R.F. (1989) Cytokines: their relevance to nutrition. Eur. J. Clin. Nutr. 43:217-230.

Grimble, R.F. (1990) Nutrition and cyrokine action. Nutr. Res. Rev. 3:193-210.

Hagan, P., Behnke, J.M. and Parish, H.A. (1981) Stimulation of immunity to *Nematospiroides dubius* in mice using larvae attenuated by cobalt 60 irradiation. Parasite Immunol. 3:149-156.

Halsted, J.A., Ronaghy, H.A., Abadi, P., Haghenass, M., Amirhakimi, G.H., Barakat, R.M. and Reinhold, J.G. (1972) zinc deficiency in man. Am. J. med. 53:277-284.

Hambidge, K.M., Casey, C.E. and Krebs, N.F. (1986) Zinc. In: Mertz, W. (ed.), Trace Elements in Human and Animal Nutrition vol. 2, pp. 1-137, Academic Press, Inc. N.Y.

Harland, B.F., Spivey-Fox, M.R. and Fry, B.E. Jr. (1975) Protection against zinc deficiency by prior excess dietary zinc in young japanese quail. J. Nutr. 105:1509-1518.

Hildebrandt, K.M., Luecke, R.W. and Fraker, P.J. (1982) Effects of maternal dietary zinc deficiency on growth and mitogenic responsiveness in suckling mice. J. Nutr. 112:1921-1928.

Hunt, J.R. and Johnson, L.K. (1992) Dietary protein as egg albumen: effects on bone composition, zinc bioavailability and zinc requirements of rats, assessed by a modified broken-line model. J. Nutr. 122:161-169.

Hurley, L.S. and Swenerton, H. (1971) Lack of mobilization of bone and liver zinc under teratogenic conditions of zinc deficiency in rats. J. Nutr. 191:597-604.

Hurley, L.S. and Tao, S. (1972) Alleviation of teratogenic effects of zinc deficiency by simultaneous lack of calcium. Am. J. Phisiol. 222:322-325.

Hurley, J.C. and Vadas, M.A. (1983) Eosinophils and acquisition of resistance to *Nematospiroides dubius* in mice sensitized with adult worms. Aust. J. Exp. Biol. Med. Sci. 61:1-9.

Iwata, T. Incefy, G.S., Tanaka, T. Fernandes, G., Menendez-Botet, C.J., Pih, K. and Good, R.A. (1979) Circulating thymic hormone levels in zinc deficiency. Cell. Immunol. 47:100-105.

Jackson, M.J., Jones, D.A. and Edwards, R.H.T. (1982) Tissue zinc levels as an index of body zinc status. Clin. Physiol. 2:333-343.

James, S.J., Swendseid, M. and Makinodan, T. (1989) Macrophage-mediated depression of T-cell proliferation in zinc deficient mice. J. Nutr. 117:1982-1988.

Johnson, W.T. and Evans, G.W. (1984) Effects of the interrelationship between dietary protein and minerals on tissue content of trace metals in streptozotocin-diabetic rats. J. Nutr. 114:180-190.

Jose, D.G. and Welch, J.S. (1970) Growth retardation, anemia and infection, with malbsorption and infestation of the bowel. The syndrome of protein-calorie malnutrition in Australian aboriginal children. Med. J. Aust. 1:343-355.

Katona, I.M., Urban, J.F. and Finkelman, F.D. (1988) The role of $L3T4^+$ and $Lyt\text{-}2^+$ T cells in the IgE response and immunity to *Nippostrongylus brasiliensis*. J. Immunol. 140:3206-3211.

Keen, C.L., Golub, M.S., Gershwin, M.E., Lonnerdal, B. and Hurley, L.S. (1988) Studies of marginal zinc deprivation in rhesus monkeys III. Use of liver biopsy in the assessment of zinc status. Am. J. Clin. Nutr. 47:1041-1045.

Kelly, J.D. and Dineen, J.K. (1972) The cellular transfer of immunity to *Nippostrongylus brasiliensis* in inbred rats (Lewis strain). Immunology 22:199-210.

Kenny, M.A., Roderuck, C.E., Arnich, L. and Piedad, F. (1968) Effects of protein deficiency on spleen and antibody formation in rats. J. Nutr. 95:173-177.

Kerboeuf, D. and Jacob, C. (1983) Rythmic egg release by *Heligmosomoides polygyrus* (*Nematospiroides dubius*), parasitic Trichostrongylida of the murine gut. Chronobiologia 10:255-267.

Keymer, A. (1985) Experimental epidemiology: *Nematospiroides dubius* and the laboratory mouse. in: Rollinson, D. and Anderson, R.M. (ed.), Ecology and genetics of host-parasite interaction, pp. 55-75. London: The Linnean Society.

Keymer, A.E. and Tarlton, A.B. (1991) The population dynamics of acquired immunity to *Heligmosomoides polygyrus* in the laboratory mouse: strain, diet and exposure. Parasitology 103:121-126.

King, J.C. (1990) Assessment of zinc status. J. Nutr. 120:1474-1479.

King, L.E. and Fraker, P.J. (1991) Flow cytometric analysis of the phenotypic distribution of splenic lymphocytes in zinc-deficient adult mice. J. Nutr. 121:1433-1438.

Korsrud, G.O. (1966) Nutritive value of solvent-extracted crambe and camelina meal. M.S. thesis. University of Saskatchewan Canada.

Latham, M.C. (1990) Protein-energy malnutrition - It's epidemiology and control. JEPTO 10:168-180.

Le Roith, D. and Pimstone, B.L. (1973) Bone metabolism and composition in the protein-deprived rat. Clin. Sci. 44:305-319.

Lee, R.D. and Nieman, D.C. (1993) Nutritional assessment. Brown and Benchmark publishers,

Madison, Wisconsin.

Lindsay, A.H. (1993) The nutrition CRSP: what is marginal malnutrition and does it affect human function? Nutr. Rev. 51:255-267.

Liu, S. (1965) Pathology of *Nematospiroides dubius* I. Primary infections in C3H and Webster mice. Exp. Parasitol. 17:123-135.

Lopez. M del C. and Roux, M.E. (1989) Impaired differentiation of IgA-B cell precursors in the peyer's patches of protein depleted rats. Dev. Comp. Immunol. 13:253-262.

Losson, B., Lloyd, S. and Soulsby, E.J.L. (1985). *Nematospiroides dubius*: modulation of the host immunological response. Z. parasitenkd. 71:383-393.

Luecke, R.W., Simonel, C.E. and Fraker, P.J. (1978) The effect of restricted dietary intake on the antibody mediated response of the zinc deficient A/J mouse. J. Nutr. 108:881-887.

Luecke, R.W. and Fraker, P.J. (1979) The effect of varying dietary zinc levels on growth and antibody-mediated response in two strains of mice. J. Nutr. 109:1373-1376.

Lunn, P.G. and Austin, S. (1983a) Excess energy intake promotes the development of hypoalbuminaemia in rats fed on low-protein diets. Br. J. Nutr. 49:9-16.

Lunn, P.G. and Austin, S. Dietary manipulation of plasma albumin concentration. J. Nutr. 113:1791-1802.

Malave, I. and Layrisse, M. (1976) Immune response in malnutrition. Differential effect of dietary protein restriction on the IgM and IgG response to alloantigens. Cell. Immunol. 21:337-343.

Malave, J.B. and Pocino, M. (1980) Abnormal regulatory control of the antibody response to heterologous erythrocytes in protein-calorie malnourished mice. Clin. Immunol. Immunopatol. 16:19-20.

Mathur, M. Ramalingaswami, V. and Deo, M.G. (1972) Influence of protein deficiency on 19S antibody-forming cells in rats and mice. J. Nutr. 102:841-846.

Mauro, N.A. and Weinstein, P.P. (1979) Effects of sterols, rat haematin and coproporphyrin on the free-living stages of *Nematospiroides dubius* and *Nippostromgylus brasiliensis*. Int. J. Parasitol. 9:421-427.

Mettrick, D.F. and Podesta, R.B. (1974) Ecological and physiological aspects of helminth-host interactions in the mammalian gastrointestinal canal. Adv. Parasitol. 12:183-278.

Michael, E. and Bundy, D.A.P. (1991) The effect of the protein content of CBA/Ca mouse diet

on the population dynamics of *Trichuris muris* (Nematoda) in primary infection. Parasitology 103:403-411.

Michael, E. and Bundy, D.A.P. (1992) Nutrition, immunity and helminth infection: effect of dietary protein on the dynamics of the primary antibody response to *Trichuris muris* (Nematoda) in CBA/Ca mice. Parasite Imuunol. 14:169-183

Minkus, T.M. (1990) The effect of low dietary zinc on outcome of primary and challenge nematode infections in mice. M.S. thesis. School of Dietetics and Human Nutrition, Macdonald College, McGill University, Montreal.

Minkus, T.M., Koski, K.G. and Scott, M.E. (1992) Marginal zinc deficiency has no effect on primary or challenge infections in mice with *Heligmosomoides polygyrus* (Nematoda). J. Nutr. 122:570-579.

Mitchell, G.F. (1980) T-cell dependent effects in parasite infection and disease. Prog. Immunol. 4:794-808.

Mittal, A., Woodward, B. and Chandra, R.K. (1988) Involution of thymic epithelium and low serum thymulin bioactivity in weanling mice subjected to severe food intake restriction or severe protein deficiency. Exp. Mol. Pathol. 48:226-235.

Molinari, J.A., Ebersole, J.L. and Cypess, R.H. (1978) Specific antibody levels in the serum of *Heligmosomoides polygyrus*-infected mice. J. Parasitol. 64:233-238.

Monroy, F.G., East, I.J., Dobson, C. and Adams, J.H. (1989) Immunity in mice vaccinated with a low molecular weight 60,000 glycoprotein secreted by adult *Nematospiroides dubius*. Int. J. Parasitol. 19:71-76.

Monroy, F.G. and Enriquez, F.J. (1992) *Heligmosomoides polygyrus*: a model for chronic gastrointestinal helminthiasis. Parasitol. Today 8:49-53.

Moqbel, R. (1986) Helminth-induced intestinal inflammation. Trans. Roy. Soc. Trop. Med. Hyg. 80:719-727.

Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. 7:145-173.

Moynahan, E.J. and Barnes, P.M. (1973) zinc deficiency and a synthetic diet for lactose intolerance. Lancet 1:676-677.

Murray, E.J. and Messer, H.H. (1981) Turnover of bone zinc during normal and accelerated bone loss in rats. J. Nutr. 111:1641-1647.

National Research Council (1978) Nutrient requirements of laboratory animals. Third revised edition, National Academy of Sciences, Washington, D.C.

Nimmanwudipong, M.D., Cheadle, W.G., Appel, S.H. and Polk, H. (1992) Effect of protein malnutrition and immunomodulation on immune cell populations. J. Surg. Res. 52:233-238.

Noble, E.R., Noble, G.A., Schad, G.A. and MacInnes, A.J. (1989) Parasitology: the biology of animal parasites. 3rd ed. Lea and Febiger Publ., Philadelphia.

Orwoll, E., Ware, M., Stribrska, L., Bikle, D., Sanchez, T., Andon, M. and Li, H. (1992) Effects of dietary protein deficiency on mineral metabolism and bone mineral density. Am. J. Nutr. 56:314-319.

Pawlowski, Z.S. (1984) Implications of parasite-nutrition interactions from a world perspective. Fed. Proc. 43:256-260.

Pentilla, J.A., Ey, P.L. and Jenkin, C.R. (1983) Adherence of murine peripheral blood eosinophils and neutrophils to the different stages of *Nematospiroides dubius*. Aust. J. Exp. Biol. Med. Sci. 61:617-627.

Petro, T.M. (1985) Effect of reduced dietary protein intake on regulation of murine in vitro polyclonal T lymphocyte mitogenesis. Nutr. Res. 5:263-276.

Petro, T.M., Chien, G. and Watson, R.R. (1982) Alteration of cell-mediated immunity to *Listeria monocytogenes* in protein-malnourished mice treated with thymosin fraction V. Infection and Immunity 37:601-608.

Prasad, A.S., Mial, A., Farid, Z., Sandstead, H.H. and Schulert, A.R. (1963) Zinc metabolism in normals and patients with the syndrome of iron deficiency anemia hepatosplenomegaly, dwarfism and hypogonadism. J. Lab. Clin. Med. 61:537-549.

Prasad, A.S. (1983) Clinical, biochemical and nutritional spectrum of zinc deficiency in human subjects: an update. Nutr. Rev. 41:197-207.

Price, P. and Bell, R.G. (1975) The toxicity of inactivated bacteria and endotoxin in mice suffering from protein malnutrition. J. Reticuloendothel. Soc. 18:230

Price, P. and Turner, K.J. (1983) Immunological consequences of intestinal helminth infections in C57BL mice. The effect on lymphoid tissue and reticuloendothelial function. Aust. J. Exp. Biol. Med. Sci. 61:371-382.

Pritchard, D.I., Williams, D.J.L., Behnke, J.M. and Lee, T.D.G. (1983) The role of IgG1 hypergammaglobulinaemia in immunity to the gastrointestinal nematode *Nematospiroides dubius*: the immunochemical purification, antigen-specificity and in vivo anti-parasite effect of IgG1 from

immune serum. Immunology 49:353-365.

Pritchard, D.I., Ali, N.M.H. and Behnke, J.M. (1984a) Analysis of mechanisms of immunodepression following infection with *Nematospiroides dubius*. Immunology 51:633-642.

Pritchard, D.I., Behnke, J.M. and Williams, D.J.L. (1984b) Primary infection sera and IgG1 do not block host-protective immunity to *Nematospiroides dubius*. Immunology 51:73-81.

Prowse, S.J., Ey, P.L. and Jenkin, C.R. (1978) Immunity to *Nematospiroides dubius*: cell and immunoglobulin changes associated with onset of immunity in mice. Aust. J. Exp. Biol. Med. Sci. 56:237-241.

Prowse, S.J., Mitchell, G.F., Ey, P.L. and Jenkin, C.R. (1979) The development of resistance in different inbred strains of mice to infection with *Nematospiroides dubius*. Parasite Immunol. 1:277-288.

Purtilo, D.T., Randall, S.R., Richard, E. and Neafie, R.C. (1976) Humoral immunity of parasitized, malnourished children. Am. J. Trop. Med. Hyg. 25:229-232.

Robinson, M., Wahid, F.N., Behnke, J.M. and Gilbert, F.S. (1989) Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): dose-dependent expulsion of adult worms. Parasitology 98:115-124.

Roitt, I. (1994) Essential immunology (8th ed.) Oxford, Blackwell Scientific Publications, London. pp. 263-268.

Roth, H.P. and Kirchgessner, M. (1980) Zn metalloenzyme activities. Wld. Rev. Nutr. Diet. 34:144-160.

Rudman, D. and Feller, A.G. (1990) Liver disease. in: Brown, M.L. (ed.), Present knowledge in nutrition, 6th edition, pp. 385-394. International Life Science Institute, Nutrition Foundation, Washington, D.C.

SAS Institute Inc. (1985) SAS user's guide: statistics, version 5 edition. SAS Institute Inc., Cary, N.C.

Scott, M.E. (1991) *Heligmosomoides polygyrus* (Nematoda): susceptible and resistant strains of mice are indistiguishable following natural infection. Parasitology 103:429-438.

Scrimshaw, N.S., Taylor, C.E. and Gordon, J.E. (1968) Interactions of nutrition and infection, WHO monograph series No. 57.

Sher, A. and Coffman, R.L. (1992) Regulation of immunity to parasites by T cells and T cell-derived cytokines. Ann. Rev. Immunol. (1992) 10:385-409.

Sherman A.R. (1992) zinc, copper and iron nutriture and immunology. J. Nutr. 122:604-609.

Shi, H.H., Scott, M.E., Stevenson, M.M. and Koski, K.G. (1994a) Zinc deficiency impairs T cell function in mice with primary infection of *Heligmosomoides polygyrus* (Nematoda). Parasite I mmnunol. 16:(in press).

Shi, H.H., Scott, M.E., Koski, K.G., Boulay, M. and Stevenson, M.M. (1994b) Zinc deficiency and energy restriction enhances development and survival of *Heligmosomoides polygyrus* (Nematoda) during primary and challenge infections in mice. Parasitology (submitted)

Slater, A.F.G. and Keymer, A.E. (1986a) *Heligmosomoides polygyrus* (nematoda): the influence of dietary protein on the dynamics of repeated infection. Proc. R. Soc. Lond. B229:69-83.

Slater, A.F.G. and Keymer, A.E. (1986b) Epidemiology of *Heligmosomoides polygyrus* in mice: experiments on natural transmission. Parasitology 93:177-187.

Slater, A.F.G. (1988) The influence of dietary protein on the experimental epidemiology of *Heligmosomoides polygyrus* (Nematoda) in the laboratory mouse. Proc. R. Soc. Lond. B234:239-254.

Slater, A.F.G. and Keymer, A.E. (1988) The influence of protein deficiency on immunity to *Heligmosomoides polygyrus* (Nematoda) in mice. Parasite Immunol. 10:507-522.

Slobodianik, N.H., Cosarinsky, R.C., Langin, S.H., Roux, M.E. and Sanahuja, J.C. (1984) Effect of severe protein deficiency on surface and intracellular markers of growing rats' lymphoid organs. Nutr. Rep. Int. 29:957-964.

Smith, N.C. and Bryant, C. (1986). The role of host-generated free radicals in helminth infection: *Nippostrongylus brasiliensis* and *Nematospiroides dubius* compared. Int. J. Parasitol. 16:617-622.

Snedeker, S.M. and Greger, J.L. (1983) Metabolism of zinc, copper and iron as affected by dietary protein, cysteine and histidine. J. Nutr. 113:644-652.

Solomons, N.W. and Keush, G.T. (1981) Nutritional implications of parasitic infections. Nutr. Rev. 39:149-161.

Spurlock, G.M. (1943) Observations on the host-parasite relations between laboratory mice and *Nematospiroides dubius* Baylis. J. Parasitol. 29:303-311.

Stadnyk, A.W., Baumann, H. and Gauldie, J. (1990) The acute-phase protein response in parasite infection. *Nippostrongylus brasiliensis* and *Trichinella spiralis* in the rat. Immunol. 69:588-595.

Stephenson, L.S. (1987) The impact of helminth infections on human nutrition. Taylor and Francis, London.

Sukhedeo, M.V.K. and Mettrick, D.F. (1984) *Heligmosomoides polygyrus* (=*Nematospiroides dubius*) (Nematoda): distribution and net fluxes of glucose, H_2O, Na^+, K^+, and cl^- in the mouse small intestine. Can. J. Zool. 62:37-40.

Svetic, A., Finfelman, F.D., Jian, Y.C., Diefenbach, C.W.M., Scott, D.E., McCarthy, K.E., Steinberg, A.D. and Gause, W.C. (1991) Cytokine gene expression after in vivo primary immunization with goat antibody to mouse IgD antibody. J. Immunol. 147:2391-2397.

Svetic, A., Madden, K.B., Zhou, X.D., Lu, P., Katona, I.M., Finkelman, F.D., Urban, J.F. and Gause, W.C. (1993) A primary intestinal helminthic infection rapidly induces a gut-associated elevation of Th2-associated cytokines and IL-3. J. Immunol. 150:3434-3441.

Systat Inc. (1990) Systat: the system for statistics. Wilkinson, Leland. Evanston, IL: Systat Inc.

Taike H., and Schubert, G.E. (1965) Enzymatische Harnstoffbestimmung im blut und serum im optischen test nach warburg. Klin. Wochschr 43:174.

Tanguay, G. and Scott, M.E. (1987) A technique for determining *Heligmosomoides polygyrus* (Nematoda) worm burden following anthelimintic treatment in mice. J. Parasitol. 73:834-844.

Tao, S-H. and Hurley, L.S. (1975) Effect of dietary calcium deficiency during pregnancy on zinc mobilization in intact and parathyroidectomized rats. J. Nutr. 105:220-225.

Thompson, R.P.H. (1991) Assessment of zinc status. Proc. Nutr. Soc. 50:19-28.

Tilkian, S.M., Conover, M. and Tilkian, A.G. (1987) Clinical implications of laboratory tests, 4th edition. The C.V. Mosby Company, ST. Louis.

Urban, J.F., Katona, I.M. and Finkelman, F.D. (1991a) *Heligmosomoides polygyrus*: CD4+ but not CD8+ T cells regulate the IgE response and protective immunity in mice. Exp. Parasitol. 73:500-511.

Urban, J.F., Katona, I.M., Paul, W.E. and Finkelman, F.D. (1991b) Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice. Proc. Natl. Acad. Sci. USA 88:5513-5517.

Urban, J.F., Madden, K.B., Svetic, A., Cheever, A., Trotta, P.P., Gause, W.C., Katona, I.M. and Finkelman, F.D. (1992) The importance of Th2 cytokines in protective immunity to nematodes. Immunol. Rev. 127:205-220.

Wahid, F.N. and Behnke, J.M. (1993) Immunological relationship during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): parasite specific IgG1 responses and primary response phenotype. Parasite Immunol. 15:401-413.

Wahid, F.N., Robinson, M. and Behnke, J.M. (1989) Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): expulsion of adult worms from fast-responder syngeneic and inbred strains of mice. Parasitology 98.459-469.

Wakelin, D. (1986) Genetic and other constraints on resistance to infection with gastrointestinal nematodes. Trans. Roy. Soc. Trop. Med. Hyg. 80:742-747.

Wakelin, D. and Lee, T.D.G. (1987) Immunobiology of *Trichuris* and capillaria infections. In: Soulsby, E.J.L. (ed.), Immune responses in parasitic parasitic infections: Immunology, immunopathology and immunoprophilaxis, vol. 1, pp. 61-88. Boca Raton: CRC Press.

Walravens, P.A., Krebs, N.F. and Hambidge, K.M. (1976) Growth of infants fed a zinc supplemented formula. Am. J. Clin. Nutr. 29:1114-1121.

Walravens, P.A., Krebs, N.F. and Hambidge, K.M. (1983) Linear growth of low income preschool children receiving a zinc supplement. Am. J. Clin. Nutr. 38:195-201.

Walravens, P.A., Hambidge, K.M. and Koepfer, D.M. (1989) Zinc supplementation in infants with nutritional pattern of failure to thrive: a double-blind, controlled study. Pediatrics 83:532-538.

Watson, R.R., Chien, G. and Chung, C. (1983) Thymosin treatment: serum corticosterone and lymphocyte mitogenesis in moderately and severely protein-malnourished mice. J. Nutr. 113:483-493.

Williams, D.J. and Behnke, J.M. (1983) Host protective antibodies and serum immunuglobulin isotypes in mice chronically infected or repeatedly immunized with the nematode parasite *Nematospiroides dubius*. Immunology 48:37-47.

Williams, R.B. and Mills, C.F. (1970) The experimental production of zinc deficiency in the rat. Br. J. Nutr. 24:989-1003.

Wirth, J.J., Fraker, P.J. and Kierszenbaum F. (1984) Changes in the levels of marker expression by mononuclear phagocytes in zinc deficient mice. J. Nutr. 114:1826-1833.

Woodward, B. (1992) Influence of wasting protein-energy malnutrition on apparent thymic T cell inductive capacity and on recirculating lymphocyte pool sizes in the weanling mouse. In: Chandra, R.K. (ed.), Nutrition and Immunology, pp. 163-177. ARTS Biomedical Publishers, St-John's Newfoundland.

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

Woodward, B., Dwivedi, A. and Nangpal, A. (1992) Mechanisms of thymic epithelial involution in weanling protein-energy malnutrition. Nutr. Res. 12:1253-1261.

World Health Organization. (1987) Report of a WHO committee: prevention and control of intestinal parasitic infections. WHO technical report series 749.

Zanzonico, P., Fernandes, G. and Good, R.A. (1981) The differential sensitivity of T-cell and B-cell mitogenesis to in vitro zinc deficiency. Cell. Immunol. 60:203-211.

Zhou, J.R., Canar, M.M. and Edman, J.W. (1983) Bone zinc is poorly released in young, growing rats fed marginally zinc-restricted diet. J. Nutr. 123:1383-1388.

Zwickl, C.M. and Fraker, P.J. (1980) Restoration of the antibody mediated response of zinc/caloric deficient noenatal mice. Immunol. Commun. 9:611-626.