THE EFFECT OF PROTEIN MALNUTRITION ON THE POPULATION DYNAMICS OF *TRICHURIS MURIS* (NEMATODA) IN CBA/Ca MICE

by

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ABSTRACT

The effect of protein malnutrition on the population dynamics of gastrointestinal nematode infections was examined using the *T. muris*- CBA/Ca mouse model. Semi-synthetic, theoretically, isoenergetic diets containing either 2%, 4% or 16% casein were used to model severe, moderate and normal mouse protein status, as assessed by body weights and immune competence. Host serological responses, both quantitative (antibody titres) and qualitative (immunorecognition), were assayed during infection to ascertain the role of specific antibody responses in the transmission of *T. muris* in well- and malnourished mice.

Dietary protein was not found to influence the establishment rate of larvae during primary infection. On the other hand, *T. muris* survival was markedly prolonged in mice fed the 4% protein diet, resulting in the establishment of chronic, long-term, patent infections. Helminth fecundity results suggest that dietary protein may not affect the *per capita* egg production of *T. muris*. However, while the mean net egg output per mouse was observed to increase with the infection dose in the deficient group, it was significantly lower and unrelated to exposure in well-nourished mice.

Host protein nutrition exerted a major impact on the survival of *T. muris* repeated infections. Thus, whereas well-nourished mice expelled their parasite burdens well before patency during repeated infection; deficient hosts, both severely and moderately malnourished, gained adult worms in proportion to their rate of infection. Both increased larval recruitment (establishment) and survival of adult worms may underlie the observed persistence and built-up of *T. muris* trickle infections in deficient hosts.

The serological analyses showed that, in both single and trickle infections, malnourished mice contained higher parasite-specific antibody titres to *T. muris* E/S antigen in sera, as quantified by ELISA, and also recognised a broader range of E/S antigen components, as assessed by Western blotting. Despite this, the results indicated that the increase in specific antibody observed during infection may correlate with protection against *T. muris* only in the case of the well-nourished hosts. This finding is consistent with the hypothesis that protein malnutrition associated susceptibility to *T. muris* may be due to nutrient deficiency-induced impairment in cellular immunity rather than defects in serum antibody response.

In conclusion, this study has shown that the nutritional status of a host can influence the population dynamics of an intestinal nematode infection. It is suggested that this is mainly caused by the deleterious effect of protein malnutrition, including moderate nutrient deficiency, on host immunocompetence, particularly cell-mediated immunity, against infection.

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CHAPTER 1: INTRODUCTION

1.1 CONTEXT AND BACKGROUND OF RESEARCH

Soil- transmitted intestinal helminth infection and human malnutrition are among the most prevalent, chronic conditions affecting human health globally (Pawlowski, 1984; Stephenson, 1987; Bundy & Golden, 1987). It is estimated that between 500 and 1000 million people are currently infected, either singly or multiply, with roundworm (*Ascaris lumbricoides*), hookworm (*Ancylostoma duodenale* and *Necator americanus*) and whipworm (*Trichuris trichiura*) (Peters, 1978; Walsh & Warren, 1979; Warren & Mahmoud, 1989; Bundy & Cooper, 1989). Although geohelminth infections are not uncommon in temperate regions (see Bundy & Cooper, 1989), the major soil-transmitted helminthiasis today are most prevalent among communities of poor and socio-economically deprived people in the countries of the tropics and subtropics, where favourable climactic conditions for transmission and low standards of hygiene, sanitation and education prevail (Arfaa, 1986; Mata, 1982). One recent estimate of the global distribution of geohelminthiasis is given in Table 1.1.

Intestinal helminths, being chronic agents of disease, are primarily a cause of morbidity as opposed to mortality. This factor, together with the difficulty in measuring morbidity in chronic helminth infection, has largely led to the traditional underestimation of the medical significance of geohelminthiasis in endemic areas (Bundy & Cooper, 1989). Further waning in interest in intestinal helminth infection in the West followed the failure of the Rockefeller Foundation hookworm campaign to eradicate ancylostomiasis in the 1920s. Consequently, the study of direct life-cycle helminth epidemiology and control has tended to be somewhat neglected by comparison with the degree of attention devoted to the more obvious acute diseases such as diarrhoea, malaria and schistosomiasis. In recent years, however, there has been a resurgence of interest in the research and control of geohelminths (Warren, 1981; Davies, 1985; WHO, 1987). This resurgence in interest is a consequence of many factors : these include the increasing recognition of intestinal helminthiasis, following the advances in the control of mortality from acute infectious diseases, as significant morbidity factors of continued child ill-health and retarded development in endemic areas (APCO, 1980a,b; Crompton, 1986; Stephenson, 1987; Pawlowski & Davies, 1989); the introduction of major control activity in South east Asia (reviewed by APCO, 1980a,b); the availability of cheap

Table 1.1 *Estimates of the number of people infected with various intestinal nematode infections by regions of the world (from Anderson, 1982a).*

	Number of cases (millions)					
Parasite	Africa	Asia (excluding USSR)	America	Europe (excluding USSR)	USSR	World total
Ascaris lumbricoides	159	931	109	39	30	1269
Hookworm (two species)	132	685	106	2	4	932
Enterobius vermicularis	24	136	115	75	48	353
Trichuris trichiura	76	433	135	41	41	687

and effective broad-spectrum anthelmintics; the global expansion of primary health delivery infrastructure (Walsh & Warren, 1979); and the increasing appreciation of the role of population ecology and transmission dynamics in allowing the design of more rational control programmes for direct life-cycle helminths (Anderson & May, 1985; Anderson, 1987a, 1989; Anderson & Medley, 1985).

It is widely recognized that improvements in sanitation and socio-economic standards offer the ideal methods for the reduction and eventual eradication of intestinal helminth parasites. However, due to cost considerations, this prospect is manifestly unlikely to occur in a significant number of endemic countries in the foreseeable future. On the other hand, the recent expansion in global primary health care, the development of broad spectrum anthelmintics, and the advances in parasite population ecology, provide new opportunities to integrate the control of helminthiasis in existing health care programmes (Walsh & Warren, 1979; Pawlowski, 1987). Recent theoretical studies in parasite ecology have indicated that the success of this community approach to control will ultimately depend on a precise and detailed understanding of the determinants of endemic infection, particularly the factors that predispose to individual host infection (Anderson & May, 1985; Anderson, 1989; Anderson & Medley, 1985).

Global estimates of the amount of malnutrition in the world today indicate prevalences almost as high as those recorded for geohelminth infections (Latham, 1984). Protein-energy malnutrition (PEM) - the commonest form of malnutrition throughout the developing world, for instance, is estimated to affect almost 500 million persons, mainly children, with about 5 to 10 million clinical cases occurring annually (Latham, 1984; Stephenson, 1987). Since the common socio-economic factor predisposing a community to both helminth infection and malnutrition is poverty, with its attendant compromised diet and inadequate sanitation (Bundy & Golden, 1987), it is perhaps not surprising to find these two afflictions frequently co-existing, often in the same individuals in similar geographical regions of the world (Crompton, 1986). One aspect of this cointensive distribution, the contribution of helminth infection to human malnutrition has been the focus of extensive studies by parasitologists, and have been comprehensively reviewed (Keusch, 1982; Crompton, 1986; Stephenson, 1987). By contrast, the role of host nutrition as a determinant of helminth transmission rates has attracted little attention. This is despite (1) the numerous studies by nutritionists and immunologists associating human malnutrition (mainly PEM) with reduced immunocompetence and concomitant enhanced susceptibility to many microparasitic infections, including tuberculosis, diarrhoeal disease and respiratory

illness (Suskind, 1977; Chandra & Newberne, 1977; Isliker & Schurch, 1981, Chandra, 1980, 1983a; Tomkins, 1986), (2) the demonstrated importance of immune responses, both humoral and cellular, in host resistance to geohelminths (Soulsby, 1987; Lloyd & Soulsby, 1988; Wakelin, 1984), and (3) the severe deleterious effect of P.E.M. on many of the mammalian immunological components likely to be of importance in immunocompetence to geohelminths, especially intestinal IgA secretions and cell-mediated immunity (Chandra, 1984). These relationships suggest that nutrient deficiency- induced alterations in host immunocompetence may be an important determinant of helminth infections in endemic communities. It is possible that malnutrition, via the suppression of host immunity, may potentiate geohelminth transmission in host communities. This topic is the focus of the present research.

Human studies into the nutrition-immunity-infection dynamics are complicated by the uncertainties of infection history, the multivariable determinants of nutritional status (type and duration), human immune status (genetic variability), and by the confounding effects of polyparasitism (Beisel, 1982; Pawlowski, 1984). For these reasons, controlled laboratory experimentation, using animal analogues of human infection, offers a more tractable system for both dissecting the specific nutrient-immunity interaction (Isliker & Schurch, 1981; Gross & NewberneC, 1980; Chandra, 1980) and estimating the parasite population consequences of host nutrition (Slater & Keymer, 1986a,b; Berding *et al.*, 1986). The research described in this thesis extends the experimental studies of Slater & Keymer (1986a) on the impact of protein malnutrition on geohelminth population dynamics.

The present studies were conducted using *Trichuris muris* infections in populations of mice, a well established laboratory analogue of human trichuriasis (Lee & Wakelin, 1983). These studies differ from previous studies of the nutrition-infection -interaction in attempting to determine the immunological basis of the nutrition-helminth dynamics interaction.

The dissertation is organized as follows. The present chapter will briefly review what is known concerning the determinants of geohelminth infection in human communities (section 1.2.1), the effect of P.E.M. on mammalian immunocompetence (section 1.2.2) and the association between P.E.M. and intestinal helminths, particularly deficiency-induced susceptibility to infection (section 1.2.3). The final section (1.3) describes the *T. muris* - mouse model of human *T. trichiura* infection (Lee & Wakelin, 1983) and discusses its relevance to studies on the nutrition-infection-helminth dynamics interaction.

Chapter 2 describes the parasitological and immunological methods used here to study the population biology and immunology of *T. muris* infections in the mouse, and details the formulation and preparation of the semi-purified synthetic mouse diets used in the study.

The results of a phenomenological study of the population biology of *T. muris* primary infection in CBA/Ca mice is given in Chapter 3, focusing on the density dependent regulation of *T. muris* populations in both responder and drug induced immunosuppressed mice.

The next two Chapters present analyses of the effect of murine protein malnutrition on *T. muris* populations during primary (Chapter 4) and trickle infection (Chapter 5). Changes over time in worm numbers, egg production, host antibody levels and antigen recognition are described, and associations between host infection rates, nutrition and immunological status in relation to the transmission dynamics of *T. muris* are presented.

Finally, the General Discussion (Chapter 7) summarizes the major results and considers how the nutrition deficiency - helminth dynamics interaction may be relevant to the design and implementation of helminth control strategies for human populations in endemic areas.

1.2 DETERMINANTS OF GEOHELMINTH INFECTION

The study of parasite population dynamics and epidemiology concerns the quantitation of processes which govern the transmission of disease (or infection) at the population level. Recent advances in these studies (both theoretical and field research) have indicated that many factors act to determine the rate at which people living in areas of endemic infection may acquire geohelminth infection. These are thought to include factors defined by the biology of the parasite's life cycle, such as worm fecundity, host-mediated factors such as acquired immunity, environmental factors such as temperature and humidity plus behavioural and sociological attributes of the human community which relate to the degree of contact with infective parasite stages. A better understanding of these factors which regulate parasite abundance, and contribute to the observed stability of helminth populations is not only of academic interest, but is relevant to the design of optimal control strategies (Anderson & May, 1979; May & Anderson, 1979; Anderson & May, 1985).

Table 1.2 summarizes the biological features of the four major gastrointestinal helminths of humans. Two features of the life cycles are of central relevance to population biology. Firstly, the common geohelminths are all directly transmitted, *i.e.*, they involve only one definitive host in their respective life cycles (Table 1.2). Secondly, the major geohelminths are all macroparasites and do not replicate directly within the host (Anderson & May, 1979). Reproduction therefore is essentially by the invasion of new hosts either by the ingestion of, or skin penetration by, infective stages which develop in the external environment (Table 1.2). These attributes of the biological structure of the common geohelminth life-history act to determine many features of the population biology, geographical distribution and control aspects of human infection.

1.2.1 Population biology of direct Life-cycle geohelminths

The life cycles of directly transmitted helminths consist of two principal populations : the adult parasitic worms carried by the hosts, and the free-living infective stages. These two principal populations play a central role in determining the overall transmission success of the parasite and, to a large extent, control parasite population growth and stability throughout the entire parasite life cycle. The adult worms are responsible for reproduction, while the infective stages determine the rate at which new hosts are "colonized" and the rate of recruitment to established parasite populations. The dynamics of parasite transmission can therefore be envisaged in terms of gains and losses for the population of adult worms within the

Table 1.2 *Biological features of the common species of nematodes found in the human alimentary tract (based on Anderson, 1982a; Crompton, 1987; Bundy & Cooper, 1989).*

Nematode species	Life history	Infective stage	Usual route(s) of infection	Life expectancy		Fecundity (eggs/worm/day)
				Infective stage	Adults	
Ascaris lumbricoides	D,d	2nd stage larva in egg	Faeco-oral	1-3 mth	1 yr	240,000
Ancylostoma duodenale	D,d	3rd stage larva	Cutaneous and oral	unknown	1 yr	10,000-25,000
Necator americanus	D,d	3rd stage larva	Cutaneous	3-10 days	3-5 yr	5,000 -10,000
Trichuris trichuira	D,d	1st stage larva in egg	Faeco-oral	11-30 days	1-3 yr	2,000 -15,000

D= direct; d= dioecious

host and the population of infective stages in the external environment of the host (see Anderson, 1982a; Anderson & May, 1985). A diagrammatic representation of the flow of parasites between these two populations is portrayed in Fig. 1.1.

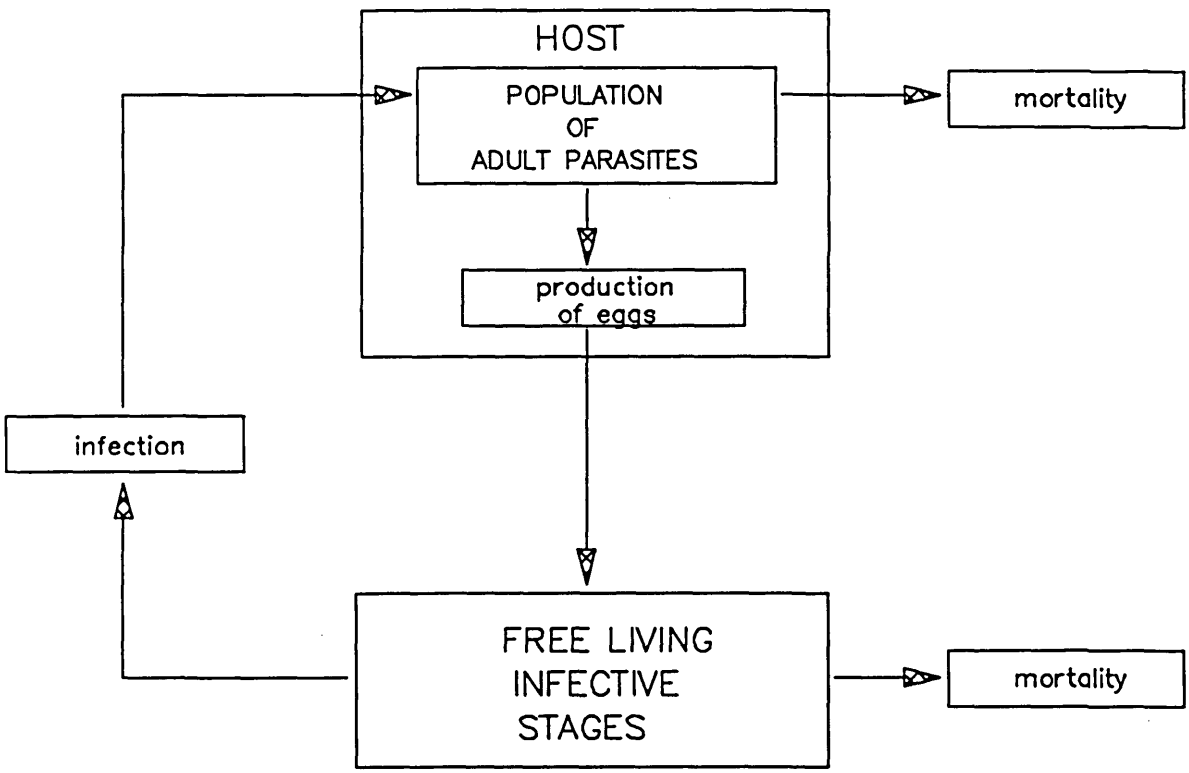

Figure1.1 *Diagrammatic flow chart of the principal populations and rate processes involved in the life cycles of directly transmitted nematode parasites (from Anderson, 1982a).*

Recent theoretical work has identified five principal factors controlling the population dynamics of direct life cycle geohelminths (Anderson, 1982a; Anderson & May, 1982, 1985).

(i) Time scales

Recent research reveals that the importance of the different developmental stages in the helminth life cycle, as determinants of the net force of transmission within the host community, scales in rough proportion to their expected life spans in relation to the host life expectancy (Anderson & May, 1982, 1985). For the direct life cycle geohelminths, the life span of the host is typically much longer than the life span of the parasites. In turn, the expected life span of the mature parasites is also many orders of magnitude greater than that of infective stages, whether larvae or

eggs (Table 1.2). As such, the major determinants of helminth transmission success, under the assumption of a constant host population, are thought to be the rates of establishment, survival and fecundity of the adult parasite in the host (Anderson, 1987b).

Adult worm life expectancy also has implications for the extent of climate induced disruption of population stability. In general, climatic variation would be of limited significance if the adult worm in the host has a long life expectancy relative to seasonal fluctuations, as appears to be the case for geohelminths.

(ii) Regulation

Helminth populations both within individual hosts and the community as a whole, appear to be regulated by density dependent constraints which act to reduce parasite establishment, survival and fecundity as worm burden per host rises (recently reviewed by Keymer, 1982). Such constraints may arise either as a result of competition between parasites for limiting resources, the effects of host responses (either non-specific or immunological) to parasite invasion, or a combination of both processes. In addition, parasite induced host mortality may also act to regulate helminth abundance (Crofton, 1971 a,b; Anderson & May, 1978).

(iii) Worm distributions

Observed distributions of worm numbers per host are typically highly aggregated. The generative mechanisms of such patterns are thought to include genetic, nutritional, behavioural, social and spatial factors. The high degree of contagion enhances the density-dependent regulation of parasite populations, and hence is considered to confer stability to helminth population growth.

(iv) The reproductive biology of geohelminths

The major geohelminths are dioecious (Table 1.2). The production of fertile eggs therefore depends on the probability that a female worm is mated. Theoretically, there exists a critical mean worm burden, termed the breakpoint, below which mating frequency is too low to maintain transmission (May, 1977). However, since this breakpoint average worm burden is found to be typically very low (close to zero worms per host) in human helminth infections, due in part to the high degrees of worm aggregation within human populations, the concept of transmission

breakpoints does not appear to be as significant to the helminth population dynamics as was originally thought.

The prodigious fecundity of geohelminths (Table 1.2), has, in addition, important implications for the threshold host densities required for parasite transmission and persistence. Theoretical studies have indicated that human helminths manage to persist in low density communities mainly due to the enormous reproductive capabilities of such parasites (Anderson, 1982a).

(v) The reproductive or transmission potential of helminth parasites

The basic reproductive rate, R_o, is used to measure this potential, and is defined as the average number of sexually mature female offspring produced throughout the life time of a female worm in the absence of any constraints on population growth. Its value is determined by many individual components such as the birth, death and transmission rates of the numerous developmental stages n the parasites' life cycle. Within a stable population, the effective or realised reproductive rate, R, is essentially equal to unity since the female parasite must exactly replace itself in the next generation. The magnitude of the difference between R_o and R thus largely determines the average abundance and prevalence of helminth infection within host communities (Anderson, 1986).

In addition, recent theoretical studies have shown the potential of parasite-induced host mortality to regulate host-helminth interactions (Anderson & May, 1979). Both parasite contagion and non-linearity in the relationship between the rate of parasite-induced host mortality and burden per host are thought to exert a stabilizing influence. As stated earlier, the impact of parasite-induced host mortality on the dynamics of the parasite is enhanced by overdispersion in parasite numbers per host (Crofton, 1971a,b; Anderson & May, 1979). On the other hand, high levels of parasite aggregation, as commonly observed in helminth infections, may tend to reduce any regulatory impact of the parasite on the growth of its host population, since the death of a few heavily infected hosts will result in the concomitant loss of a high number of parasites (Anderson & May, 1978). More broadly, it is likely that an interplay between the pathogenicity of helminth infection and the nutritional status of the host contributes importantly to the density-dependent regulation of natural populations (Anderson, 1982b), with the parasites greatly amplifying the effects of low levels of nutrition (Puffer & Serrano, 1973; Bundy & Golden, 1987).

(vi) Implications for eradication and control

The involvement of distinct free-living infective stages in geohelminth life cycles, together with their mode of infection (Table 1.2), implies that the transmission potential of geohelminths in human communities is critically dependent upon (1) the degree of environmental contamination with parasite eggs and (2) the appropriateness of local climatic factors (temperature, humidity, soil-type) favouring the development and maturation of viable infective stages (Brown, 1927). Together, these factors explain the current global prevalence of geohelminthiasis among communities of poor and socio-economically deprived people of the tropics and subtropics, where both the requisite climatic factors for parasite egg development in the soil (warm, humid conditions) and high human faecal contamination of the environment (due to low prevailing standards of hygiene, education and sanitation) co-exist (Arfaa, 1986). The implications for control are clear : long-term cessation of helminth transmission and eventual eradication is plainly contingent upon the development and successful implementation of strategies which aim to both prevent continued environmental contamination and remove or exhaust existing reservoir of infective stages in the environment (Seo, 1980). The realization of such methods, however, eventually depends upon the overall economic development of the endemic regions with its attendant enhanced levels of sanitation, living standards and education. In many developing countries this is unlikely to occur in the foreseeable future. Medium-term gains in the control as opposed to eradication of infection, however, may be achievable by the methods of chemotherapy, nutrition and even vaccination provided these methods are based on sound epidemiological principles.

1.2.2 Observed patterns of Geohelminth Infection

A comparison of the epidemiological trends recorded in a large number of studies of Ascaris, Trichuris and hookworm infection in human communities throughout the world has revealed a series of common features. This section aims at a brief discussion of the typical infection patterns, the factors which are thought to govern them and their relevance to helminth control.

(i) Age-related changes in prevalence and intensity of infection

Horizontal cross-sectional studies of the major geohelminths within human communities have consistently revealed contrasting patterns of change in prevalence and average worm intensity with host age (Anderson, 1986). While prevalence is commonly shown to rise rapidly and remain at a plateau in the

teenage and adult age classes, average intensities of infection typically demonstrate a marked convex relationship with host age : worm loads rise rapidly to a peak in the child (*A. lumbricoides* and *T. trichiura*) or teenage (hookworms) age classes but decline to low levels in the adult groups, although the convexity may be less marked for hookworms. The later age at which the hookworms attain asymptote prevalence in human communities has been attributed to their greater life expectancy (Table 1.2), and illustrates an example of the role of the parasite life history in determining helminth transmission (Anderson, 1986).

The discrepancies between the age-prevalence and intensity profiles are thought to be a consequence of the aggregated distribution of worm numbers per person within infected communities (Anderson, 1982a; Anderson & May, 1985). For an aggregated or clumped probability distribution (as it is for most helminths), it has been shown that large changes in intensity may have little impact on prevalence (Anderson, 1982a; Anderson & May, 1985).

(ii) Frequency distribution of worm burdens

As mentioned earlier, helminth parasites of humans are invariably aggregated in their distribution within infected communities (Croll & Ghadirian, 1981; Croll *et al.*, 1982; Anderson, 1982a; Anderson & May, 1985). Most people harbour few worms and a few harbour many. The trend is observed not only for the overall community but also within age-groups (Croll *et al.*, 1982; Thein Hlaing, 1985; Elkins *et al.*,1986; Bundy, 1988). This suggests that heterogeneity in intensity of infection within communities is generated by both the characteristics of the individual host, as well as by group factors such as age and sex (Anderson & Medley1985; Anderson, 1986). The negative binomial probability distribution provides a convenient, though empirical, description of the observed trends, permitting the severity of aggregation to be expressed in a single parameter, k, of the exponent of the negative binomial expansion (Seo, 1980; Anderson & May, 1982; Elkins *et al.*, 1986; Bundy & Cooper, 1989). Available studies suggest there is a remarkable degree of uniformity in the value of the k parameter (typically in the range of 0.1 to 0.9) between parasite species, host classes, and geographical locations (Bundy & Cooper, 1989). Since heterogeneity in the rate of gain or loss of parasites among hosts has been shown to be major cause of aggregation in parasite populations (Anderson & Gordon, 1982), the observed constancy of k values may further suggest a degree of uniformity in the processes which act to create heterogeneity and hence helminth aggregation within human communities.

As mentioned, such patterns enhance the severity of density dependent checks on the growth of adult parasite populations in areas where infection intensity is high (Anderson & May, 1985; Keymer, 1982). In low intensity areas, the aggregation of parasites is advantageous to the success of the parasite because the probability of mating may be enhanced (Anderson & May, 1985). Another effect of helminth aggregation is that the few heavily infected hosts make a disproportionately large contribution to environmental contamination (Bundy & Cooper, 1989), a fact which is of considerable significance to the design of optimal control strategies.

(iii) Predisposition to Infection

Perhaps the most significant empirical observation in recent years is that individuals are predisposed to a particular infection intensity. After drug treatment, heavily infected individuals tend to reacquire higher than average intensity infection, while lightly infected individuals reacquire lighter than average infections. This relationship has been demonstrated for *A. lumbricoides* (Croll *et al.*, 1982; Thein Hlaing, 1985; Elkins *et al.*, 1986) mixed hookworm infection (Schad & Anderson, 1985); *T. trichiura* (Bundy, 1986), and *Enterobius vermicularis* (Haswell-Elkins *et al.*, 1987). Furthermore, Haswell-Elkins *et al.* (1987) have also provided evidence for multiple species predisposition to human helminth infections. Bundy *et al.* (1988) showed that the positive association between an individual's pre- and post treatment infection intensity is further a consequence of a direct relation between the rate of reinfection and initial infection status. Thus it was pointed out that in an endemic community, an individual will consistently express an higher (or lower) average intensity of infection, with the relative magnitude of the worm burdens scaling according to age (Bundy *et al.*, 1988). This pattern also appears to be operative at the family level, as indicated by a recent study of *A. lumbricoides* in Mexico which showed that heavily infected families, on an average, tend to reacquire heavy infections even after all the family members are successfully treated (Forrester *et al.*, 1988).

(iv) Re-infection following chemotherapy

Field-based reinfection studies, using chemotherapy as a research tool, have provided valuable information on rates of helminth reinfection and how these vary according to initial intensity of infection and demographic parameters such as host age. Three general conclusions emerge from such work.

First, rates of reinfection vary greatly with host age; children tend to more rapidly reacquire infection than do adults (Bundy *et al.*, 1988). Second, reinfection rates are directly and positively related to initial intensity of infection (see above), the basis for the phenomenon of predisposition in helminthiasis. Third, recovery to pretreatment infection levels in areas of moderate to high transmission, appears to be inversely correlated with the life expectancy of the adult parasite in the human host (Anderson & May, 1985; Anderson, 1986). If life expectancy is short, the return time is fast and *vice versa*. In addition, Monte Carlo simulation studies, based on probability models of helminth transmission, suggest that the average rate of reinfection of a particular helminth is most rapid under conditions of differential host susceptibility rather than under differential host exposure (Anderson & Medley, 1985).

1.2.3 Factors generating the distribution of infection intensity

(i) The distribution of infection intensity and host age

As mentioned earlier, helminths typically exhibit convex age - intensity patterns of infection in human communities. Since infection with helminths is essentially a pure immigration - death process (i.e there is no multiplication within the host) the convex age dependent pattern of helminth acquisition has been generally thought to reflect age-related changes in exposure to infection, the acquisition of immunity which acts to reduce parasite establishment or survivorship or a some combination of both processes (Warren, 1973; Bradley & McCullough, 1974, Anderson & May, 1985).

Exposure to infection with geohelminths has proved difficult to quantify. This is in contrast to field studies on schistosomiasis, which have demonstrated some agreement between age - related changes in water contact and average intensity of infection within human communities (Dalton & Pole, 1978). However, recently (Bundy, 1988) comparing the amount of soil - derived silica in the stool (Wong *et al.*, 1988) with the pattern of *T. trichiura* intensity in a West Indian endemic population has indicated that the intensity of geohelminth infection may rise with increasing practice of geophagia in children, and decline as geophagia decreases in adults. These studies suggest that behaviorally mediated reduction in exposure with age may be determinants of age - intensity profiles for both geohelminths and schistosomes. However, other lines of evidence suggest that acquired immunity, acting to decrease rates of parasite establishment and survival in a manner related to past experience of infection, may also play a role in generating typical convex age - helminth intensity profiles.

Firstly, the rate of decline of infection intensity with age from peak worm loads for both schistosomes and hookworms have been shown to be maximal in communities with high overall rates of transmission (Anderson & May, 1985; Anderson, 1987b). Theory and experimental studies have suggested that acquired immunity is the more likely candidate mechanism for this phenomenon than reduction in exposure (Anderson & May, 1985; Crombie & Anderson, 1985; Keymer, 1985).

Secondly, field studies have revealed a trend for the individuals' reinfection rates to decrease with increasing age for both geohelminthiasis (Anderson, 1986; Elkins, *et al.*, 1986; Thein Hlaing *et al.*, 1987; Bundy *et al*, 1987) and schistosomes (Wilkins *et al.*, 1987; Butterworth *et al.*, 1985). For the schistosomes, this has been shown to occur independent of individual host water contact rates, suggesting that host resistance induced by past experience of infection may have a limiting effect restricting adult worm intensity in older age classes (Wilkins *et al.*, 1984). The recovery of juvenile worms from all age classes following chemotherapy (indicating continuous exposure) within communities with endemic *A. lumbricoides* infection (Anderson, 1989) may be taken to suggest that acquired immunity may also operate in a similar manner to regulate the adult human worm intensity in geohelminthiasis. Further insights into the operation of acquired immunity in controlling geohelminth infections derives from the work of Thein-Hlaing *et al.* (1987), who observed that in children under 10 years old experiencing a 6- monthly anthelmintic regimen, the prevalence of *A.lumbricoides* reached a level higher than both pre-treatment and that of similar children on a 12- monthly regimen. This finding, corroborating numerous laboratory results, may indicate that a stable worm population (in children of the 12- monthly group) might stimulate some protection against new waves of infective stages.

Thirdly, although detailed immuno-epidemiological studies of geohelminthiasis are limited at present, available studies suggest that humans can mount immunological responses to intestinal helminths, whether measured via serum or secreted antibodies or cellular activity (Ogilvie & De Savigny, 1982; Pawlowski, 1982; Soulsby, 1987; Bujis & Ruitenberg, 1987, Bundy, 1988; Haswell-Elkins *et al.*, 1989). Bundy (1988) showed that antigen-specific IgG levels to *T. trichiura* exhibited a marked age-dependency in a West Indian endemic community closely mirroring the prevailing infection intensity profile. Recently, Haswell-Elkins *et al.* (1989) found a similar age- and infection- dependent profile for IgG responses against *A.lumbricoides* in a South Indian fishing community. These latter findings, together with the observation that parasite recruitment appears to take place in all age classes in geohelminthiasis (Elkins, *et al.*, 1986) suggest that acquired immunity to

intestinal helminths is either inefficient in controlling infection intensity or resident in immune parameters other than humoral responses. On the other hand, it is also possible that the discrepancy between activated humoral immune responses and the intensity of helminth infection may be due to other confounding factors such as age-related changes in human physiology and nutrition, and the presence of concurrent infections, which may modulate the expression of immune responses in children (Wakelin, 1987, 1989; Tomkins, 1986; Chandra, 1984; Behnke, 1987). Further work is clearly required to determine the effects of factors such as age, nutrition and polyparasitism on the relationship between infection exposure (antigen load) and the immune response (humoral & cellular) in order to explain the observed mismatch between intact humoral responses and increased helminth burdens in children.

Finally, studies of mice exposed to repeated levels of infection ("trickle infections") have provided supportive evidence for acquired immunity as an epidemiological determinant of helminth age-intensity profiles in host communities (Anderson & Crombie, 1985; Keymer & Hiorns, 1986b; Maema, 1986). These studies, in which the rates of exposure were held constant, have consistently demonstrated that helminth establishment declines as the duration of exposure to infection increases, essentially in a manner related to the accumulated sum of past experience of infection. Such studies have also revealed that the degree of convexity induced in experimental age-intensity profiles in populations of mice is dependent on the intensity of exposure to infection. This observation suggests that both exposure and acquired immunity may play important roles in shaping age-intensity profiles. In areas of intense transmission, it is possible that individuals will build up resistance at an earlier age, and thereby induce a greater degree of convexity in the age-intensity profile, than in areas of low transmission.

In conclusion, these studies indicate that present knowledge of the relative roles of exposure and immunology as determinants of helminth age-intensity profiles in man is contradictory and remains unresolved. Further progress in this area is clearly contingent upon both a better understanding of the immunology of geohelminthiasis and the development of effective immunological and exposure-related markers of infection to track age-related changes in worm intensity of individual patients living in endemic areas. On current evidence, however, it may be concluded that both exposure rates and immunology may act concomitantly to shape age-intensity patterns, with exposure driving the rate at which individuals are subjected to infection and hence able to build up infection-specific resistance (Anderson, 1987b).

(ii) The distribution of infection intensity between individuals

As stated elsewhere, age is not the only source of variation in helminth infection intensity: considerable individual heterogeneity in worm burden also occurs within all age-classes of an endemic population (see above). Reinfection studies suggest that the heterogeneity in infection intensity within age-classes may be a consequence of individual host predisposition to either a high or low average intensity of infection (Anderson, 1986; Bundy, 1988).

The factors governing host predisposition to infection are thought to be associated with some characteristic or characteristics specific to an individual host. Individual differences in susceptibility or environmental exposure, or a combination of both processes are generally considered as the likely generative mechanisms (Schad & Anderson, 1985).

The importance of environmental factors, particularly sanitation, in generating differences in helminth infection intensity has been demonstrated by numerous community studies (recently reviewed by Feachem *et al.*, 1983a; Henry 1981, 1988). Individual or family behaviours also appear to influence exposure and have been implicated in the general failure to demonstrate any positive association between infection status and the availability of sanitation facilities (Feachem*et al.*, 1983b). Quantitative studies of one such behaviour, geophagia, indicate that the heterogeneity in geohelminth infection status in children at least may be correlated with individual differences in such exposure-related behaviour (Wong *et al.*, 1988). In addition, the observation that familial predisposition to *Ascaris* and *Trichuris* infection is significant only for the larger households in the community (Forrester *et al.*, 1988) may also be suggestive of focal transmission. Further support for the hypothesis that predisposition is caused simply by differential exposure derives from a recent comparison of *T. trichiura* reinfection between institutionalized and village children (Bundy & Cooper, 1988). While it was shown that the village children living in different dwellings (encountering variable foci of transmission) had consistent differences in infection intensity (as assessed by reinfection following chemotherapy), no such consistency was observed in the population of children, living and playing together in the same institutional environment.

Although these observations relating to predisposition are consistent with heterogeneity in exposure as a major determinant of infection status, evidence also existsto suggest a complementary role for variability in host immunocompetence. As discussed, immunity acquired as a result of prior experience of infection may play a

role in shaping age-intensity profiles of geohelminth infection. This means that the resistance mechanisms involved in predisposition cannot primarily depend on acquired immunity because the evidence for predisposition implies that it is precisely the individuals with the greatest prior experience of infection who subsequently reacquire intense infection. Immunological predisposition, therefore, is thought to be based upon intrinsic host factors which consistently depress (or enhance) immunocompetence at the individual or family level. Two candidate factors generally thought to be important in this respect include host nutritional status and genetic control of susceptibility to infection.

Genetic restriction in immunocompetence to helminth infection is well established (Wakelin, 1978a, 1985a,b, 1988, 1989; Behnke, 1987). Both MHC restricted (H-2 in the mouse) and background genes (non-MHC), controlling various immune parameters, have been shown to be implicated in this form of susceptibility to helminths in murine models (reviewed by Wakelin, 1978a,1985a,1988,1989; Dargie, 1982). In man, analogous HLA correlates of disease have been demonstrated for both schistosomes (Sasazuki *et al.*, 1980; Hirayama *et al.*, 1987) and trichuriasis (Bundy, 1988). A similar immuno-genetic control of ascariasis is also suggested by the recent findings of Haswell-Elkins *et al.* (1989) that infected persons in an endemic community may vary considerably in their ability to recognize *Ascaris* antigens, a process which has been demonstrated to be MHC restricted in mice (Kennedy *et al.*, 1987).

The role of nutrition as a determinant of human and animal immunocompetence to infection is well established (see Isliker & Schurch, 1977; Suskind, 1977; Chandra & Newberne, 1977; Chandra, 1983; Tomkins, 1986). Studies of animal models have shown that malnutrition may influence the ability of a host to resist geohelminth infection (see Hunter, 1953; Gibson, 1963; Bawden, 1969; Bolin *et al.*, 1977; Duncombe *et al.*, 1979; Storey, 1982; Cummins *et al.*, 1986; Slater & Keymer, 1986a,b; Bundy & Golden, 1987; Slater & Keymer, 1988; Slater, 1989). Despite this, and the widespread global co-occurrence of malnutrition and geohelminths, human studies into this host-parasite interaction remain limited. Preliminary studies carried out by Bundy & Golden (1987) attest to the potential importance of host nutrient-mediated susceptibility in generating predisposition to intense infection in endemic areas. Individuals with low levels of plasma zinc, a trace element that is essential to thymus-mediated immune responses (Beisel, 1982b), were shown to have above-average *T. trichiura* worm burdens. As pointed out by Bundy (1988), this result could imply that plasma trace element concentration, which is determined by factors that are primarily socio-economic (diet) or genetic

(physiology), may modify a genetically determined factor (immune competence) to create heterogeneity in infection intensity. A further insight into the potential interactive relationship between host genotype and diet derives from the observation by Slater & Keymer (1986a) that while variability in *Heligmosomoides polygyrus* burdens increased with repeated infection dose in outbred MF1 mice fed a high protein diet, it was consistently random in mice fed a low protein diet. This suggests that protein malnutrition, by suppressing the development of acquired immunity in the malnourished mice, overrides the role of host genotypic variability in generating parasite overdispersion. Thus while the transmission rate of *H. polygyrus* was significantly raised in a semi-naturally infected community of protein malnourished outbred CD1 mice, a greater degree of parasite overdispersion and concomitant lower rate of *H. polygyrus* transmission were observed in the corresponding well nourished mouse populations (Slater & Keymer, 1986b). These studies indicate that nutrition - induced susceptibility to infection intensity, by influencing the dispersion of parasite numbers within endemic communities, may play an important complementary role in the stable transmission, persistence and control of geohelminth infection.

From the above discussion, it is obvious that the aetiology of differential susceptibility to infection is complex. An improved understanding of the controlling factors, however, is not only of academic interest but of great potential significance to the design of community-based intervention programmes to control helminthic transmission and morbidity. Individuals predisposed to intense infection are the major contaminants of the environment and the most at risk of morbidity from helminthic disease. If it is found that genetic factors are pre-eminent in predisposition then these individuals could be selectively treated and clinically monitored (Anderson & Medley, 1985). If, on the other hand, environmental exposure factors are of greater significance then better morbidity control could be achieved by targeting treatment at the most heavily exposed age-classes in the community (Warren & Mahmoud, 1976). Nutrition-induced predisposition, however, may have rather more implications for control. Firstly, it would suggest that predisposition to intense infection may be transient, and is likely to decline as children (who often form the biggest nutrient-deficient group in a community (Keller & Fillmore, 1984)) grow into the more nutritionally adequate adult age-classes. This factor may pose severe complications for selective drug treatment as it would mean repeated identification (at great logistic and financial costs) of heavily infected individuals at each treatment interval (see Anderson & Medley, 1985). Secondly, nutrient-induced susceptibility may also suggest that chemotherapy alone would not be sufficient to control helminthiasis, since

reinfection to pre-control levels will occur rapidly due to the continued susceptibility of the malnourished fraction of the population (Anderson & Medley, 1985). An integrated approach, combining the concomitant treatment of both the infection (chemotherapy) and malnutrition (nutrient supplementation), of the susceptible population may well be indicated, therefore, in helminth control.

Although host genetic restriction and nutrition may constitute the most consistent mechanisms of reduced immunocompetence to helminth infection, it is worth noting that other less well- known constraints on host immunocompetence, such as the suppressive effects of concurrent infections with viral (Tomkins, 1986), protozoan (Urquhart *et al.,* 1973; Philips *et al.,* 1974; Philips & Wakelin, 1976) and other helminth parasites (Behnke & Wakelin, 1973; Behnke, *et al.,*1984), and the physiological changes associated with pregnancy and lactation in the mammalian female (Selby &Wakelin, 1975; Behnke, 1987; Lloyd & Soulsby, 1988), may also be related to individual human susceptibility to helminthiasis in endemic areas.

1.3 MALNUTRITION AND INTESTINAL HELMINTHS

The central theme of this thesis is the effect of protein malnutrition on the transmission dynamics of geohelminths. The population aspects of geohelminthiasis has been considered in preceding section. In this section, a brief overview of human malnutrition, the association between protein energy malnutrition and susceptibility to helminth infection, the likely mechanisms underlying this relationship, and the effect of P.E.M. on mammalian immunocompetence is presented.

Available estimates of the prevalence rates of the most important forms of malnutrition suggest that there are four major nutritional deficiency diseases of human populations (see Table 1.3) (Latham, 1984). Of these, protein-energy malnutrition (PEM), because of its high prevalence and relationship with child mortality rates and impaired physical growth, is widely considered the most important (Torun & Viteri, 1989).

Table1.3 *Gross estimate of total number of people affected by the various forms of malnutrition in the world today (adapted from Latham, 1984).*

Deficiency	Morbidity	Number of cases
Protein and energy	Stunted growth	500 million
	Clinical cases of Kwashiorkor & marasmus	1million
Iron	Anaemia	35 million
Vitamin A	Blindness	6 million
Iodine	Goitre	150 million
	Cretinism	6 million

The causes of PEM include both direct effects, such as low food availability, and infectious disease stress on adequate but marginal diets, notably diarrhoeal loss of nutrients and reduction in appetite (anoxeria) (Torun & Viteri, 1984; Rosenberg & Bowman, 1984; Mata, 1989; Nesheim, 1987). Since both infection and low food availability are associated with poverty, it is perhaps unsurprising that recent

studies have invariably indicated the importance of social, economic, cultural and educational deprivation as primordial determinants of human malnutrition (Latham, 1984; Torun & Viteri, 1989).

PEM may affect all age groups but has been demonstrated to be most frequent among children under 5 years of age, and particularly between the ages of 1 and 1.99 (Keller & Fillmore, 1983), when growth increases nutritional requirements and who cannot ensure adequate food intakes by their own means (Puffer & Serrano, 1973; Chen *et al.,* 1980, Keller & Fillmore, 1983). A conservative estimate by WHO is that about 100 million such children currently suffer from moderate-severe P.E.M. manifesting as marasmus and kwashiorkor (Chandra, 1980). Older children generally exhibit milder forms of PEM mainly because they are thought to cope better with social and food availability constraints, and because infections are less severe. Pregnant and lactating women in the community may also have PEM but the consequences are mainly seen in the growth, nutritional status and survival rates of their foetuses, newborn babies and infants. Adult men and nonpregnant, nonlactating women normally are shown to have the lowest prevalence and the mildest form of the disease (see Torun & Viteri, 1989).

The age distribution of human PEM may have significance for the epidemiology of infectious disease in developing countries. It is widely recognized that PEM is associated with depressed immunity to infectious disease (see section 1.2.2.3). Given that children under 5 years of age invariably constitute the group most prone to PEM, it is pertinent that most childhood viral infections, including viral-associated diarrhoeas and measles, have been shown to possess an early peak age incidence, generally around 12 months (McFarlane, 1976). It is also possible that the impact of early episodes of acute infection or malnutrition or both in children, particularly during foetal and suckling periods may lead to long-lasting or permanent depression in immune function (McFarlane, 1976). In particular, depressed T-cell number and function may persist for several months or years (Chandra, 1975a; Dutz *et al.,* 1976; Ferguson, 1978). This may be important in the context of enhanced individual susceptibility to the later chronic infections of children, such as geohelminthiasis.

1.3.1 Protein - energy malnutrition (PEM) and susceptibility to infectious disease

The association between malnutrition and infectious disease has been long recognized (Scrimshaw & Taylor, 1968; McFarlane, 1976; Suskind, 1977; Chandra & Newberne, 1977; Isliker & Schurch, 1980; Chandra, 1983a). The early interest in the nutrition-infection relationship arose from the clinical impressions of physicians and other health workers dealing with underfed communities, of the often increased severity and occurrence of various infectious diseases, particularly acute infections such as bacterial and viral diarrhoeas and respiratory diseases among the malnourished (reviewed by Gershwin *et al.*, 1985; Chandra, 1980, 1983a). This led to systematic epidemiological and microbiological investigations in many parts of the developing world that have confirmed the intimate association between nutritional deficiencies, growth failure and infectious disease, notably acute microparasitic disease (Scrimshaw & Taylor, 1968; Mata, 1972; Puffer & Serrano, 1973; Alleyne *et al.*, 1977; Chandra, 1979a, 1980, 1983a; Brown, 1981; Keusch, 1982; Gershwin *et al.*, 1985; Tomkins, 1986)

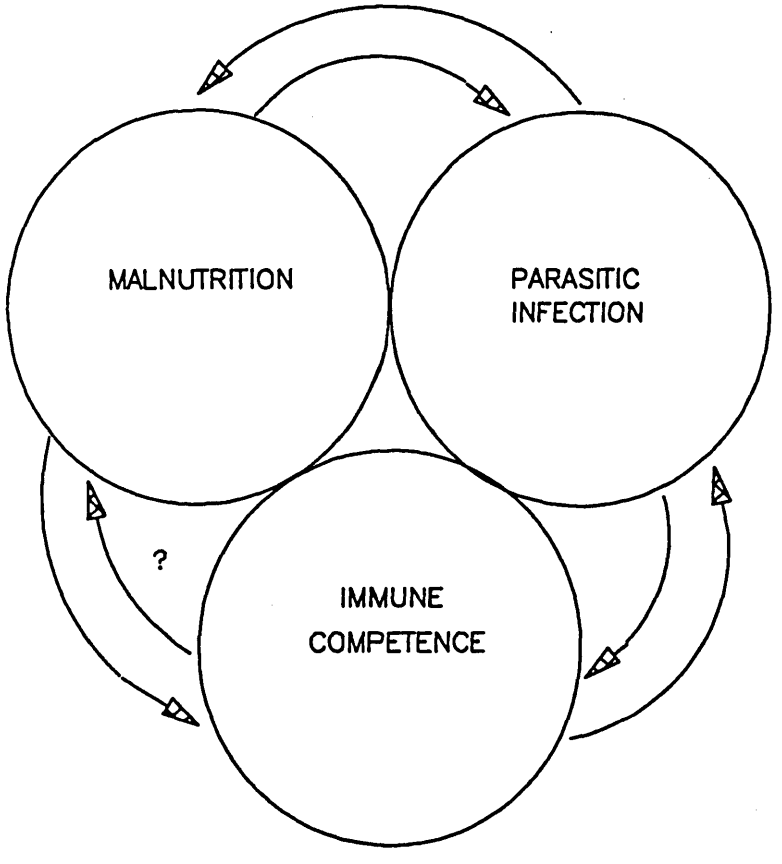

Figure1.2 *Interactions between malnutrition, immunity and parasitic infection (modified from Chandra, 1980).*

Following the advances in immunology and molecular biology, a complex three - sided interrelationship has been postulated as occurring between infectious disease, individual host nutritional status and the function of the immune system (Fig. 1.2) (McFarlane, 1976; Chandra & Newberne, 1977). According to this scheme, nutritional status influences host immunological function and the response to pathogenic challenge; conversely, infectious disease, whether acute or chronic, has a detrimental effect on the nutritional state. In addition, infection and immune functions are also closely linked and have reciprocal interactions. Accordingly, infection associated with malnutrition may act to accentuate the immunodepressive effect of nutritional deficiency *per se* (Beisel, 1982, Chandra, 1984). Fig. 1.3 portrays a simple model of the possible mechanisms which could underlie the three - way interaction between infection, nutritional status and immunity (Chandra, 1980).

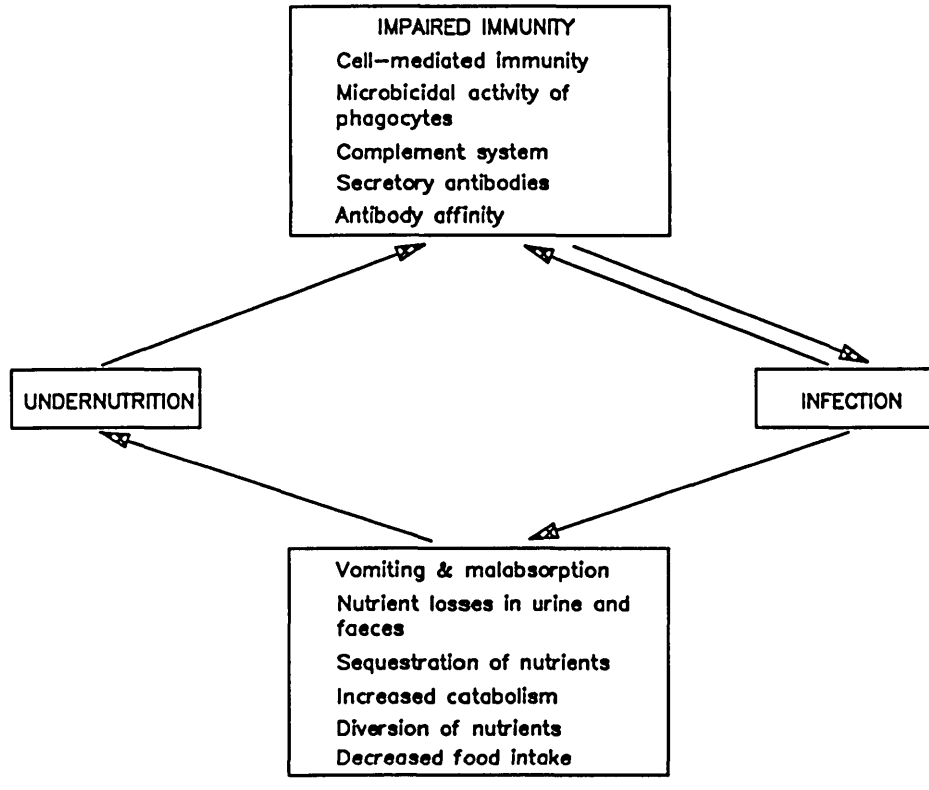

Figure1.3 *Potential mechanisms underlying the malnutrition-immunity-infection interaction (adapted from Chandra, 1980).*

Although the concept that malnutrition may enhance individual host susceptibility to infection is generally accepted, it is pertinent to note that there are reports in the literature to suggest that the reverse interaction may also occur and that the treatment of the deficiency may even precipitate a clinical infection. Thus both clinical amoebiasis and malaria have been shown to be associated with iron

supplements in anaemic African nomads and pastoralists (Murray *et al.,* 1978a; Murray *et al.,* 1978b). However, as pointed out by Beisel (1982), it is unclear from the published data whether these situations arise as a direct effect of the increased availability of a key nutrient for parasite replication, or indirectly because of an unbalanced recovery of both important iron-requiring cellular enzymes and defensive responses during the refeeding regimes.

To date, much of the quantitative analysis of the interaction between infection, immunity and nutrition has been concerned mainly with acute infectious diseases, particularly diarrhoeal diseases and measles (Chandra, 1983a). Such studies have led to the formulation of various intervention strategies to treat the malnutrition-infection complex, such as oral rehydration in the treatment of diarrhoeas, the promotion of breast feeding, immunopotentiation by cell-extracts or pharmacological agents, and short-term nutritional supplements to increase resistance to infection (Chandra, 1979a; Keusch & Scrimshaw, 1986).

The relation of malnutrition to chronic infection, such as geohelminthiasis, is less clear. In their classical review, Scrimshaw, Taylor & Gordon (1968), through the clinical concepts of synergism and its converse - antagonism, showed that pre-existing protein deficiency may enhance susceptibility to both acute and chronic diseases, including helminthic infection. Mata *et al.* (1978) also demonstrated an association between PEM and increased infection with *A.lumbricoides*, *T. trichiura* and *Strongyloides stercoralis* in undernourished Gautemalan children. By contrast, other workers have found little evidence to suggest a significant relationship between nutrition status and infection with geohelminths in human populations (Newmann *et al.,* 1975; Henry, 1981; Brown *et al.,* 1981; Harland, 1986). These studies have illustrated that while the mutually aggravating relationship between infectious disease and human malnutrition is well-established for acute microparasitic infections, the evidence for chronic infections, such as intestinal helminthiasis, is rather more tenuous. This situation may exist as a result of the difficulty in demonstrating the importance of the chronic diseases in the human malnutrition-infection complex. Human studies have invariably tended to be constrained by the uncertainties of nutrient and infection history, and the effects of polyparasitism, all of which are likely to determine infection intensity in the helminth-malnutrition interaction (Pawlowski, 1984). It is significant that animal studies (in which infection and nutrient parameters can be precisely controlled) have consistently shown that protein malnutrition invariably induces greater host susceptibility to helminth infections (Table1.5).

1.3.2 Malnutrition and mammalian immunocompetence

It is widely held that the most likely mechanism which underlies the increased susceptibility to infectious disease associated with PEM is altered immunity (Scrimshaw & Taylor, 1968; McFarlane, 1976; Chandra & Newberne, 1977; Chandra 1979a, 1983a). The extensive literature on the effect of PEM on various parameters of the mammalian host immunocompetence has been reviewed (Suskind, 1977; Chandra & Newberne, 1977; Alleyne *et al.*, 1977; Beisel, 1979; Isliker & Schurch, 1981; Chandra, 1980; Gerswhin *et al.*, 1985; Tomkins, 1986). Some of the major findings on the effects of PEM on mammalian immunity are outlined in Table 1.4. In brief, these results indicate that the most consistent changes in immunocompetence induced by PEM are in cell-mediated immunity (CMI), the bactericidal function of neutrophils, the complement system, and the secretory IgA antibody response.

A summary of the major changes induced by PEM in cellular and humoral immunity, the components of relevance to immune protection against *T. muris* (Wakelin & Lee, 1987), is given below.

(i) Cellular immunity

CMI in PEM has been tested by dermal delayed sensitivity to a battery of ubiquitous recall antigens or after deliberate sensitization with chemical agents such as 2-4-dinitro-chlorobenzene (DNCB), the proportion and number of circulating T-lymphocytes, lymphocyte transformation induced by mitogens and antigens, and by the production of soluble mediators of immunological reactivity in response to mitogens and antigens (Chandra, 1983a).

The delayed cutaneous hypersensitivity is depressed (see Suskind *et al.*, 1977; Alleyne *et al.*, 1977; Chandra & Newberne, 1977; Beisel, 1979; Chandra, 1980; Gerswhin *et al.*, 1985). Since this reaction is a composite of several sequential steps (such as sensitization via macrophagic antigen presentation, recognition of specific antigenic determinants (memory), proliferation and elaboration of lymphokines by T-cells, vascular changes, and migration of inflammatory cells to the local site), reduction in cutaneous reactivity may be suggestive of impairment of several cellular processes in PEM. Skin anergy, however, may not be absolute and a higher dose of the test antigen can occasionally elicit a positive response (Chandra, 1983a).

Table 1.4 *Immune deficiency in PEM (Based on Cunningham-Rundles, 1982; Chandra, 1980; Gerswhin et al., 1985).*

Parameter	Humans	Animals
1. Size of lymphoid organs	Thymus most affected, lymphoid organs also also reduced in size.	Early deficiency leads to thymic atrophy and depressed spleen size.
2. Morphology of lymph nodes	Depletion of paracortical in undernourished children	Germinal center maintained. Stem cells, and nonmigratory T cells most affected. Regrowth delayed but possible.
3. Gut-associated lymphoid tissue, tonsillar and adenoid tissue	Size reduction; depletion of paracortical areas and germinal centers.	Mesenteric lymph node less affected than thymus, atrophy of Peyers patches, tonsillar and adenoid tissue.
4. Humoral immune response	Serum immunoglobulin levels normal or increased. Antibody response to viral immunization may be depressed. Elevated IgE.	Variable, may be reduced to T cell-dependent antigens. Intrinsic B cell function relatively intact. Increased response to T-independent antigens.
5. Secretory antibody response	Poor secretory IgA function after immunization. Serum IgA normal. Secretory IgA in tears and saliva reduced, IgG increased.	Suppressed but may be related to maturation delay.
6. T cell number	Depressed	Depressed

Table 1.4 *cont'd*

7. Delayed cutaneous hypersen-	Depressed secondary response. Weaker response may be elicited with stronger dose. Impaired primary response.	Depressed primary and secondary responses.
8. In vitro response to T lymphocyte mitogens	Slight to severe depression reversible with nutritional repletion. Some cases normal response.	No effect. Protein deprivation alone causes enhancement.
9. Lymphokine production	Few studies, interferon production reduced in vitro.	Interferon production decreased. Migratory inhibitory factor production depressed.
10. Phagocytic cell function	Defective mononuclear cell chemotaxis, normal neutrophil chemotaxis in some studies. Impaired killing demonstrated in some cases.	Reduction of marrow neutrophil pool, reduced neutrophil mobility.
11. Complement	Total heamolytic complement activity depressed. C3 and factor B reduced.	Normal response. C3 levels reduced.

The most significant change in PEM is the marked reduction in the proportion and absolute number of T-lymphocytes, identified by their ability to form rosettes with sheep red blood cells, in the peripheral blood (Chandra 1974; Ferguson *et al.,* 1974) There is some evidence that autologous serum inhibits rosette formation. Among others, alpha-feto protein, an embryonal immuno-suppressive protein, raised IgE, endotoxin, antigen-antibody complexes, and C-reactive protein (CRP) have significant inhibitory function in this phenomenon (Chandra, 1980). Elevated levels of free cortisol (Parent *et al.,* 1974) may also exert a lympholytic effect. Since there is little change in the proportion of B cell numbers (Bang *et al.,* 1975; Steihm, 1980), the "null" cells, i.e. lymphocytes that do not bear markers of either T or B lymphocytes, are relatively increased (Chandra, 1977; Beisel, 1979) and have subpopulations which exhibit cytotoxic and suppressor activity (Chandra & Newberne, 1977). It has been proposed that null cells may be undifferentiated T-lymphocytes (Chandra, 1980), a theory supported by the findings of (1) reduced serum thymic factor activity (required for normal maturation and differentiation of T-lymphocytes) in children with PEM (Chandra, 1979b), (2) of an increased number of rosetting cells on addition *in vitro* of thymus products (Jackson & Zaman, 1980; Olusi *et al.,* 1980) and (3) of the raised activity of leucocyte terminal deoxynucleotidyl transferase (TdT); an enzyme present in moderate amounts in the early stages of T cell development (Chandra *et al.,* 1983b). These findings suggest that PEM impairs the terminal differentiation and maturation of T-cell precursors. Apart from circulating lymphocytes, both intraepithelial lymphocytes (IEL) and other mucosal T cells are also reduced in PEM (Allardyce & Bienenstock, 1984), although loss through the intestine has been ascribed for this phenomenon by one study (Maffei *et al.,* 1980).

Alterations in T-cell subsets have also been noted in PEM (Chandra, 1979d). The ratio of T4+(helper) : T8+(suppressor/cytotoxic) cells is reduced, largely due to a decrease in the number of T4+ cells. This finding may imply a reduction in either the absolute number of such cells or the density of antigen molecules on each cell surface (Chandra, 1983c). It is recognized that cell-surface glycoproteins may be altered in PEM.

The *in vitro* proliferative capacity of lymphocytes to mitogens and antigens in PEM has been variously reported to be either impaired or normal (see Chandra, 1980; Schlesinger & Stekel, 1974; Rafii *et al.*, 1977). It is possible that the main reason for diminished *in vitro* lymphocyte transformation is due to the reduced number of T lymphocytes in culture (Chandra, 1980). Furthermore, it has been revealed that autologous serum may contain factors capable of inhibiting immunological responses such as *in vitro* lymphocyte transformation (Chandra, 1980; Gerswhin *et al.*, 1985). Such factors are thought to be related to those responsible for decreased rosetting of T cells in PEM (see above). PHA-stimulation of lymphocytes in mitotic activity are consistently decreased in animal models of PEM (McFarlane & Hamid, 1973; Ashkenasy, 1974).

Data on the production of soluble mediators by sensitized lymphocytes in PEM are limited and conflicting. While assays for macrophage migration inhibition factor (MIF), show a variable reduction in PEM (Chandra, 1980), reports concerning leukocyte MIF have been mixed, with some investigators reporting low levels of activity (Lomnitzer *et al.*, 1976) and others reporting levels within normal ranges (Gershwin *et al.*, 1985). Interferon production is also shown to be either lower than normal (Schlesinger *et al.*, 1976), or unaffected in PEM (Gerswhin *et al.*, 1985).

(ii) Humoral immunity

Polyclonal hyperimmunoglobulinaemia is common in patients with PEM (McFarlane *et al.*, 1970; Alvarado & Luthringer, 1971; Purtilo *et al.*, 1976). Marked increases are observed in IgA and IgE, the latter particularly in individuals with helminthic infections (Purtilo *et al.*, 1976). The hyper immunoglobulinaemia in PEM is considered most likely to be the direct result of repeated infections of the skin, gastrointestinal and respiratory systems in the malnourished (Chandra, 1980, 1984; Gerswhin *et al.*, 1985). Elevated IgE levels in PEM may also reflect an abnormality in T cell regulation, particularly alterations in the T- suppressor cells (Steihm, 1980). Occasional infants with early - onset malnutrition, however, may have low serum IgG and IgA concentrations (Aref *et al*, 1970; McFarlane *et al.*, 1970; Chandra, 1972). This is thought to reflect reduced synthesis and/or gastrointestinal loss (Chandra, 1980).

Serum antibody responses (to specific antigens) in PEM are usually normal (not surprising since B cell numbers are unaffected in PEM), but may be submaximal for some thymus- dependent or macrophage- processed antigens. Reduction in antibody affinity may also underlie this depressed antibody response in PEM (Chandra *et al.*, 1984), and may contribute to the frequent occurrence of immune complexes in the serum of the malnourished and to the development of immunopathology in infections such as malaria and schistosomiasis (Chandra, 1980). There are limited data on the effect of PEM on immune response to parasites. Available studies indicate that the rate of parasite- specific antibody production may be either normal or slightly depressed (Saowakontha, 1975; Jayapragasam *et al.*, 1977; Wagland *et al.*, 1984; Slater & Keymer, 1988).

Perhaps the most consistent change affected by PEM in humoral immunity is in the secretory antibody response. In general, the levels of secretory IgA and lysozyme are low in a variety of bodily secretions, tears, mucous, saliva, etc. (Chandra, 1975; Watson & McMurray, 1979)). The mechanisms involved may include decreased synthesis of the secretory component, an alteration of immune cell populations in the GALT, especially T4+ helper cell populations (Alleyne *et al.*, 1977); decreased numbers of mucosal plasma cells producing IgA (Chandra, 1983) and/or changes in localization of IgA-producing lymphoblasts in the gut (McDermot *et al.*, 1982). In addition, recent research in rodents has indicated that reduced IgA secretion in intestinal fluids may also be related to the retarded maturation of the local synthesis of these proteins (Watson, 1984). Diminished IgA secretion is recognised to have serious clinical significance in terms of incidence of septicaemia, absorption of macromolecules leading to increased immune complex formation and allergic reactions (Chandra, 1975; Gershwin *et al.*, 1985), severity of local gastrointestinal infections and effectiveness of vaccine-induced immunity (Chandra, 1980).

In general, these findings suggest that while PEM may invariably impair the cellular immune response, the concomitant effects on the humoral immune system is variable, but may be reduced to T-dependent antigens. Secretory IgA levels are significantly depressed in PEM, with implications for gastrointestinal infections.

1.3.3 Malnutrition and parasite population parameters

So far, the discussion on PEM-related susceptibility to infectious disease has focused upon the individual host as the unit of study. In order to examine the parasite population consequences of host undernutrition, however, it is necessary to study the effects of the nutrient deficiency on various aspects of the parasite population parameters, such as the transmission, mortality and reproductive rates involved in the parasite life cycle. For geohelminths, as discussed elsewhere in this chapter, these parameters are the rates of worm establishment, survival over time and fecundity (Anderson, 1987b). Comparable data in this area are currently limited (Slater & Keymer, 1986a,b). However, re-evaluation of some recent data on the relation between protein nutrition and intestinal helminthiasis suggests that protein/protein-energy malnutrition may indeed have a profound effect on the transmission success of intestinal helminth parasites (Table 1.5) (Bundy & Golden, 1987). It is apparent that dietary protein deficiency may enhance helminth intensity, presumably as a consequence of increased parasite survival rates in malnourished hosts. However, the consequences of protein malnutrition for helminth reproduction are variable, and may depend upon the particular host-parasite system. Further, although initial parasite establishment may be unaffected by host nutritional status, it appears that subsequent host reinfection rates may be greatly facilitated by an associated dietary protein deficiency. These latter findings, which have been recorded for both acute (*Nippostrongylus brasiliensis*) and chronic (*Heligmosomoides polygyrus*) nematode infections (Behnke, 1987), indicate that protein deficiency-induced defects in acquired immunity to infection may be the likeliest mechanism underlying the enhanced transmission of helminths in malnourished hosts.

There may be additional underlying complexities in the parasite-nutrition interaction which may affect helminth population dynamics. Firstly, as discussed elsewhere, nutritional stress may increase the rate of parasite-induced host mortality. Thus, Gordon (1960) found that sheep fed a low protein diet and infected with *Trichostrongylus colubriformis* had a higher rate of mortality than their well-nourished infected counterparts. Similar relationships between dietary status, infection and host mortality rates have also been obtained with *T. axei* (Gibson, 1955) and *H. polygyrus* infections (Ehrenford, 1954). The main population effect of this phenomenon would be to enhance the stability of the host helminth interaction (Anderson & May, 1979).

Table 1.5 *Effect of deficient protein nutrition on intestinal nematode populations in mammalian hosts.*

			Population parameter					
Host	Parasite	Nutritional factor	Worm burden	Initial establishment	Survival	Fecundity	Reinfection	Source
Man	G.I. heminths	P.E.M.	+					Jose & Welch (1970) Mata (1972,1978)
Rat	*Nippostrongylus brasiliensis*	General low protein	+	+	+	no effect	+	Donaldson & Otto (1946)
		Low protein		+/no effect	+	+	+	Bolin *et al.* (1977) Duncombe *et al.* (1979) Ash *et al.* (1985)
Mouse	*Heligmosomoides*	Low protein		+/no effect	+/no effect	+/no effect	+	Bawden (1969) Slater & Keymer (1986) Slater (1987) Brailsford & Mapes (1987)
Dog	*Ancylostoma caninum*	General low protein	+		+	+	+	Foster & Cort (1932,35)
Sheep	*Oesophagostomum columbrianum*	Low protein		no effect	+	+		Dobson & Bawden (1974)
	Haemonchus contortus	Low protein		no effect	no effect	+/no effect		Abbott *et al.* (1985,1986)
	Trichostrongylus axei	General low protein	+					Gibson (1963)
Lamb	*T. colubriformis*	Low protein			+	+		Wagland *et al.* (1984)
	Bunostomum trigocephalum	General	+			+		Lucker & Nuemayer (1947)

+= enhanced; absence of result indicates not measured

Density-dependent fecundity may be another helminth regulatory mechanism which could be influenced by the nutrition of the host. It has been demonstrated that specific carbohydrate deficits may abolish this regulatory mechanism in some acanthocephalan and cestode infections of rodents (Crompton *et al.,* 1983; Keymer *et al.,* 1983a; Boddington & Mettrick, 1981). As pointed out by Bundy & Golden (1987) the potential removal of this constraint on parasite population growth may have major consequences for the subsequent stability of the worm population.

Finally, the role of host nutrition in inducing differential host susceptibility to helminth infection intensity has already been discussed. The population consequences of this will be to increase the degree of parasite overdispersion and hence the stability of parasite populations within the host community.

The most eloquent and complete investigation to date into the influence of protein malnutrition on geohelminth population dynamics is that of Slater & Keymer (1986a,b). Their experimental studies clearly established that dietary protein deficiency may significantly enhance the transmission of chronic *H. polygyrus* infection in malnourished laboratory mice. It was found that the major impact of protein malnutrition was to increase worm survival, although worm fecundity was also raised in the malnourished mice (Slater & Keymer, 1986b). Analysis of the data from repeated infection studies (Slater & Keymer, 1986a) indicated that while *H. polygyrus* abundance in malnourished mice was directly related to the rate of host infection, parasite population growth was influenced by an additional immunity-induced mortality factor in well-nourished hosts (Berding *et al.,* 1986). The present research extends the above study, using the *Trichuris muris* - mouse analogue of human *T. trichiura* infection (Lee & Wakelin, 1983), as an experimental test system. The present studies will in addition also focus on likely nutrient-induced differences in mouse serological responses as a potential mechanism underlying the differential transmission of *T. muris* between well- and malnourished CBA/Ca mice.

The majority of studies on the relation between malnutrition and infectious disease have tended to concentrate on the severely or acutely malnourished host. Comparable data on the relative risks of infection in hosts with milder or chronic malnutrition remains limited despite the fact that moderately malnourished children generally represent the majority of the pre-school population in many developing countries (Whitehead, 1980; Goldsmith, 1974). Further, it is also possible that the outcome of a particular parasite-nutrition interaction may depend critically on the degree of host malnutrition (Bundy & Golden, 1987). The latter

authors describe three mechanisms by which host nutrition status may affect helminth parasites: (1) alteration of host immune defences, (2) induced parasite malnutrition, and (3) modification of the parasite environment within the host. Since these conditions have conflicting consequences for helminth populations and appear to be related to the degree of malnutrition, it was proposed that changes in host nutritional insult may have paradoxical consequences for intestinal helminths. Thus, while a well-nourished host may be expected to provide abundant specific nutrients for parasite growth, an intact immune response may act to severely limit worm intensity. On the other hand, although immuno-competence may be lowered as the degree of nutritional insult increases, at extreme levels of malnutrition, significant changes in host gut physiology may prove inimical to parasite establishment and survival. The authors therefore proposed that high intensity intestinal helminth infections may typify a moderately malnourished individual with impaired immune competence but minimal alterations in gut physiology.

Because of these considerations, one secondary aim of the present research was to examine the interaction between the degree of mouse protein nutriture (normal, moderate and severe malnutrition) and *T. muris* population dynamics.

1.4 *TRICHURIS MURIS* - MOUSE MODEL

Like the major human soil- transmitted geohelminths, *T. muris* has a simple, direct life cycle, the murine host being infected by ingestion of a fully embryonated egg. Development of eggs to infectivity under laboratory conditions has been shown to be relatively slow, taking about two months at 20°C (Wakelin, 1969a). It is believed that the infective larva within the egg is always a first stage (L_1) larva, no moults occurring prior to infection (Wakelin, 1969a). Hatching of eggs and all subsequent post- embryonic worm development, from larval establishment through to adulthood and sexual reproduction, occurs predominantly in the mouse caecum (Panesar & Croll, 1980). The larvae first penetrate the epithelium in the crypts of Lieberkuhn, and then migrate to the superficial luminal epithelium, undergoing cuticular moulting (Panesar & Croll, 1980; Panesar, 1981). Altogether, three larval moults occur during the course of maturation: on days 9- 11 (Wakelin, 1969a), 20- 23 (Fahmy, 1954; Panesar & Croll, 1980), and on day 30 post- infection (Panesar & Croll, 1980). By adulthood, the worms are typically observed to lie with their filamentous anterior ends threaded through the mucosa of the surface epithelium while the thicker posterior ends, containing the reproductive organs, protrude into the caecal lumen. *T. muris* is dieocious, and sexual maturity is generally reached by days 32- 37 p.i. (see Pike, 1969), after which eggs are produced and released by the female worms. The life expectancy of *T. muris* in primary infections is estimated to be in the order of 12- 13 weeks (Worley *et al.*, 1962; Pike, 1963).

1.4.1 Dynamics of *T. muris* Infection

Murine trichuriasis is an acute nematode infection (Behnke, 1987). In the majority of the mouse strains, strong spontaneous cure responses, resulting in the complete removal of the worms are stimulated during initial infection (Wakelin, 1975a). The time taken to mount the primary spontaneous cure may vary considerably between mouse strains, but the majority expel single infections well before patency (Wakelin, 1975a; Wakelin & Lee, 1987). In some outbred strains (e.g. Schofield, MF1), a proportion of mice will support adult worm infections, whereas the remainder become immune (Wakelin, 1969b). Recent work suggests that *T. muris* survival in primary infections may also depend upon the distinct effects of different mouse MHC (H-2) haplotypes, certain haplotypes being associated with slower expulsion patterns than others (Else & Wakelin, 1988). Apart from mouse genetics, the spontaneous cure of *T. muris* is also shown to be infection- dose dependent. Generally, it appears that there is a threshold level of infection (approximately equivalent to 10 worms) required to elicit an effective immune response (Wakelin, 1973). Thus while lower- level infections normally survive to patency and are fertile,

infections above the immune threshold level stimulate immunity and are expelled (Wakelin, 1973). Superficially, therefore, this situation appears to correspond to the population regulation type 3 (at the individual host level) considered by Bradley (1972), but the difference here is that all parasites are expelled when the threshold is exceeded. However, as suggested, the immune threshold is strain and haplotype variable, and some mice can therefore support larger subthreshold infections than others. In addition, the threshold level may also be influenced by factors such as host physiological state (Selby & Wakelin, 1975) and the presence of concurrent infection (Behnke *et al.*, 1984).

Challenge infections with *T. muris* are normally expelled extremely rapidly from all mouse strains tested, with parasite loss being virtually complete by 24h post infection (Lee & Wakelin, 1982). Furthermore, solid immunity to secondary and tertiary infections may be induced in mice as effectively with small immunizing infections of 10 worms as with worm infections 10 or 20 times as many (Wakelin, 1973). However, in non- responsive (e.g. some outbred Schofield mice) and immunocompromised mice, challenge infections may establish readily (Wakelin, 1967).

Trickle or repeated infections with *T. muris* do not cause stable worm burdens in responder mouse strains such as NIH or CFLP, unless the mice are immunocompromised or concurrently infected with *H. polygyrus* (Behnke & Wakelin, 1973; Behnke *et al.*, 1984). This situation contrasts sharply with that seen in *Nippostrongylus brasiliensis*, a nematode causing acute infection in the rat, in which it is possible to circumvent the development of a spontaneous cure process in this manner and thereby accumulate large and persistent burdens (Jenkins & Phillipson, 1970). In *T. muris*, therefore, it appears that regardless of the mode of infection (whether by primary or trickle infection methods) once the strain- specific immune threshold level is exceeded, strong immune expulsion responses are triggered which removes the worm population. However, in trickle infections, not all the worms may be eliminated and a small number of early infections may become sexually mature (Behnke & Wakelin, 1973).

1.4.2 Immunity to *T. muris*

(i) Stimulation and targets of immunity

As mentioned, *T. muris* is an highly potent stimulator of host protective immunity. Infections with as few as 10 worms may elicit measurable protection (Wakelin, 1973). Several studies have seeked to isolate and characterize the antigens involved

in such immunity (reviewed by Wakelin & Lee, 1987). In brief, these studies have demonstrated that the major protective antigen in *T. muris* immunity is a stable protein of molecular weight of approximately 43kD (as analysed by SDS-PAGE separation): carbohydrate components were not found by the workers to be essential for its immunogenicity (Jenkins & Wakelin, 1977). In addition, the protective component was found to be conserved, being present in antigen prepared directly from adult worm stichocytes, preparations of adult excretory- secretory products (ES) collected during short- term *in vivo* maintenance, homogenates of 14 day- old larvae and in embryonated eggs (Jenkins & Wakelin, 1977,1983). It has been suggested that the antigenic material in ES products may originate within the stichocytes and are released into the surrounding milieu via the mouth, probably for the extra- corporeal digestion of cellular material (Wakelin & Lee, 1987). Given the intra- epithelial attachment site of *T. muris*, such a method of feeding may therefore facilitate the presentation of large quantities of antigenic material to the immunocompetent cells of the gut associated lymphoid tissue (GALT) in the mucosa (Wakelin & Lee, 1987). Antigen presentation in murine trichuriasis is thus likely to be extensive and continual.

A recent study has dealt with the role of surface antigens in the *T. muris*- host interaction (Preston *et al.*, 1986). The authors demonstrated that the larval parasite surface (days 5 to 25 p.i.) was able to elicit stage- specific IgG binding, induce eosinophil adherence (in part by promoting adherence antibodies), and activate complement via the alternate pathway, with subsequent elaboration of chemotactic C3b molecules. Despite adherence, however, eosinophils were not observed to effect parasite killing in *in vitro* experiments. Further, it was shown that the stage-specificity of the surface IgG binding may be related to larval moulting, with the older stages casting earlier cuticles at each parasitic moult. The different larval stages were also found to vary in their ability to bind specific lectin probes, suggesting the probable occurrence of qualitative changes in the expression of surface carbohydrate moieties between moults (Preston *et al.*, 1986). The significance of these changes at the parasitic surface to *T. muris* protective immunity is unclear, but it is possible that carbohydrates may serve to mask other antigenic molecules, and the stage- specific turnover of surface antigens at each larval moult may constitute an evasive stratagem to avoid host immunity (Preston *et al.*, 1986).

(ii) Effectors of immunity

Immunity to *T. muris* has been extensively reviewed (Wakelin & Lee, 1987). Serum transfer experiments suggest that the immune expulsion of *T. muris* may involve both humoral and cellular components, notably mesenteric lymph node cells (Selby & Wakelin , 1973; Wakelin, 1975b). Wakelin (1975b) also showed that these components acted sequentially in order to affect worm expulsion, the humoral response preceeding the cellular activity. Thus, immune mesenteric lymph node cells (MLNC) transferred late in infection (d.7-9 p.i.), presumably after a period of active humoral response, to irradiated NIH mice were more effective than cells transferred immediately before infection (Wakelin, 1975b).

Although antibody was required for initiating the expulsion response, transfer experiments showed that worm expulsion may be affected only in the presence of an intact MLNC related component (Wakelin, 1975b; Wakelin & Selby, 1976). Immune serum given to animals whose cellular response had been ablated by irradiation did not confer significant immunity.

Few attempts have been made to date to identify the humoral factors responsible for the protective activity of immune serum. A recent study by Else & Wakelin (1989), however, showed that the IgG1 response may predominate over IgM and IgA in murine antibody responses to *T. muris* E/S antigen.

As mentioned, MLNC populations may confer significant immunity to *T. muris*. This is borne by not only the effectiveness of immune and non-immune MLNC preparations to transfer immunity to recipient hosts (Selby & Wakelin, 1973; Wakelin, 1975b), but also by temporal correspondence of the kinetics of cell increase in MLN of CBA/Ca mice and the time of worm expulsion by such mice (Lee *et al.*, 1983). This increase in cellularity, furthermore, paralleled the increase in the ability of cells isolated from the node to transfer immunity to naive hosts (Wakelin & Lee, 1987).

Efforts to examine the characteristics of the cells present in the MLN at the time of maximal ability to transfer immunity, using the techniques of nylon wool separation and anti Thy 1.2 treatment, indicated that the T cell fraction, but not the b cell fraction, may be responsible for the protective activity of MLNC (Lee *et al.*, 1983).

Attempts to assess the importance of other intestinal cellular components such as bone marrow and mucosal mast cells indicated that these cells may not be relevant

in host immune protection against *T. muris* (Wakelin & Selby, 1976; Lee & Wakelin, 1982). Gross inflammation of the gut is also not evident in murine response to *T. muris* (Wakelin & Lee, 1987). Increased goblet cell numbers, usually associated with intestinal inflammation are not normally seen in *T. muris* infected mice (Lee, 1982).

These findings indicate that there may be a more direct interaction between effector T cells and *T. muris* during worm expulsion from the mouse large intestine (Wakelin & Lee, 1987). It has been suggested that intraepithelial lymphocytes (IEL), bearing the ly2+ markers and derived from the MLN and Peyers patches, may play an important role in this context (Wakelin & Lee, 1987).

The features of murine trichuriasis outlined above indicate that the host immune response may represent the most potent regulatory mechanism controlling worm population growth. This may make the *T. muris*- mouse model a particularly suitable experimental system for studies on the nutrition- immunity- helminth population interaction, and forms the major rationale for using the model in the present study.

However, a number of important points should be made at this juncture concerning the relevance of animal nutrition- infection studies with regard to human disease. Firstly, it must be recognised that human malnutrition is more complex than experimental deficiencies in animal models. For example, malnutrition in humans is rarely of a single nutrient, is most probably complicated by multiple infection stress, and may develop irregularly over time, unlike the steady and controlled process imposed upon an experimental system. Secondly, the type, dose, specificity, and route of infection used in animal models may not approximate those occurring naturally in humans. Finally, it must be borne in mind that the specific nutritional requirements of humans and experimental animals in relation to immune responses may well be substantially different (Isliker & Schurch, 1981).

Nevertheless, it is recognized that model systems may remain the most important source of data concerning not only immune responses to helminth parasites (Wakelin, 1978b) and the dissection of the specific nutrient/immunity interaction (see Isliker & Schurch, 1981), but also for estimating the parasite population consequences of host nutritional status (Slater & Keymer, 1986a,b; Slater, 1988).

CHAPTER 2 : MATERIALS AND METHODS

2.1 HOST AND PARASITE STRAINS

All experimental infections were performed on inbred, male CBA/Ca mice, purchased from Harlan Olac (Bicester, U.K) or obtained from small breeding stocks maintained at the Department of Pure and Applied Biology, Imperial College. Routine passaging of *T. muris* was carried out using both CBA/Ca and *in situ* bred BALB/c mice.

The parasite *Trichuris muris* was obtained from Prof. D. Wakelin (University of Nottingham) in September 1986, and continuously passaged at Imperial College for the duration of the study.

2.2 PARASITOLOGICAL METHODS

2.2.1 Mouse maintenance

All mice were maintained under standard animal house conditions. Mice were housed either in cages lined with wood chippings (routine passage and experiments reported in chapter 3) or in grid-floored cages (to reduce coprophagy) in the nutrition experiments (chapters 4, 5 and 6). All mice were treated with 0.2mls of Piperazine citrate (Sigma Chemical Co. Ltd) at 12.5% in water twice, a week apart, to rid any existing pinworm infections, before infection with *T. muris* eggs. Mice used for routine parasite passage and for the experiments reported in chapter 3 were fed on pelleted animal diet (Oxoid 4B). Mice utilized for the nutrition experiments were fed on special, semi-purified, casein based diets in powder form (see below). Both the diets and water were provided *ad libitum* at all times.

2.2.2 Parasite maintenance

The methods used for the passage of *T. muris* were as described by Wakelin (1967). Mice at 6-8 weeks of age were infected with approximately 400 embryonated eggs by gavage. On days 7, 9, 11, 13, and 15 post-infection (p.i) the infected mice were injected subcutaneously (peritoneal region) with 0.05-0.075ml of cortisone acetate (25mg/ml, Sigma Chemical Co. Ltd) using a 1ml syring and a 25 gauge needle. Mice

were also given Oxytetracycline hydrochloride (Terramycin, Pfizer) in drinking water at 165 mg/litre from day 7 when cortisone acetate was first administered.

On day 40 p.i. the infected mice were killed by cervical dislocation and the large intestines containing the adult worms were dissected out into plastic petri dishes (Sterilin, U.K) containing Phosphate Buffered Saline (PBS). The caecum and colon were carefully cut open and the contents gently flushed out. Following this, the organs with the attached worms were transferred to another petri dish with PBS and the adult worms were carefully teased out from tunnels in the intestinal mucosa using fine forceps. The mature female worms were separated, placed in a glass tissue homogenizer containing a small volume of PBS, and then ground together with a size small plunger so as not to rupture the eggs. The resulting suspension was then strained through a fine muslin or wire mesh (size 250 um) into a clean beaker. After allowing for sedimentation, the eggs were washed once by removing the supernatant and resuspending with distilled water. The wash supernatant was then discarded and the egg extraction was resuspended in distilled water containing 0.25% formalin and Fungizone at 250ug/ml (GIBCO), in tissue culture flasks (NUNC).

The above egg suspension was cultured by incubating at room temperature in the dark for at least 60 days to facilitate the development of embryonated eggs. Once sufficient time had elapsed for the embryonation of eggs (verified by the presence of vermiform larvae in the eggs), the cultures were stored in the dark at 4^0C until used for inoculations.

2.2.3 Host infection

Mice were infected by gavage (stomach intubation). The desired concentration of embryonated eggs made up in 100 ul of distilled water was administered to the mouse by means of a 1ml syringe fitted with a blunted 35 mm 19 gauge needle modified to take a small plastic "knob" at the end (to prevent injury to the gut during intubation). The mouse was held by grasping the loose skin behind the ears between the thumb and forefinger, and securing the tail under the fourth or fifth fingers. This technique restrained mouse movement while freeing the other hand for the safe and accurate introduction of the infection tube into the mouse's mouth, and subsequently its oesophagus.

2.2.4 Experimental recovery and counting of parasites

Adult worms were recovered from experimental mice using methods similar to those described in section 2.2.2, except that after the removal of worms from the large intestine, they were normally sexed, counted and stored in 10% formal saline. For experiments described in Chapter 3, following fixing in formal saline, the wet weights of the female worms were measured.

Larval recovery, and some adult worm recovery in chapters 5 and 6, entailed the post-mortem removal of the caecum and colon from experimental mice and subsequent freezing of the organs for at least 24 hs at -20^{0}c. Following this, the gut was allowed to thaw at room temperature and then spit open for worm estimation in grid-marked petri dishes containing PBS. The intestinal contents were first flushed out before the split organs were transferred into a second petri dish, in which the mucosa was scrapped to dislodge the embedded parasites. Both the intestinal wash and mucosal scrapings were then scanned under a stereo dissecting microscope, and the number of larvae and adult worms present in the debris enumerated.

2.2.5 Mouse faecal egg counts

(i) Faecal collection

24h faecal specimens to estimate *T. muris* fecundity were collected either from individual mice (chapters 3 & 4) or from groups of mice (chapter 6). In the former procedure, the infected mice were separated and housed individually in grid-floored cages in racks which facilitated the fitting of collection trays underneath each cage. This arrangement reduced coprophagy and allowed efficient 24h faecal collections. The collection tray was lined with absorbent paper and dampened with 25ml of water at the beginning of each 24h faecal collection period. All mice were allowed *ad libitum* access to both food and water during the period of collection. Exactly 24h after the start of faecal collection, all faeces present in the trays were removed, and each tray relined with fresh, dampened paper for further faecal collections as required by the experimental design (see chapters 3 & 4 for details of the experiments). With regard to mouse group faecal collections (chapter 6), trays lined with moist absorbent paper as described above, were fitted under the grid-floored mouse cages containing the appropriate treatment groups, and faecal samples from each group collected according to experimental needs.

(ii) Egg count technique

The egg count technique used was a modification of the method of Stoll (Cheesbrough, 1987). Each 24h faecal specimen was dried, collected in a pre-weighed clean universal container (Sterilin, U.K) and its weight determined. Enough 0.1M solution of NaOH was then added to the sample to give a 1 in 10 dilution of the faeces. After standing overnight, the suspension was shaken with 5 glass beads (3.5 - 4.5mm diameter) until homogenous; it was then passed through a 212um sieve, and resuspended to 10ml with excess 0.1M NaOH. After thorough shaking, 0.1ml of this faecal suspension was taken for egg counting under a compound stereo microscope. Results were normally expressed as eggs per gram (epg) and eggs per day (epd) of the 24h faecal material collected from each animal or mouse group.

2.3 NUTRITIONAL METHODS

2.3.1 Experimental casein diets

Synthetic, theoretically isoenergetic diets (Crompton *et al.*, 1981) containing varying amounts of protein (casein supplemented with methionine and threonine) were used to model mouse protein status in the nutritional experiments described in this thesis. Details of the theoretical composition of the diets used are given in Table 2.1. As shown, the constituents of each diet were identical; the only variable was the weight by weight ratio of casein and maize starch.

Experimental diets were mixed in a small, hand operated cement mixer (20 litres) and were stored at 6 to 8^0C in plastic containers. All light and moisture sensitive ingredients, especially the vitamin mix components, were always stored desiccated and in the dark at 4^0C. All diets were used within 8 weeks of production.

Table2.1 *The diet mix (based on Crompton et al., 1981).*

Ingredients	*g/1000g diet*		
	2%	4%	16%
Casein (from bovine milk)	20	40	160
Hydrogenated Vegetable oil	100	100	100
a-Cellulose	100	100	100
Starch (Maize)	720.5	700.5	580.5
Threonine	1.5	1.5	1.5
Methionine	1.5	1.5	1.5
Choline chloride	1.5	1.5	1.5
Mineral mix[a]	45	45	45
Vitamin mix[b]	10	10	10
Vit. B12 soln.	.2ml	.2ml	.2ml

Mineral Mix[a]

Ingredients	*g/100g*
CaCO3, precipitated heavy	18.4
CaHPO4	9.4
Na2HPO4 anhydrous	16.9
KC1	13.0
MgSo4.7H2O	12.2
MnSO4.4H2O	.8
CuSO4 anhydrous	.03
ZnCO3 basic	.13
C6H507Fe.5H2o (ferric citrate)	.74
KI03 (potassium iodate)	.005
KHCO3	7.8

Vitamin Mix[b]

Ingredients	*g/100g*
Thiamine Hydrochloride	.1
Riboflavin	.15
Pyridoxine Hydrochloride	.15
Nicotinic acid	.5
a-Ca-pantothenate	.5
Folic acid	.1
Biotin	.01
Menadione	.02
Butylated Hydroxytoluene	1.0
Vitamin E	.0033 or 20IU
Vitamin A	.1 or 5000IU
Vitamin D3	.02 or 1000IU

Experimental diets were provided to mice in powder form in conventional mouse food hoppers, to which a three sided stainless steel casing, designed to cover the sides and bottom of the hopper, was attached to prevent excess spillage of food (fig. 2.1). A sloping perspex plane inserted into the hopper ensured that the powdered diet always fell to the uncased front end where the mice could gain ready access to the diets. Two such adapted hoppers were placed per cage for the duration of each experiment. Stale diet was removed and fresh food was added to each hopper daily. Mice were allowed *ad libitum* access to food and water at all times.

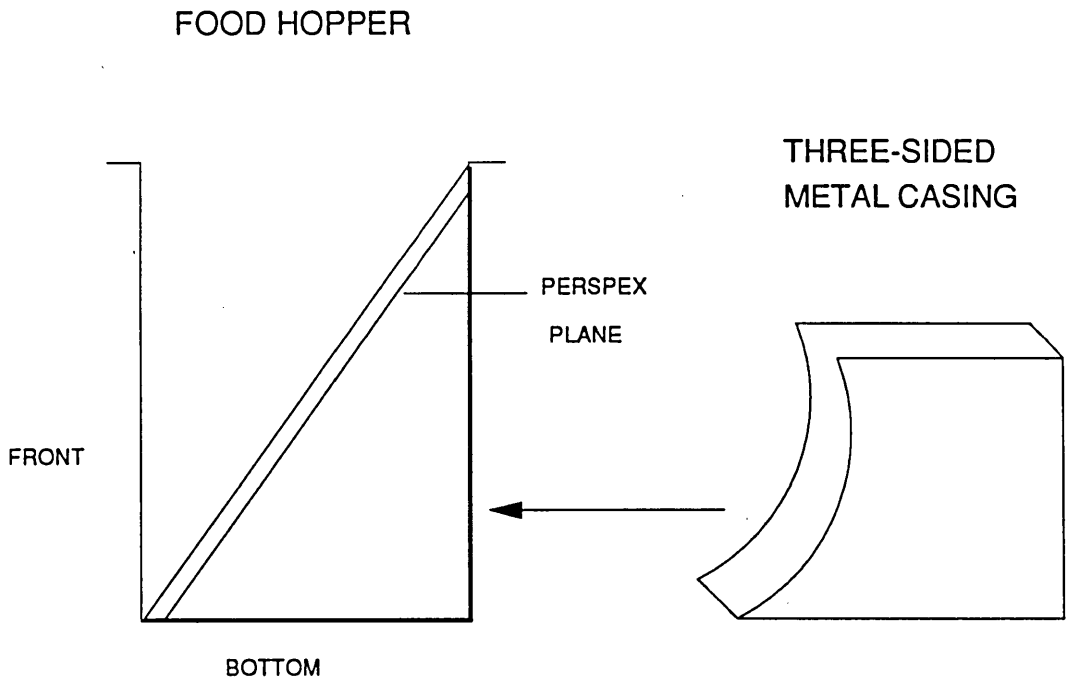

Figure2.1 *Feeding apparatus used in the nutrition experiments.*

The present synthetic mouse diets has been extensively tested for their general effects on the growth and nutritional status of rats (Crompton *et al.*, 1981) and mice (Slater, 1987). The latter work on 4 or 6 wk old CD1 mice has shown that the diet containing 2% (by mass) casein was consistently able to induce obvious mouse protein malnutrition relative to animals fed diets containing 4%, 8% and 16% (by mass) casein (see Slater, 1987). Preliminary growth trials on 4wk old male CBA/Ca mice in the present study confirmed the above earlier observations (fig. 2.2). While mice fed the 2% casein diet had almost no weight increase over a 5-week trial

period, those fed 4% or 16% casein exhibited near normal weight gain over the same period. No significant differences in weight gain were observed between mice fed 4%, 16% and the standard Oxoid 4B pellets, although the mice fed the 16% diet grew slightly faster than those fed the 4% casein diet (fig. 2.2).

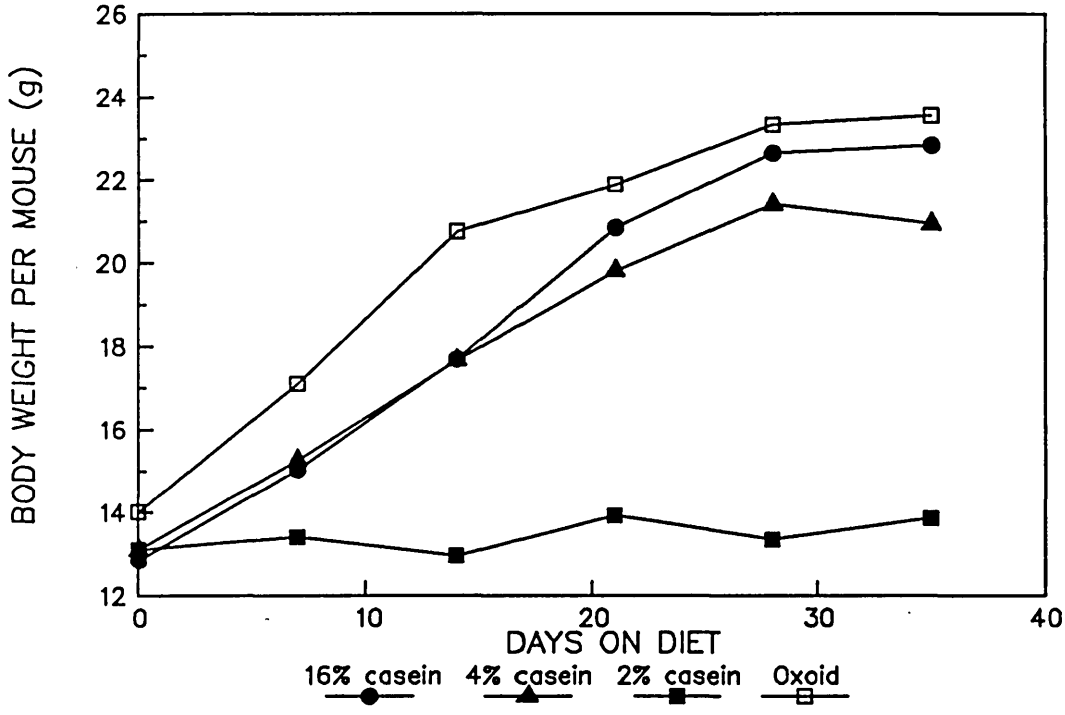

Figure2.2 *The effect of various diets on the growth rate of male CBA/Ca mice.*

On the basis of these findings, it was decided to model normal, moderate and severe protein malnutriture in CBA/Ca mice by feeding mice with 16%, 4% and 2% casein diets respectively.

2.3.2 Mouse food consumption

The daily food consumption of each mouse (chapters 3 & 4) was measured by weighing all the food placed in the hoppers at the beginning of each 24h period, and weighing uneaten pellets as well as fragments in the collection tray at the end of the 24h period.

2.4 IMMUNOLOGICAL METHODS

2.4.1 Collection of mouse sera for serological analyses

Blood for serological analyses was collected from mouse tail veins following standard prodecures (Johnston & Thorpe, 1987). Tail bleeds were facilitated by first warming the mouse for 5 to 10 minutes in a jar onto which a lamp with a 60 to 100 watt light bulb was directed. The mouse was then restrained in a narrow plastic chamber, and the exposed tail vein incised with a razor blade. The venous blood was collected in a 1.5ml Eppendorf tube, allowed to clot at room temperature for 1h and then left overnight at 4^0C to facilitate maximum clot shrinkage. Following this, it was centrifuged at 2000 rpm for 10 minutes at 4^0C, the serum pipetted off, and placed into a clean, labelled 1.5ml Eppendorf tube. The labelled mouse serum was then stored at -20^0C until required.

2.4.2 *T. muris* Excretory/secretory (E/S) antigen preparation

The methods used to collect *T. muris* E/S antigen were adapted from those of Else & Wakelin, 1989). Adult male and female *T. muris* were removed individually from the large intestines of mice as previously described (section 2.2.4), and placed in sterile RPMI 1640 medium containing 2nm glutamine (GIBCO). The culture medium was further supplemented with 500 ug/ml of penicillin/streptomycin (Penstrep) and 1% glucose. After washing for 2 - 3h in this medium at 37^0C, worms were put into fresh medium in 1.5ml microtitre wells at 20 worms/ml/well and left overnight at 37^0C for collection of E/S products. The culture supernatant was removed, centrifuged at 200g for 5 minutes to remove eggs and then vacuum-dialysed overnight at 4^0C against two-three changes of PBS at pH 7.2. Following dialysis, the antigen solution was analysed for protein concentration by a Bradford protein assay (Biorad, U.K) using Bovine serum albumin (BSA) as the standard, aliquoted into 0.1ml portions, and then stored at -70^0C until used.

2.4.3 Serological analysis by ELISA

An indirect Enzyme Linked Immunosorbent Assay (ELISA) was used to quantify the amount of antibodies present in mouse serum samples against *T. muris* E/S antigen. Assays were developed to estimate the levels of parasite (E/S products)-specific total IgGAM, IgA, IgM and subclass IgG1 immunoglobulins present in mouse sera. The protocol used was an adaptation of that given by Lillywhite *et al.*, (LSHTM int. pub.).

Table2.2 *ELISA buffers.*

1. PBS	85g NaCl 1.28g Na_2HPO_4 0.156g $NaHPO_4.2H_2O$ 1*l* dist. H_2O
2. Incubation buffer	100ml PBS 0.05ml Tween 20 (Sigma Ltd, U.K)
3. Coating buffer	1.59g Na_2CO_3 8.9g $NaPHCO_3$ 1*l* dist. H_2O pH 9.6
4. Washing buffer	9g NaCl 0.5ml Tween 20 1*l* dist. H_2O
5. Substrate buffer	7.19g Na_2HPO_4 5.19g Citric acid 1*l* dist. H_2O
6. Stock substrate	100mg orthophenylamine diamine 10ml methanol
7. Substrate solution	100 ml substrate buffer 1ml stock substrate 10*ul* 30 H_2O

Briefly, 100ul of *T. muris* E/S antigen, at a concentration of 10 ug/ml made up in coating buffer (Table 2.2) was placed in each well, except those in the first column, of a 96-well microtitre plate (Linbro, Flow Ltd, U.K). In the first column, only coating buffer was added and this served as control to assess non-specific binding. The plate and contents were then incubated overnight at room temperature in plastic sandwich boxes to enable binding of antigen to well surface. Following overnight incubation, unbound antigen was removed by washing three times with washing buffer (Table 2.2). Unoccupied binding sites on the plastic surface were then blocked by adding 100ul of 2% BSA (BSA, Sigma) to each well and incubating the plate for 1h at room temperature. After three washes in washing buffer to remove any unattached BSA, 100ul of test, and reference positive and negative sera (pooled from separately infected and naive male CBA/Ca mice respectively) at a dilution of 1/100 in incubation buffer (Table 2.2) were added to the appropriate wells, and the plate incubated for 2hs at room temperature. The plates were then

washed thrice to remove the excess sera. The bound mouse immunoglobulins were then probed with the addition 100ul of either Horse radish peroxidase labelled goat anti-mouse IgGAM (Dako), goat anti-mouse IgA (a chain specific) (Sigma Chem Co. Ltd) or goat anti-mouse IgM (u chain specific) (Sigma), all diluted 1/1000 in incubation buffer, to each well. The plate was then incubated overnight at 4^0C. In the case of IgG1 estimation, direct labelling of primary antibody was not possible due to non-availability of the corresponding enzyme-labelled reagent. Thus, 100ul of unlabelled goat anti-mouse IgG1 (Tago), at a 1/1000 dilution in incubation buffer, was initially added to each well, and the plate incubated for 3 hs at room temperature. The bound unlabelled antisera was then labelled by overnight incubation at 4^0C with 100 ul per well of a 1 in 1000 dilution of peroxidase labelled rabbit anti-sheep immunoglobulins (Dako). The plates were washed extensively between the latter two incubation steps. Following the final incubation, plates were washed again and 100ul of substrate buffer (Table 2.2) containing o-phenylenediamine/H_2O_2 (substrate solution (Table 2.2)) was added to each well. The reactions were stopped by adding 25ul of 2.5M H_2SO_4 per well and read spectrophotometrically at 490nm on an automated ELISA reader (Flow Ltd., U.K), the machine being blanked on the first column.

2.4.4 Determination of optimal antigen and serum dilutions for the assay of mouse immunoglobulin levels in sera by ELISA

The optimal concentrations of *T. muris* E/S antigen and mouse sera required for the ELISA detailed above were determined by checkerboard titration as described by Lillywhite *et al.* (LSHTM int. pub.). The antigen was double diluted in coating buffer down the rows of the micrititre plate from an initial concentration of 20 ug/ml through to 0.195 ug/ml, and the plate then incubated overnight at 4^0C. After washing and then blocking with 2% BSA, positive sera (pooled from 10 male CBA/Ca mice infected singly with 100-200 *T. muris* eggs for 3-5 weeks) was doubly diluted across half the plate (columns 2-6) from an initial dilution of 1/50 through to 1/800. Negative sera (pooled from 5 naive male CBA/Ca mice) was similarly diluted across the other half from columns 8-12. Columns 1 and 7 were unused and served as controls for non-specific binding. The ELISA protocol outlined in section 2.4.3 was then followed for secondary antibody addition (at 1/1000 dilution), development and reading of the enzymatic reactions.

From the results obtained, the concentration at which antigen was no longer in excess was found to be 10 ug/ml and the serum dilution at which positive and negative sera could be distinguished was 1/100. Thus these dilutions were used in all assays to determine antibody levels in mouse sera.

2.4.5 Storage of mouse serum samples for repeated serological assays

Prior to the serological assays carried out in this study, the relevant mouse sera originally stored at -20^{0}C were retrieved, diluted 1/25 in 50% glycerol/PBS and restored at -20^{0}C until required. This treatment is thought to prevent the freezing of the immunoglobulins present in the serum at the storage temperatures chosen, thus minimizing damage caused by the freeze-thaw cycles that would be otherwise required for repeated serological analyses (Kemeny & Chantler, 1988). The glycerol diluent has been found to have little effect on the reactivity of human sera in the ELISA system even after prolonged storage (8 months) (M.J. Cox, pers comm).

2.4.6 Immunoblot analysis

T. muris E/S antigen recognition by individual mouse serum was qualitatively analysed by the Western blot technique (Towbin *et al.*, 1979). The technique entailed the following steps. Components of the E/S antigenic mixture were first separated by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)(Laemmli, 1970). The antigen components, thus separated and maintaining their spatial relationships, were then transferred electrophoretically onto sheets of nitrocellulose. Finally, immunorecognition was visualized by treating the NC blot with the appropriate antibody solution, and the presence of bound antibodies detected by an enzymatic antibody probe.

(i) Preparation of SDS-PAGE gels

Reagents used are given in Table 2.3. The gel mould was first assembled by clamping together two glass plates separated by the perspex spacers on either sides. Before use the glass plates, 0.7 mm spacers and combs were cleaned with detergent, distilled water and alcohol. Plates were then sealed at the sides and the bottom by adding a 10% separating gel solution along the sides. 100-200ul TEMED (*N,N,N',N'*-tetramethylethene diamine) was added to the solution immediately before pouring.

Gradient gels were poured into the top of the gel mould thus assembled y loading 8 mls each of 8 and 20% separating gels prepared as above (with 250ul TEMED per gel mix) into separate chambers of a linear gradient maker (Pharmacia) and pumping at 10 ml/min using a Gilson Minipuls. The ammonium persulphate was

Table2.3 *Reagents for the preparation of SDS-PAGE gels.*

1. Stock solutions:

a. 50% Acrylamide: 48.75% (w/v) acrylamide grade 1
1.25 % (w/v) bis-acrylamide

b. Ammonium persulphate: 1.5% (w/v) (10.5mg/ml) solution in water prepared fresh as required.

c. Tris-HCl: 1M Trizma-base (Tris, Sigma)(121.14g in 1litre of water) adjust pH with conc. HCl.

2. Gel mix:

a. Separating gel- volume (ml) to make 40ml

		Final polyacrylamide concentration		
		8%	10%	20%
Acrylamide stock (50% w/v total)		6.4	8.0	16.0
1M Tris-base (tris)- HCl pH 8.8		15.0	15.0	15.0
Water		17.9	15.7	7.7
			degas and add	
10% w/v SDS	0.4	0.4	0.4	
1.5% w/v ammonium persulphate		0.9	0.4	0.9

b. Stacking gel- volume (ml) to make 10ml

	Final polyacrylamide concentration	
		3%
Acrylamide stock (50% w/v total)		0.6
1M Tris-HCl pH 6.8		1.25
water		7.55
10% w/v SDS	0.1	
1.5% w/v ammonium persulphate		0.5

added to the respective solutions in the chambers just before the commencement of pumping. The gradient was poured into the mould leaving at least 3 cm space at the top for the stacking gel. Gels were overlaid with water to ensure level surface and left to polymerize. The overlay was removed from the gels; and the 3% stack solution (to which 10-20ul TEMED was added) was then poured upto the top of the gel mould. A wide-slotted perspex comb was pushed slowly into the solution and the gel left to polymerize (10-20 min).

Table2.4 *SDS-PAGE running buffers.*

1. Electrophoresis buffer:	0.38 M Glycine 0.05 M Tris-base 0.1% SDS pH 8.3
2. PAGE sample buffer:	0.125 M Tris pH 7.0 4% SDS 20% Glycerol 6% (w/v) 2-mercaptoethanol

(ii) Running SDS-PAGE gels

SDS-PAGE was performed using a BRL Vertical Gel Electrphoresis system (Cambridge, U.K). The comb was carefully removed, along with the bottom clips and spacers from the polymerized gels. The gel mould was then clamped to the electrophoresis tank containing the electrode buffer using bulldog clips. E/S antigen was diluted in sample buffer, mixed and boiled at 100^0C for 4-5 minutes. Approximately 2.5ug per track of protein was then applied to each gel, and the samples electrophoresed at 4-5 mA overnight. Molecular weight standard proteins (Sigma Chem Co. Ltd) were loaded at the edges of the gels for each run. Bromophenol blue was added to the electrode buffer to act as dye marker.

(iii) Immunoblotting from polyacrylamide gels

The electrophoretic transfer of the resolved antigens from PAGE slabs to nitrocellulose paper was achieved by semi-dry blotting using a LKB Multiphor II Novablot electrophoretic transfer kit. Immunorecognition of transfer antigens was then visualized by using an enzyme labelled second antibody.

The buffers and reagents used for Western blotting are given in Table 2.5. Six pieces of Whatman 3MM filter paper cut to the size of the gel were first soaked in anode soln 1 and placed on the lower (+) graphite electrode. This was followed by a further three pieces of 3MM paper soaked in anode soln 2. The Nitrocellulose (NC) paper (Schleicher and Schull (BA 85), wet in distilled water, was then added to the stack. The PAGE gel, run as above, was recovered and placed on top of the NC. Care was taken to eliminate air bubbles. Another three pieces of wet filter paper soaked

Table2.5 *Reagents and buffers for immunoblotting*

1. Anode solution 1:	36.3g Tris-base 200ml Methanol Make up to 1 litre with distilled water.
2. Anode solution 2:	3.63g Tris-base 200ml Methanol Make up to 1 litre with distilled water.
3. Cathode solution :	1.05g Aminocuproic acid 0.605g Tris-base 40ml Methanol Make up to 200ml with distilled water
4. Amido black stain:	1% (w/v) Amido black 45% (v/v) Methanol 10% (v/v) Acetic acid
5. TBS:	8.75g/l NaCl 2.42g/l Tris-base 0.1% Tween 20

in cathode soln 1 were placed on top and the apparatus assembled. Transfer was carried out for 2 hs at 194 mA.

After transfer, the NC paper was marked for gel outline and molecular weight lanes. Transfer and molecular weight migration were then checked by staining the marked strip of NC, containing the molecular weight lanes, with amido black for 5 mins and destaining in water as required. The rest of the NC membrane was blocked at 4^0 C overnight in 5% FCS/TBS, dried between absorbent paper, and stored in air-tight plastic bags at 4^0C until used.

The NC blots were washed in TBS before immunorecognition was determined by cutting the blot into approximately 3mm strips, and incubating each strip with 1 ml of individual mouse serum, at 1/200 dilution in 5% FCS/TBS, for 2hs at room temperature on a rocking platform (Orbital shaker, Luckham). Blots were then washed for 1 hour with three changes of TBS followed by 3 hs incubation with 1ml of a 1/500 dilution of goat anti-mouse IgG1 (Tago). The strips were washed as above,

and then incubated with 1ml of a 1/1000 dilution of the peroxidase labelled rabbit anti-sheep immunoglobulins (Dako) overnight at 4^0C. Strips were washed again, rinsed for 5 mins in PBS, and placed in PBS containing 50mg per 100ml of DAB chromogen (3,3' -diaminobenzidine tetrahydrochloride (DAB, Sigma) and 10ul of 30% hydrogen peroxide substrate. Incubation was continued until the reaction had proceeded sufficiently. Blots were then rinsed thoroughly in deionized water and dried between absorbent paper.

CHAPTER 3 : POPULATION BIOLOGY OF *T. MURIS* PRIMARY INFECTIONS IN NORMAL AND IMMUNOSUPPRESSED CBA/Ca MICE

3.1 INTRODUCTION

The primary aim of this thesis is to determine the effect of protein deficiency on the population biology of intestinal helminth infections, using the *T. muris* - CBA/Ca mouse model. However, in order to facilitate the interpretation of the results of the nutrition experiments, it was necessary to first elucidate the population characteristics of primary infections of *T. muris* in normally fed (with standard laboratory mouse pellets) immunologically intact and susceptible (drug induced) CBA/Ca mice.

As discussed in Chapter 1, trichuriasis in mice is an acute nematode infection, characterized by spontaneous cure and rapid expulsion responses in primary and repeated infections respectively (Behnke, 1987). However, mice may be made tolerant to *T. muris* by treatment with immunosuppressive drugs, such as cortisone acetate (Lee & Wakelin, 1983). Chronic infection resulting from such treatment has been considered by the latter authors to approximate the situation in human trichuriasis, in which infections are typically chronic and there is little evidence to date for the occurrence of effective protective immunity (Bundy & Cooper, 1989). However, despite this close homology between *T. muris* immunotolerance models and human trichuriasis, and the value of experimental models in parasite epidemiology (Keymer, 1985; Anderson & Crombie, 1985; Anderson, 1986), few attempts have been made to utilize *T. muris* infections in tolerant mice in order to examine the population biology of chronic trichuroid infections (Pike, 1969). The use of such immunotolerance models may, in addition, also yield useful information concerning the roles of intraspecific parasite competition and host immune response as density dependent regulatory factors controlling parasite population growth in chronic helminth infections (see Keymer, 1982).

The aims of the research presented in this chapter were therefore twofold. The first was to provide baseline information concerning the population biology of primary infections of *T. muris* in order to aid in the interpretation of the results of the subsequent nutrition experiments. The second aim of the study was to identify potential regulatory factors, particularly those related to parasite density, which may govern parasite population numbers in both acute and chronic primary infections of *T. muris*.

3.2 EXPERIMENTAL DESIGN

Mice used in the experiments reported in this chapter were housed 5-6 to a cage on wood shavings and were all fed water and a standard laboratory mouse diet (Oxoid 4B) *ad libitum*.

Experiment 1: Effect of infection dose on *T. muris* establishment in normal male CBA/Ca mice.

In this experiment, six week-old male CBA/Ca mice were randomly assigned to 6 infection groups of 5-6 mice each and given either 5, 10, 20, 50, 100 or 200 *T. muris* infective eggs respectively. Worm establishment rate at each infection dose was assessed by killing all mice on day 44 p.i., and counting the number of worms present in the large intestine of each mouse.

Experiment 2: Population biology of chronic primary infections of *T. muris* in CBA/Ca mice.

The experimental protocol followed in this study is summarized in Table 3.1. Individual six week-old male CBA/Ca mice were given between 10 and 1000 infective *T. muris* eggs to form 6 groups (n 5) of mice, each group having a different intensity of infection (Table 3.2). On days 7, 9, 11, 13, and 15 p.i., all mice were administered cortisone acetate injections in order to induce immunotolerance to *T. muris*. To examine *T. muris* fecundity relationships, each mouse was placed in a separate faecal collection cage and 24 hr faecal specimens for egg counts were collected daily from individual mice from day 36 to day 42 p.i. (6 consecutive days). The collections made on day 36 were not used for analysis, to allow for a period of acclimatization by the mice to the experimental procedure. Each 24 hr faecal specimen was weighed at the time of collection and egg numbers in the faecal

samples were determined using the method outlined in section 2.2.5. It has been recently suggested that there may be a causal relationship between temporal helminth egg production and host faecal output, which may be related to parasite-induced changes in host food intake (Keymer & Hiorns, 1986b). This possibility was evaluated by simultaneously monitoring mouse food consumption/24 hr over each of the five days on which faecal collections were made. On day 44 p.i. all mice were killed and individual mouse worm burdens were determined by examination of the caecum and colon. The worms were counted, sexed and stored in 10% formal saline before the wet weights of the females were measured.

Table3.1 *Experimental protocol.*

Day post infection	Experimental procedure
0	Infection by gavage
9-15	5 * cortisone acetate injection (1.25 mg)
36	Acclimatization
37-42	5 * 24h faecal collection 5 * 24h food intake
44	Assessment of worm burden

3.3 RESULTS

3.3.1 Experiment 1

The results of experiment 1 are shown in Fig. 3.1, in which the mean number of *T. muris* recovered per mouse group, expressed as a percentage of the infection dose, is plotted against the amount of infective eggs given. The results confirm previous studies (Wakelin, 1973; Behnke & Wakelin, 1973) and clearly support the suggestion that there exists a threshold infection level in normal murine trichuriasis above which *T. muris* is expelled from the mouse host (Wakelin, 1973). For CBA/Ca mice, it would appear that this infection level is approximately between 10 and 20 eggs. While a large percentage of infective eggs matured into

adult worms at the small infection doses of 5 and 10 eggs (68% and 30% respectively), mean worm recoveries above infection levels of 20 eggs were consistently insignificant (Fig. 3.1). Furthermore, the remaining worms were also small and stunted at the latter infection levels; considered to be the characteristic situation which occurs when self-cure in *T. muris* infections is induced by much larger infections (Behnke & Wakelin, 1973).

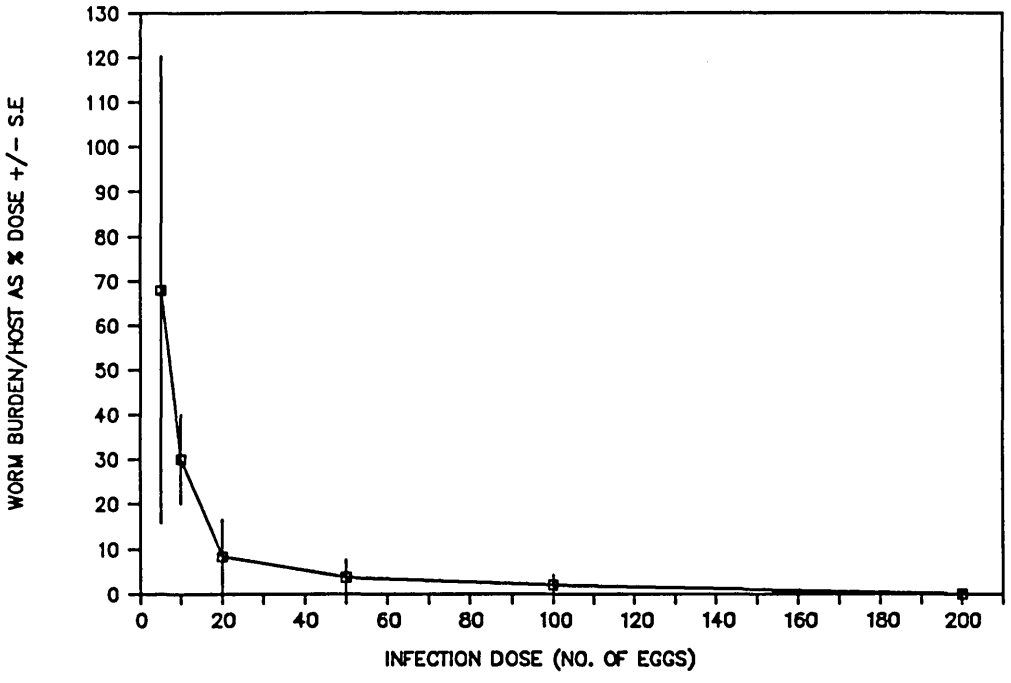

Figure3.1 *The relationship between infection dose and percentage worm recovery in normal CBA/Ca mice. The open squares represent the mean number of worms recovered for each infection group. The vertical bars denote the standard error of the means.*

3.3.2 Experiment 2

(i) Relationship between worm burden and infection dose

The number of worms recovered from the large intestines of the cortisone treated CBA/Ca mice on day 44 p.i. in Experiment 2 is shown in Table 3.2. It is evident that corticosteroid treatment facilitated the establishment of large chronic *T. muris* infections in the normally responsive CBA/Ca (Figs. 3.1 & 3.2). However, worm establishment in tolerant mice was not directly proportional to the infection dose administered. Regression of the logarithmic relationship between worm recovery

Table3.2 *Summary of the numbers of worms recovered from the large intestines of the 6 groups of mice on day 44 p.i., with their associated mean 24h faecal output, food intake and faecal egg counts on days 37-42.*

Group	No. of mice	Infection dose	Mean no. of worms	Mean g food/24h	Mean g faeces/24h	Mean epg/f* ($x10^{-3}$)
A	10	10	2.7 +/-0.85	3.63+/-0.09	0.66+/-0.02	2.16+/-0.99
B	5	50	13.0+/-1.86	3.77+/-0.21	0.77+/-0.05	5.88+/-0.75
C	5	100	32.2+/-3.64	2.99+/-0.35	0.57+/-0.09	11.16+/-1.61
D	5	200	65.8+/-7.37	4.18+/-0.14	1.01+/-0.06	3.61+/-0.57
E	6	600	72.7+/-8.45	3.61+/-0.20	0.99+/-0.07	3.51+/-0.66
F	6	1000	170.3+/-42.50	4.35+/-0.13	1.09+/-0.03	1.72+/-0.69

@All group figures are the average of the means of five values for each animal in the group +/- s.e.
*Estimated no. of eggs/g faeces/female worm.

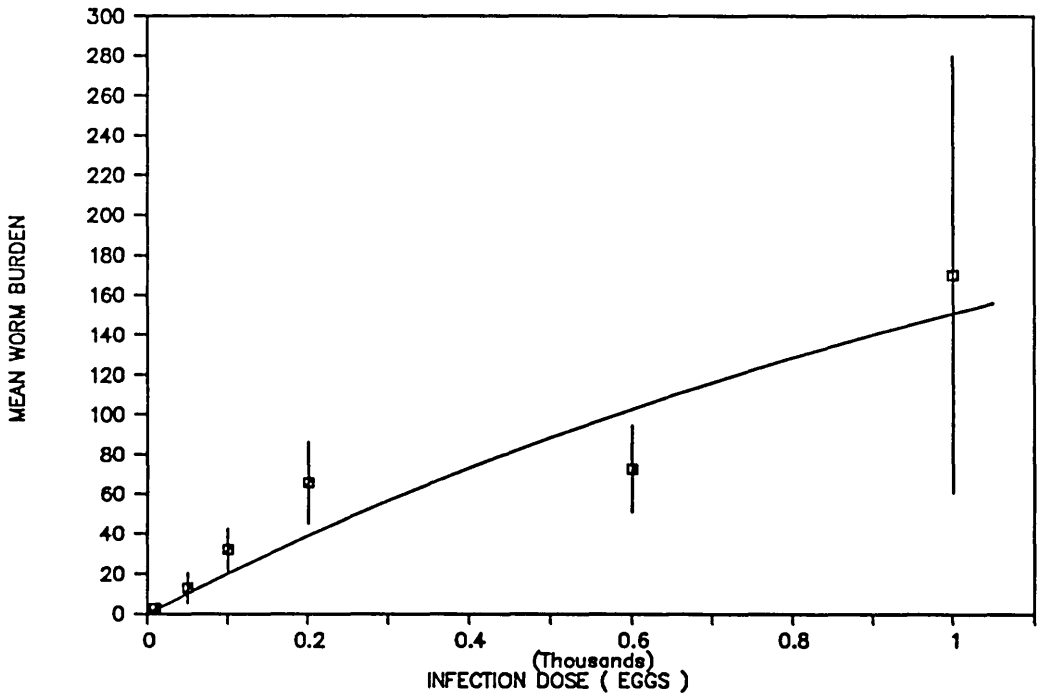

Figure3.2 *The relationship between the number of worms established in immunosuppressed hosts and the infection dose administeredc. Open squares, mean number of worms established; the bars denote the 95% confidence limits of the means. The curve represents the best-fit empirical saturation model described in the text (Eqn (1)) with parameter values K = 300 and d = 0.0007.*

and infection dose resulted in a slope that was found to differ significantly from 1 (b = 0.85, s.e. = 0.06, t = 2.5, 35 d.f., P< .02), suggesting the probable presence of a threshold effect on *T. muris* establishment in these mice with increasing exposure rates. These results are better described by an empirical saturation model of the form :

$$P(L) = K(1-\exp(-dL)) \qquad (3.1)$$

where P , is the mean number of parasites that establish in the host at each infection dose,L; K is the maximum worm burden, which in this study was found to be approximately 300 for *T. muris* in tolerant CBA/Ca mice; and d, is a shape parameter (unit = 1/burden) which is related to the dose at half-maximum burden (say L=q, and P=K/2) by the simple relation d = Ln(K/2)/q (Fig. 3.2).

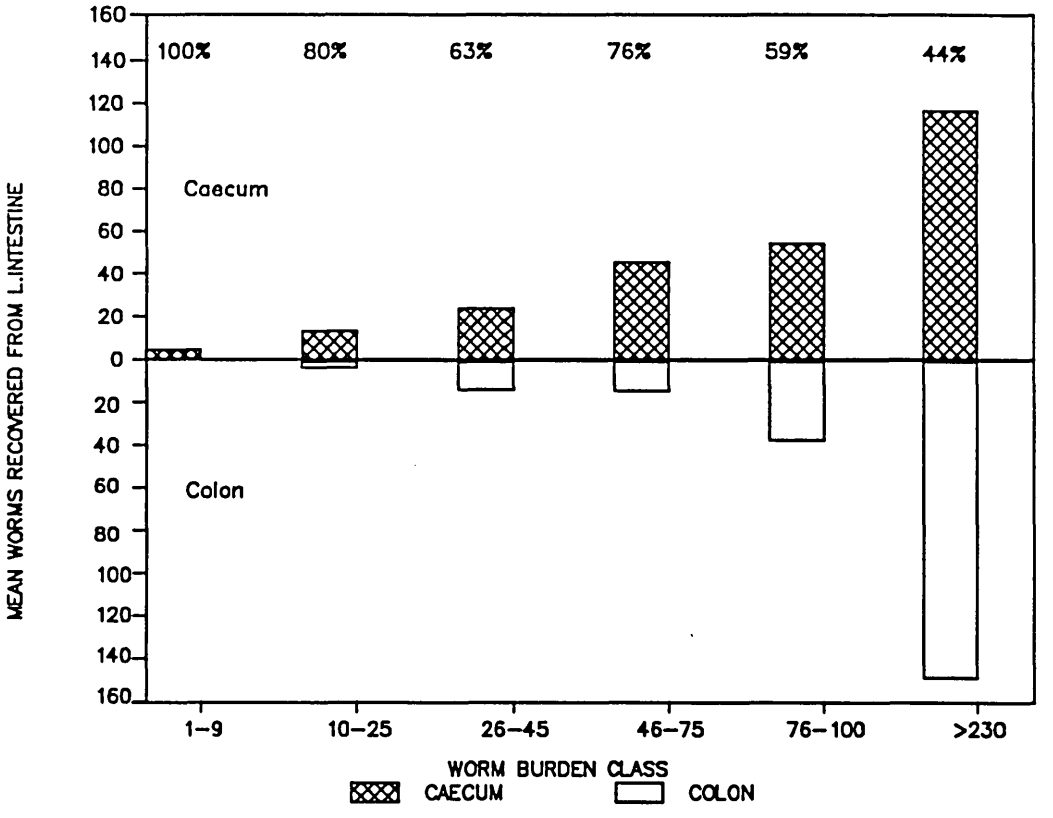

Figure3.3 *The distribution of T. muris in the murine large intestine as a function of worm density. Closed bars, mean number of worms found in the caecum; open bars, mean number of worms obtained in the colon for each of the 6 worm class described at the bottom of the graph. The figures at the top indicate the percentage of worms recovered from the caecum for each worm burden class.*

(ii) Distribution of adult T. muris in the large intestine

Fig. 3.3 illustrates the spatial location of *T. muris* recovered from the large intestine of chronically infected CBA/Ca mice in relation to worm density. The data clearly suggest the existence of a parasite density effect on the distribution of worms in the large intestine : at increasing intensities of infection, an increasing proportion of the worms colonises the less crowded colon as well as the more normal location in the caecum ($t = 7.36$, 32 d.f., $p < .001$).

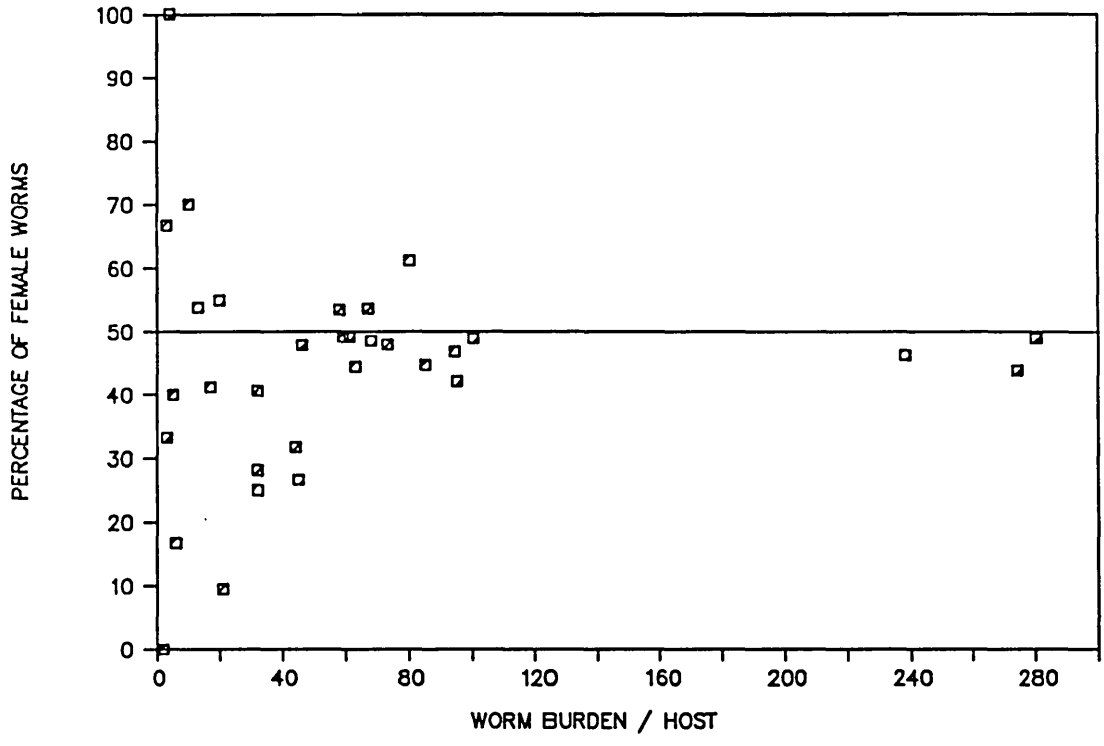

Figure3.4 *The sex ratio of worms recovered from each of 37 mice, and the density-independent relationship between sex ratio and worm burden.*

(iii) Sex ratio of T. muris

The mean female : male ratio of adult *T. muris* in chronically infected mice was found to be 0.95 : 1.00. No significant bias was detected in the sex ratio (t = 0.14, 33 d.f., P> .05). Regression analysis of the data also indicates that the sex ratio of *T. muris* is independent of worm burden (t = -.055, 32 d.f., P> .05) (Fig. 3.4). Sex ratio is therefore unlikely to have any significant influence on the relationship between worm burden and fecundity.

(iv) Day to day variability in faecal egg counts (Days 1 - 5)

Variability in replicate daily measurements is a striking feature of helminth faecal egg counts (Hall, 1981; Croll et al., 1982; Sinniah, 1982; Anderson & Schad, 1985; Keymer & Hiorns, 1986b; Bundy, Cooper, Thompson, Anderson & Didier, 1987). It was decided, therefore, in the present study, to examine this feature of helminth faecal egg counts, under the controlled experimental conditions of the laboratory. Fig. 3.5 shows that the relationship between the variance and the mean on a $\log_{10}$ scale for both epg and epd is linear, with slope b of the linear models being 1.90 and

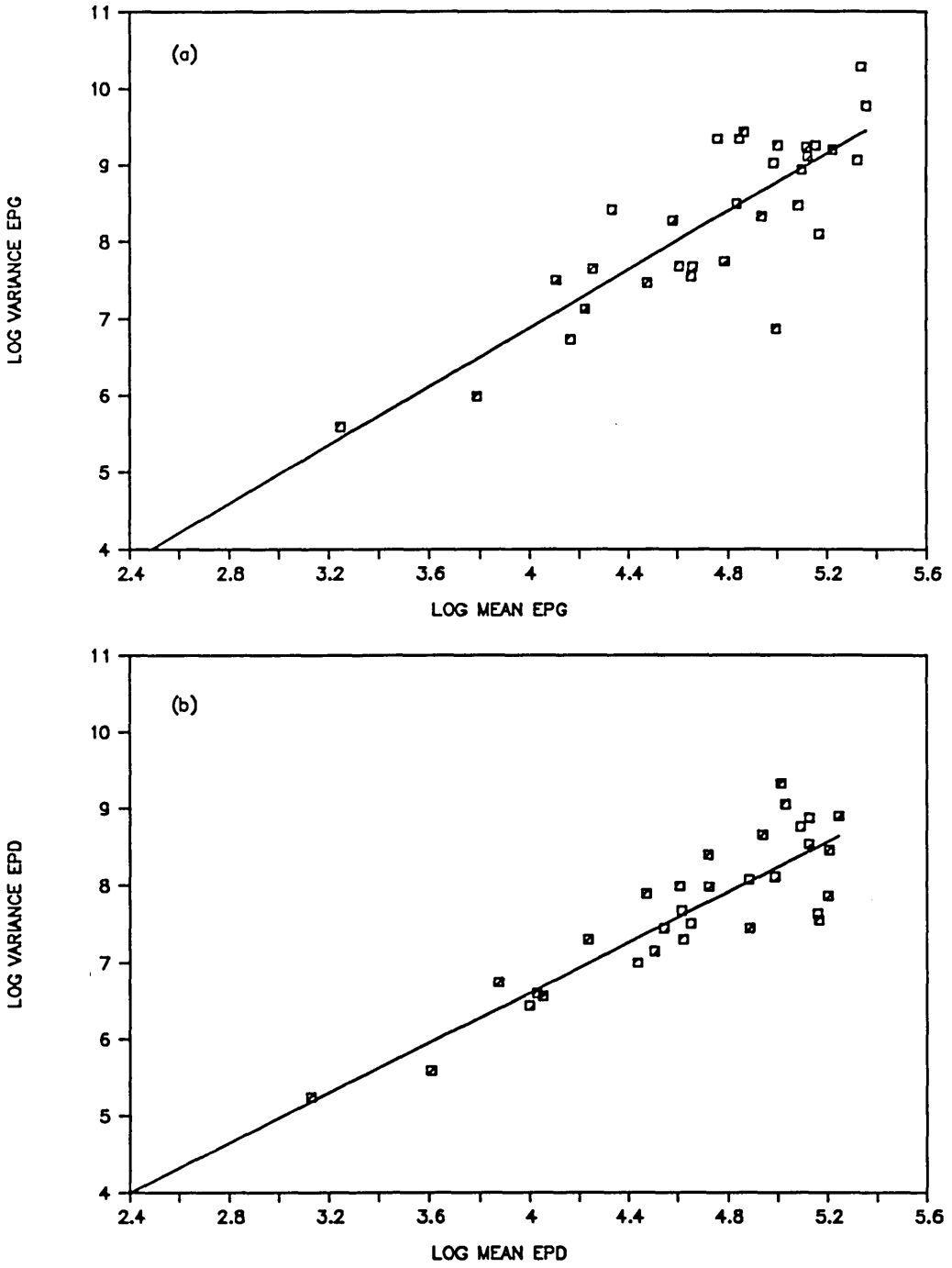

Figure3.5 *Day to day variability in T. muris egg output from individual mice. The relationship between the logarithms of the variance (V) and mean (M) epg (a) and epd (b) of 5 repeated counts form each of 37 mice. Open squares, observed values; line denotes the best-fit linear model of the form* $\log_{10}(V) = a + b \log_{10}(M)$ *where a= -0.72 and b= 1.90, and a= 0.09 and b= 1.63, for epg and epd respectively. Values of b close to 2.0 reflect a high degree of variability in the raw counts, wheras if the day to day counts were randomly distributed, the slope of the linear model would be approximately unity in value (Elliot, 1977).*

1.63 respectively. The results indicate that while both the epg and epd counts exhibit high day to day variability, epd counts (r^2 = 0.77) are marginally less variable than epg (r^2 = 0.69).

(v) Relationship between T. muris faecal egg counts and worm burden

The relationship between the mean eggs/g faeces contributed by each female *T. muris* (per capita epg) and total worm density in chronic primary infections is shown in Fig. 3.6a. The power model of the general form :

$$y = ax^b \qquad (3.2)$$

gave the best empirical fit to the observed data. Per capita epg was inversely related to worm burden (t = -3.64, 29 d.f., P< .002). The parameter *z* (Croll et al., 1982; Anderson & May, 1982; Bundy, Thompson, Cooper, Golden & Anderson, 1985), which varies inversely with the severity of the density dependent constraints on parasite fecundity ($z = \exp[-b]$, where *b* is the exponent of the relationship between parasite fecundity and worm burden), for the present chronic population of *T. muris* was found to have a value of 0.992. It is significant that this value of *z* is almost identical to that observed for *T. trichiura* and for other gastrointestinal helminths of humans (Bundy et al., 1985), suggesting that worm fecundity may indeed be a major regulatory force stabilising the growth of chronic parasite populations (Anderson, 1982a; Anderson & May, 1985).

iii) Relationships between host faecal output, worm density and faecal egg output

The relationship between mean host faecal output/24h and *T. muris* density in chronic murine trichuriasis is displayed in Fig. 3.7. The results indicate that mean faecal output/24h/host exhibited a slight, but statistically significant increase with worm density (F = 8.11, 1,29 d.f., P< .01). In order to evaluate a possible mechanism behind changes in faecal output in infected hosts, mouse food consumption/24h was also measured simultaneously over each of the 5 days on which faecal collections were made. Host food intake/24h , however, did not change significantly with worm density (F = 3.53, 1,29 d.f., P> .05). Thus, the data in the present system do not appear to support the suggestion that variations in host faecal output may be mediated by parasite-induced alterations in host food intake (Keymer & Hiorns, 1986b).

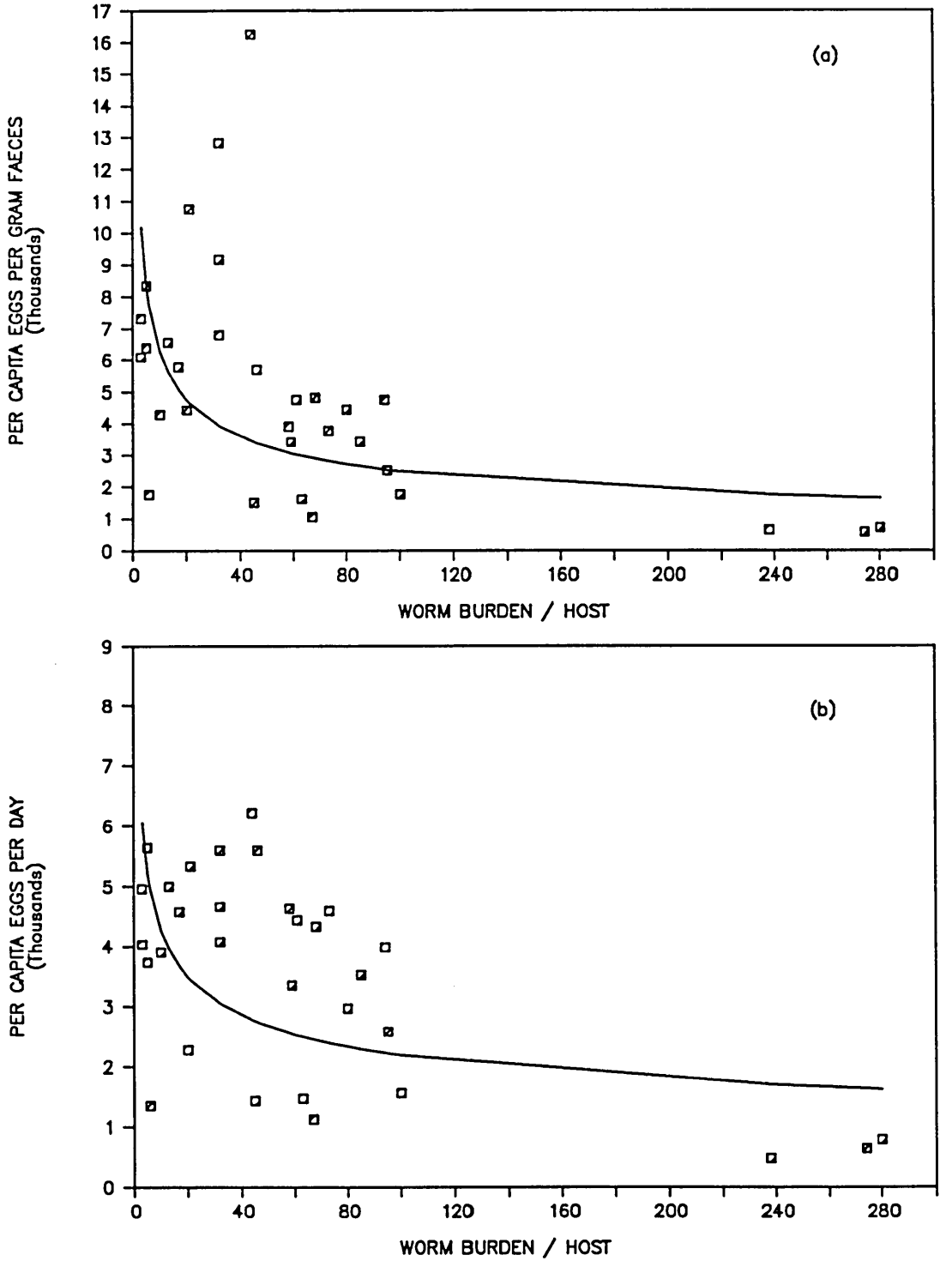

Figure3.6 *Egg output per female worm as a function of total worm burden. (a) Relationship based on epg counts; (b) based on epd counts. Open squares represent observed values (mean of 5 replicate measurements for each of 31 patent mice; curve gives the best-fit power function (Eqn (2) in the text) with parameter values a= 15,812.5, b= -0.40; and a= 8,317.64, b= -0.29, for epg / female worm and epd / female worm respectively.*

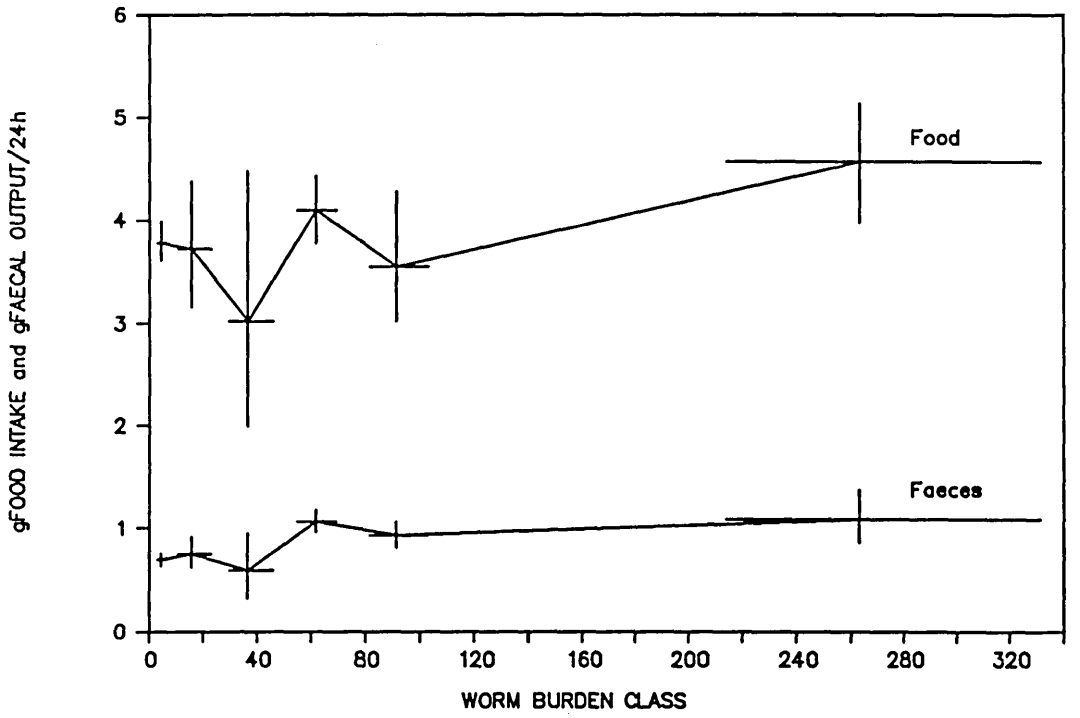

Figure3.7 *The relationship between average host food intake, faecal output per 24h and worm burden. The intersections of the vertical and horizontal bars are observed means, the vertical bars denote the asymmetric 95 confidence limits, the horizontal 95% limits arise as a result of grouping individual observations into each of 6 worm classes. The sample sizes for each worm class from left to right were 5, 5, 5, 8, 5 and 3 respectively.*

The results presented in Figs 3.6a and 3.7 may, however, suggest the possibility that the density dependence apparent in the per capita epg values could simply depict a diluting artefact of increasing host faecal output at higher intensities of worm infection (Hall,1981). This possibility was tested by considering the relationship between per capita daily egg counts (epd) and worm density. If *T. muris* fecundity was independent of worm burden, the per capita epd values, which by definition, are independent of faecal output should counteract the diluting effect of increased host faecal egestion at rising intensities of infection. The relationship between mean per capita epd and worm density is shown in Fig. 3.6b. Once again, the egg counts are found to be a inverse function of worm burden; the power model again provides the best empirical fit to the density dependent relationship ($t = -3.22$, 29 d.f., $P < .005$). Significance testing of the slope, b (=-0.29), with that obtained for per capita epg (b=-0.40) also showed no difference ($t = 0.323$, 58 d.f., $P > .05$), implying that host faecal output had little effect on faecal egg counts in chronic *T. muris* infection.

This conclusion is further supported by the results of an analysis of covariance to assess the variability in egg count and host faecal output from day to day, and from mouse to mouse. The results presented in Table 3.3 clearly demonstrate the independence of faecal egg counts from host faecal output. While the mice exhibited significant variation in faecal output from day to day, the egg counts, although describing daily fluctuations (Fig. 3.5), do not vary concomitantly with host faecal output. However, as would be expected, on the basis of the relationships shown in Figs 3.6 and 3.7 , both parasite egg counts and host faecal output varied significantly between individual mice. These results suggest that the faecal egg counts obtained in the present experiment were a true reflection of *T. muris* egg production, which varied significantly between mice as a density dependent function of their worm burdens.

Table3.3 *Variability in host faeces and egg counts between mice and between days.*

g faeces/24h	Bet. mice	F=8.85 29,119.,d.f. P < .001
	Bet. days	F=7.90 4, 144.,d.f. P < .001
epg	Bet. mice	F=65.12 29,119.,d.f. P < .001
	Bet. days	F=0.75 4, 144.,d.f. P > .05 $^{n.s.}$
epd	Bet. mice	F=134.66 29,119.,d.f. P <.001
	Bet. days	F=0.01 4, 144.,d.f. P> .05 $^{n.s}$

$^{n.s}$= not significant

(vi) Worm size and density dependent fecundity

It has been suggested that worm size may be a better predictor of helminth fecundity than worm numbers *per se* (Ratcliffe & Lejambre, 1971; Coadwell & Ward, 1982). In the context of density dependence in worm fecundity, this hypothesis would therefore predict the existence of a corresponding density dependent relationship for adult worm size.

Fig. 3.8 shows the results of the regression of mean female worm weight upon total worm burden/host recovered from tolerant CBA/Ca mice. The results indicate an highly significant decrease in female *T. muris* weight with increasing worm density ($t = -6.22$, 32 d.f., $P < .001$).

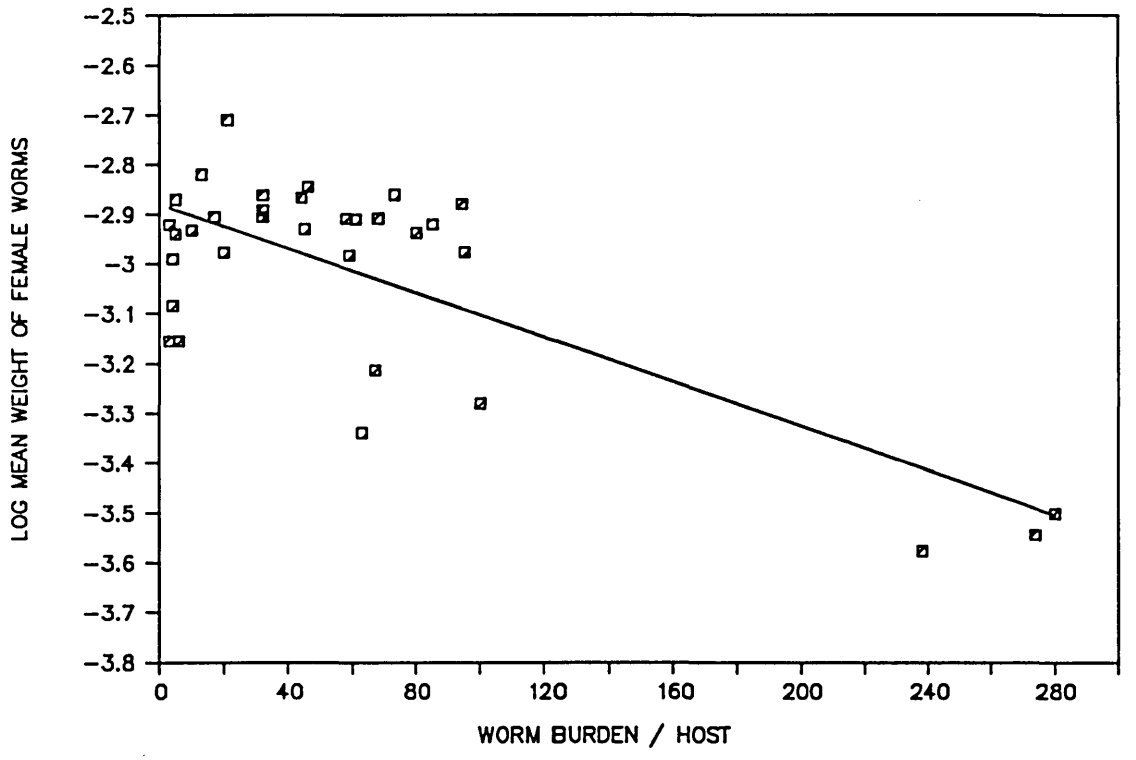

Figure3.8 *The semi-logarithmic relationship between mean worm weight and total burden. Open squares, observed values; line, best-fit linear regression of the log10 worm weight on worm burden with parameter values a= -2.88 and b= -0.002.*

To evaluate the significance of the density dependence in adult size on worm fecundity, the mean female worm weights for each burden were plotted against the respective per capita epg and assessed for relationship. As can be seen from Figure 3.9, the results portray an highly significant positive correlation ($r = 0.86$, 29 d.f., $P < .001$) between mean female worm weight and their mean egg production indicating that density dependence in helminth fecundity may well arise as a consequence of population effects on individual worm growth or size.

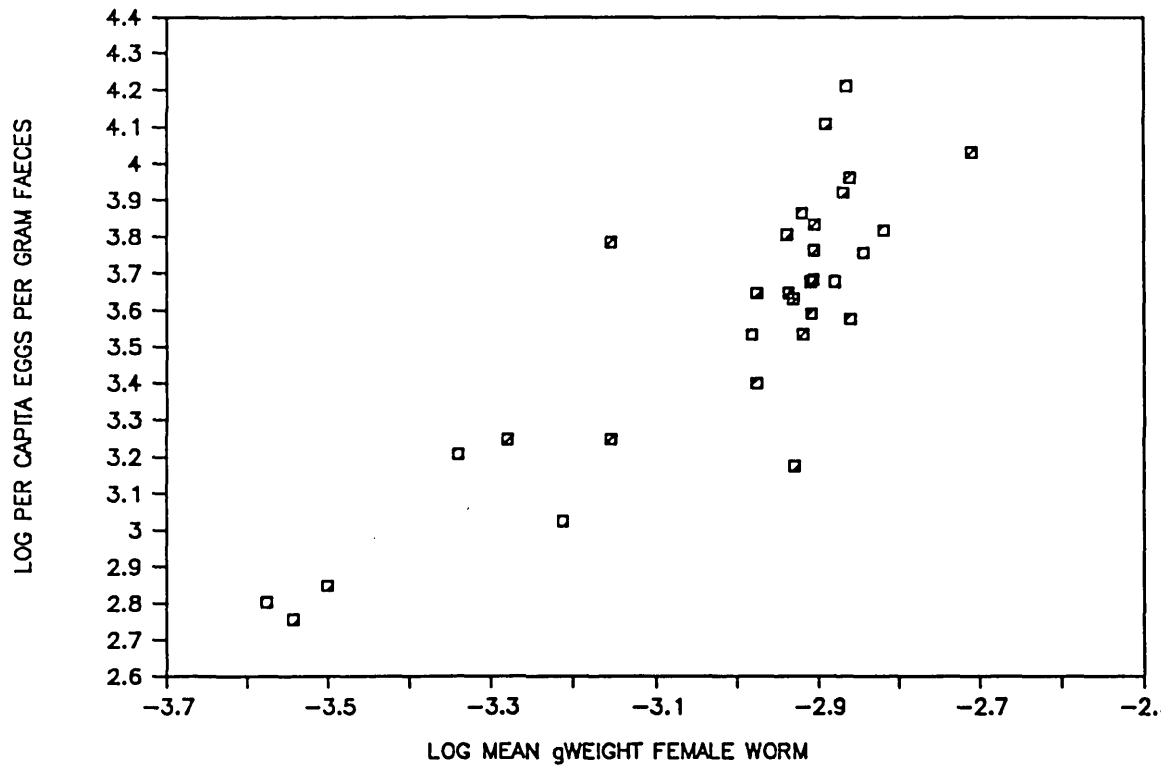

Figure3.9 *The logarithmic relationship between mean female T. muris weight and per capita fecundity. The open squares depict the mean of 5 replicate epg estimates from each patent mouse.*

This conclusion is further supported by the analyses presented in Fig. 3.10, in which the relationship of total female *T. muris* numbers and total female worm weight per burden on net *T. muris* eggs/g faeces is evaluated. The analyses were performed on log transformed data in order to normalize the variance and to preserve a linear relationship. The lines drawn represent the regression line computed by the method of least squares. The results suggest there was a better correlation between the egg counts and the total weight of female worms ($r^2 = 0.81$) than between the egg counts and the total number ($r^2 = 0.59$).

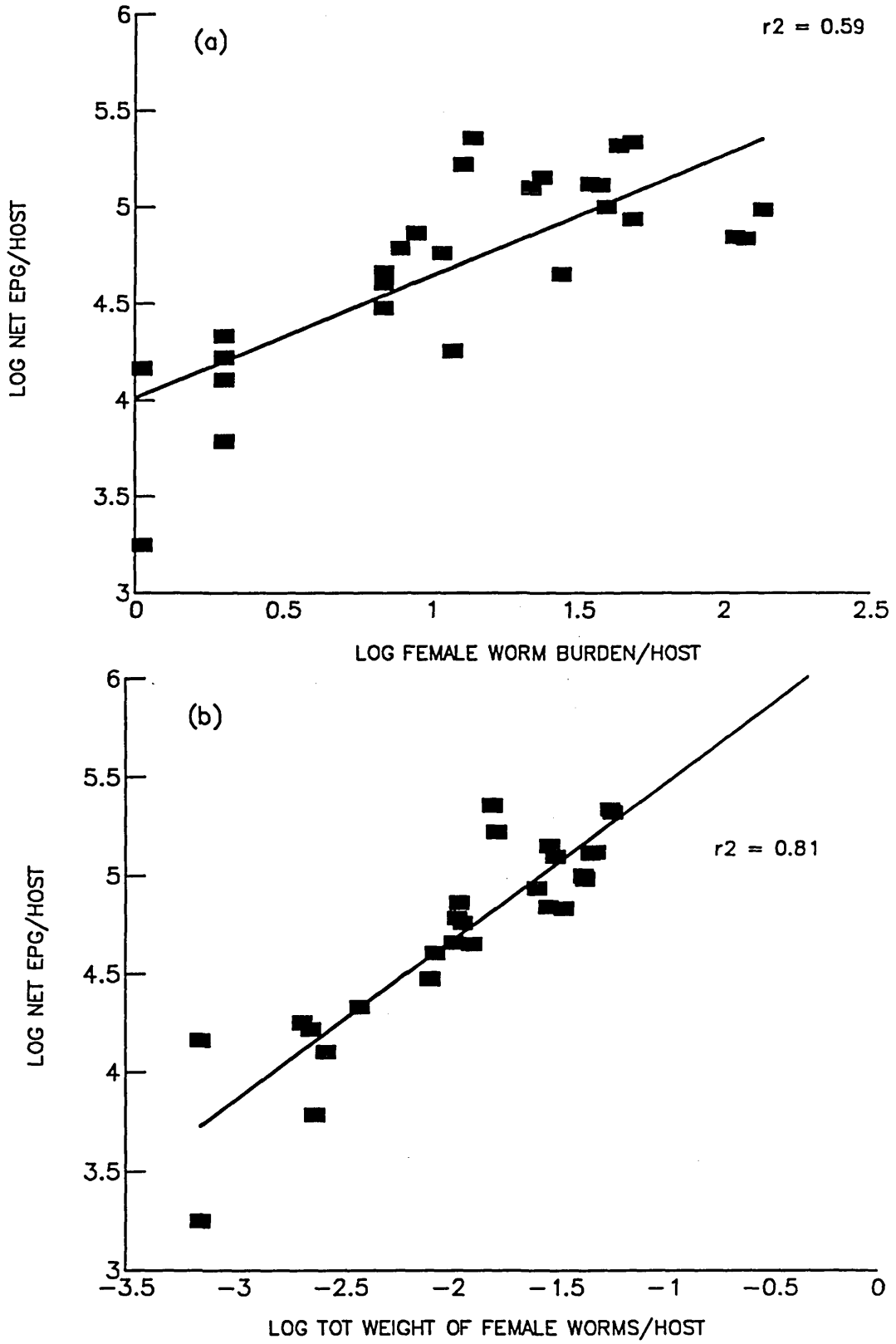

Figure3.10 *The logarithmic relationship between net T. muris eggs per g faeces and (a) female worm burden and (b) total weight of female worms per host. Closed squares represent the mean of 5 replicate epg estimates. The lines depict the best-fit linear regression model.*

3.4 DISCUSSION

It is apparent from the preceeding analyses that *T. muris* population size and activity in both acute and chronic primary infections in CBA/Ca mice are regulated by density dependent processes. Adult worm establishment in CBA/Ca mice is infective dose dependent (Figs. 3.1 & 3.2). As expected, this relationship was most dramatic for the infections given to normal, untreated CBA/Ca mice (Fig. 3.1a), in which there appears to be a threshold level of infection, approximately equivalent to 10 eggs, above which spontaneous cure responses are triggered and almost all parasites are expelled before patency is reached (Wakelin, 1973). This existence of an immunological threshold in murine trichuriasis would therefore appear to place severe restraints upon *T. muris* transmission in the wild. However, as pointed out by Wakelin (1985b), it is important to note that the threshold barrier in *T. muris* transmission may be influenced by factors such as host physiological state (Selby & Wakelin, 1975), host genotype (Wakelin, 1975a), the presence of concurrent infection (Jenkins & Behnke, 1977; Behnke *et al.*, 1984) and the immunomodulatory effects of the worm itself (Else & Wakelin, 1988) which may elevate the immunological thresholds and permit greater numbers of worms to reach sexual maturity.

It is clear that corticosteroid treatment induced significant susceptibility to primary infections of *T. muris* in the normally resistant CBA/Ca mice (Figs. 3.1 3.2). However, adult worm establishment in such mice was not a direct function of infection dose. Instead, significant non-linearity was detected in the relationship between worm establishment and infection dose in these mice (Fig. 3.2). This finding may be suggestive of a dose dependent limitation on *T. muris* establishment operating even in susceptible mouse hosts. The suggestion of a possible upper limit to worm establishment in the chronic infection model may indicate the probable existence of a physical carrying capacity in the mouse for *T. muris*. Wakelin (1975c) found 500 worms to be the maximum burden supportable by non-responder Schofield mice. In the present study, this number was detected to be around 300 for the smaller cortisone treated CBA/Ca mice. The existence of a finite capacity for chronic infections of *T. muris* in these mice may be related to the large size of the worm (around 8.8 - 10.1 mm and 14.2 - 15.8 mm in length for young males and females respectively (Fahmy, 1954)), relative to the dimensions of the mouse large intestine. A similar physical limitation on gut carrying capacity has been suggested for the large human intestinal worm, *Ascaris lumbricoides* (Beaver, 1980). This may indicate a role for parasite size in the mechanisms involved in population regulation.

The typical location for *T. muris* in the mouse host is considered to be the caecum (Panesar & Croll, 1980). However, as host burdens increase, an increasing proportion of worms are displaced to the colon such that at very high worm densities, there are more worms recovered from the colon than the caecum (Fig. 3.3). It has been suggested that newly-hatched larvae must enter caecal gland openings rapidly or risk being swept away with the luminal contents into the colon (Wakelin, 1967; Panesar, 1981). This, and competition between larvae for available caecal gland openings may account for the increasing displacement of establishing larvae to the colon at increasing infection intensities (Fig. 3.3).A similar displacement of worms, from the caecum to the colon and even to the rectum has been described in heavy *T. trichiura* infections of humans (Bundy & Cooper, 1989). This displacement of worms from presumably normal locations may have important consequences for the population dynamics of trichiuroid infections. While it is possible that the physiology of the worm may be affected by displacement from optimal sites, with adverse implications for both survival and fecundity (Krupp,1962); it is important to note that this may be offset by advantages offered for parasite growth by the less crowded conditions prevailing in the untypical locations.

In common with many studies of geohelminth infection in humans (Croll *et al.*, 1982; Martin, Keymer, Isherwood & Wainwright, 1983; Anderson & Schad, 1985; Bundy *et al.*, 1985; Elkins, Haswell-Elkins & Anderson, 1986), no significant bias was found in the sex ratio of *T. muris* in the present study. Furthermore, the sex ratios of both *T. trichiura* (Bundy *et al.*, 1985) and *T. muris* are not density related (Fig 3.4). These findings support the assumptions of equal sex distributions made for models of mating probabilities of dioecious helminths (Macdonald, 1965; May, 1977; Anderson & May, 1978).

The day to day variability recorded for mean egg counts from individual mice (Fig. 3.5) is unsurprising. Similar variability has been reported both in chronic human (Croll *et al.*, 1982; Anderson & Schad, 1985; Bundy *et al.*, 1987) and experimental (Keymer & Hiorns, 1986b) intestinal helminth infections. The present data collected under the controlled experimental conditions of the laboratory support the conclusion that daily variability in helminth egg counts is an inherent biological feature of the host - parasite interaction, and not an artefact created through estimation procedures (Keymer & Hiorns, 1986b).

In addition to density dependence in worm establishment (Fig. 3.2), the results also indicate the existence of density dependence in worm fecundity in chronic primary

infections of *T. muris* (Fig. 3.6). The analyses made in this study (Table 3) demonstrate that variations in faecal egg count between hosts are not artefacts of differences in host faecal output, but are a function of the worm burden.

The above results are in direct contrast to the observations on *H. polygyrus* infection in outbred MF1 mice (Keymer & Hiorns, 1986b), where host faecal production exhibited an inverse relationship with worm density, but showed a positive association with parasite fecundity. These findings led the authors to conclude that density dependence in intestinal helminth fecundity may be a direct outcome of differences in faecal output between infected mice, and thus that helminth fecundity may be functionally related to host faecal production (Keymer & Hiorns, 1986b). The present data (Figs 3.6, 3.7, and Table 3), however, clearly do not support this conclusion. Instead, they indicate that faecal egg counts are a true reflection of worm egg production, and that density dependence in helminth fecundity is independent of host faecal output; a conclusion which accords with the numerous observations of helminth density dependence in human and veterinary infections (Michel, 1969; Boray, 1969; Croll *et al.*, 1982; Anderson & Schad, 1985; Medley & Anderson, 1985; Bundy *et al.*, 1985; Barger, 1987; Smith , Grenfell & Anderson, 1987; Ramsay *et al.*, 1989).

The present study also provides some insight into the mechanisms which generate density dependence in worm fecundity. The density dependence of *T. muris* female weight (Fig. 3.8), and the positive association between worm weight and per capita egg production (Fig. 3.9), suggest that the density dependent decline in individual worm fecundity may be due to the stunted growth of individual worms when occurring at high population densities. This conclusion is supported by the demonstration that faecal egg counts are better correlated with female worm weights than with worm numbers *per se* (Fig. 3.10a, b). This finding also supports the suggestion that the weight or size of worms harboured by a host provides a better measure of the parasite density or relative "crowdedness" of the helminth population than the total number of worms (LeJambre *et al.*, 1971; Ractliffe *et al.*, 1971). These results show that one feature of the worm burden which is correlated with the rate of helminth egg production is its weight. It is likely that other variables, both intrinsic and extrinsic to the worm burden, such as age of the worm, and host characteristics such as genotype (Jones *et al.*, 1989) and physiological state (Swanson & Bone, 1983), are also correlated. As pointed out by LeJambre *et al* (1971), until such relationships are explicitly known, it would not be necessary to postulate that the control of egg production is independent of the worm burden.

Patterns of density dependent establishment, growth and reproduction within populations of helminth parasites are generally speculated to arise either as a result of intraspecific competition for finite resources, or through the effects of host immune responses which increase disproportionately in efficiency as parasite burden increases (Anderson & May, 1978; Keymer, 1982; Anderson & May, 1985). Although numerous workers have seeked to study density dependent phenomena in helminth infections (reviewed by Keymer, 1982; Scott & Lewis, 1987), the causal factors, whether immunology or ecology, have remained difficult to resolve. In the present study, while it is clear that host immune responses may severely restrict adult *T. muris* establishment in normal, resistant CBA/Ca mice (Fig. 3.1a), and both humoral and cellular immunity have been implicated in depressed helminth growth and fecundity (Wakelin, 1984; Lloyd & Soulsby, 1987, 1989), they are probably irrelevant to the chronic *T. muris* infection system, in which the mice were rendered immunotolerant to infection by corticosteroid treatment (Lee & Wakelin, 1983). Intraspecific competition for space or food resources, enhanced at high worm density is therefore a more probable candidate mechanism underlying the density dependent processes reported for chronic *T. muris* infections in this chapter. This finding suggests that purely ecological processes, arising from intraspecific parasite interaction, may be an important regulatory factor controlling parasite population numbers and activity in chronic helminth infections, including human intestinal helminthiasis; in which to date there is little evidence of the occurrence of effective immunity. This conclusion indicates the value of laboratory immunotolerance models in the exploration and understanding of the population processes that govern host - parasite interactions in nematode infections.

In conclusion, this study of *T. muris* provides evidence of density dependence in both worm establishment and fecundity; the latter being related to density dependence in individual worm growth and development. These observations are compatible with similar, though necessarily less controlled, studies of human and veterinary helminth infections. The results also accord with theoretical analyses which indicate that the remarkable temporal stability of helminth populations is a function of tight density dependent regulation (Anderson, 1982a; Croll *et al.*, 1982; Anderson & May, 1985; Anderson & Medley, 1985).

CHAPTER 4: THE EFFECT OF DIETARY PROTEIN ON THE POPULATION DYNAMICS AND IMMUNOLOGY OF *TRICHURIS MURIS* PRIMARY INFECTIONS

4.1 INTRODUCTION

The term "primary infection" is used in the parasitological literature to describe the infection process which arises from the exposure of immunologically naive hosts to parasite infective stages at a single point in time. Such single infection protocols have been used extensively in experimental parasitological research in order to study the effects of various factors on the inherent or intrinsic susceptibility or resistance of a host to a particular parasite. Thus, immunologists have used primary infections in conjunction with different animal host strains in order to demonstrate the importance of immunogenetics to the innate resistance/susceptibility of naive hosts to helminth infection intensity (see Wakelin, 1978a,1984, 1985a). Similarly, experimental parasitologists have used single infection protocols to show that both exposure rate and immune responsiveness may generate heterogeneity in helminth infection intensity between previously uninfected hosts (see Anderson & Crombie, 1985; Keymer, 1985, Keymer & Hiorns, 1986a).

From the viewpoint of experimental epidemiology, the value of primary infections may lie in their potential to facilitate the estimation of the intrinsic or natural population rate parameters that may govern a given host-parasite relationship. It is clear that knowledge of the intrinsic rate of parasite establishment, survivorship and fecundity in naive hosts (*i.e.* in the absence of the effects of acquired immunity) is an essential prerequisite for the interpretation and analyses of the more realistic, but more complicated, infection dynamics which may arise under conditions of repeated host-infection exposure. The characterization of the dynamics of primary infection may be considered to represent the starting point of any experimental epidemiological study, and forms the rationale for the experiments described in this chapter.

Hitherto, studies on *T. muris* primary infections have been mainly performed from an immunological point of view. These studies (reviewed in chapter 1) have clearly established that murine trichuriasis, as also in the case of *Trichinella spiralis* in mice and *Nippostrongylus brasiliensis* in rats, constitutes an acute nematode

infection, since the parasite is normally expelled from an immunologically intact mouse host well before patency during primary infections (Behnke, 1987). This situation therefore contrasts with that found in *H. polygyrus* infections in mice, for example, in which primary infections are normally long-lived and survive to form "chronic" patent infestations (reviewed by Behnke, 1987).

The immunological studies carried out on *T. muris* primary infections have, furthermore, clarified that the "spontaneous cure" response in murine trichuriasis is both infection dose and mouse strain dependent: there being a threshold infection level of above 10 eggs required to elicit the response, and also the existence of distinct host strain and haplotype determined differences in the "spontaneous cure" ability of individual mice (Wakelin& Lee, 1987; Else & Wakelin, 1988). Subsequent studies have established that the capability of individual mice to immunologically resist primary infections with *T. muris* may also be modified by both the host physiological state at the time of infection, *e.g.* lactation (Selby & Wakelin, 1975), and the presence of concurrent infections with other parasites (Behnke *et al.,* 1984). Studies into the nature of the spontaneous cure mechanisms have revealed that *T. muris* expulsion during a primary infection involves two components, an initial antibody mediated phase, as well as a subsequent lymphoid cell-mediated effector phase (Wakelin, 1975b; Wakelin & Lee, 1987).

Several studies have investigated the influence of dietary protein on various aspects of the dynamics of intestinal helminth primary infections. These studies, involving both acute and chronic nematode infection models, have provided some evidence that host protein nutriture may induce significant differences in helminth infection intensity between well- and malnourished hosts (summarized in Table 1.5). Many of these studies, however, have tended to examine the effects of host nutrition on helminth infection mainly by comparing the number of adult worms present in well- and malnourished hosts at some single time point after initial infection. Because of this approach, it is still not clear at present whether the generally observed increase in helminth infection intensity in protein malnourished hosts during primary infections is primarily due to an increase in the rate of initial parasite establishment or to malnutrition induced increase in worm survival. The data available for parasite establishment, however, suggest that protein malnutrition may either have no effect or may increase initial geohelminth establishment in primary infections (Table 1.5). Furthermore, the observations of Donaldson & Otto (1947) could indicate that the interaction between host nutritional status and initial adult helminth establishment may also be modified by the peculiarities of the parasite life cycle. Their study on the effects of dietary protein on *N. brasiliensis*

primary infection in rats clearly suggested that the increased establishment of adult worms in the guts of malnourished rats was due to the faster systemic migration (to the intestine) and maturation of infective larvae in these hosts compared to their well-nourished counterparts. As can be seen from Table 1.4, the effects of protein malnutrition on helminth fecundity in primary infections is highly variable, being increased in some host-parasite systems or unchanged in others. Worm survival rates, on the other hand, are invariably increased in protein malnutrition during primary infections (Table 1.4). This finding, which has been recorded for both acute and chronic nematode systems, suggests that the major impact of protein malnutrition on the dynamics of geohelminths during primary infections is to increase parasite survival in deficient hosts.

The most complete investigation to date into the influence of protein malnutrition on the dynamics of geohelminth primary infection is that of Slater (1987). This study, utilizing chronic *H. polygyrus* infections in mice, clearly showed that while protein nutrition may not influence either *H. polygyrus* establishment or fecundity in primary infections, the survival rate of adult worms may differ markedly between malnourished and well-nourished mice. Analysis of the worm survival data indicated that the rate of worm mortality was independent of the duration of infection in malnourished mice whereas it was significantly time dependent in their well-nourished counterparts. The author interpreted these results as being suggestive of the occurrence of a slightly faster rejection of adult *H. polygyrus* from well-nourished hosts compared to a possible delay in worm expulsion in the protein deficient mice.

It has been generally concluded that nutrient deficiency-induced impairment in immunity may be the most likely mechanism underlying the observed malnutrition associated increase in host susceptibility to parasitic infections, including helminth infections (Chapter 1). Despite this conclusion, few studies have sought to directly correlate the interrelationships between host nutritional status, specific immunity and infection intensity, especially with regard to helminth infections. Generally, studies on nutritional immunology and the relationship between host nutrition and infection, have been conducted separately, making it difficult to comment directly on the relevance of the nutrition-immunity interaction in host susceptibility to a particular infection. Recently, however, some authors have attempted to examine the interaction between host protein nutrition, specific immunity and helminth infection intensity (Cummins *et al.*, 1978; Duncombe *et al.*, 1981; Cummins *et al.*, 1987). These studies, focusing on acute *N. brasiliensis* infections, showed that the failure of protein deficient rats to expel primary infections may be associated either

with defective bone marrow cells (Duncombe *et al.*, 1981) or fewer and/or impaired response of mucosal mast cells, goblet cells and intraepithelial lymphocytes in the malnourished small intestine (Cummins *et al.*, 1987). These findings indicate that studies on the nutrition-immunity-infection interaction may not only be potentially of relevance towards our understanding of mechanisms underlying nutrient deficiency-induced susceptibility to parasitic infections, but may also be useful, in a wider context, in dissecting the nature of host protective immunity to various infections.

The present study has two major aims. The first is to characterize the effects of marginal changes in protein nutrition on the dynamics of *T. muris* primary infection in CBA/Ca mice, addressing specifically the effects on initial worm establishment, survival and fecundity. The second aim of the study is to examine the interrelationship between mouse protein nutrition, specific serological responses to *T. muris*, and the dynamics of *T. muris* primary infection in CBA/Ca mice. Mouse serological responses, both quantitative (antibody titres) and qualitative (immunorecognition), were chosen for the immunological analyses in this study not only because antibody responses have been implicated in mouse resistance to *T. muris* (Wakelin & Lee, 1987), but also because it has been suggested that differences in the timing of worm expulsion between mice may reflect a variation in the ability to produce the required level of antibody (Wakelin, 1975b). An analysis of the antigen recognition profiles of normal and protein deficient CBA/Ca mice was also carried out simultaneously to examine the possibility that, in addition to diet-induced alterations in specific antibody titres, the epitope specificity of the antibody produced may also be influenced by dietary protein, such that well nourished mice may recognize protective antigens not seen by the deficient animals.

4.2 METHODS AND EXPERIMENTAL DESIGN

Male CBA/Ca mice, at 4 weeks of age, either purchased from Harlan Olac (Bicester, U.K.) or obtained from small breeding stocks held at the Department of Biology, Imperial College, were used in the experiments described in this chapter. The mice were weighed on receipt and randomly assigned to each of two dietary groups. One group was fed a high protein diet containing 16% (w/w) casein (well nourished group) while the other was placed on a low protein diet containing 4% (w/w) casein (protein deficient group). The constituents of the casein-based diets, and the methods employed for diet preparation and feeding of mice are as given in Table 2.2

and chapter 2. All mice were acclimatized on their respective diets for two weeks before infection with *T. muris* was carried out on day 0 of the experiment. Mice were generally housed 5 to 10 in cages fitted with wire grid bottoms (to reduce coprophagy) and given the diets and water *ad libitum* at all times. On the day of primary infection (day 0), the mice in each dietary group were further subdivided into 3 treatment groups- one uninfected control group, and 2 experimental infected groups, each receiving either a low infection dose of 10 *T. muris* eggs per mouse or a high dose of 650 eggs per mouse (Table 4.1). These infection levels were chosen in order to facilitate the examination of the effects of protein nutrition on the dynamics of both a subthreshold *T. muris* infection (10 eggs), in which worm expulsion is not expected to take place, and a suprathreshold infection (650 eggs) in which *T. muris* expulsion is normally expected to take place around d.20 p.i. Prior to infection on day 0, all mice were weighed and blood collected to determine initial antibody levels. The specific parasitological and immunological techniques followed in this experiment are as described in chapter 2. The experimental programme followed and the numbers of mice used are summarised in Table 4.1.

Table4.1 *Summary of the experimental programme.*

group	number of mice per group	number of mice examined			
		d.14	d.21	d.28	d.49
4% diet					
control	8[a]	—	—	—	7
10	30	10	5	5	10
650	30[a]	10	5	5	9
16% diet					
control	10-	-	—	—	10
10	27[a]	9	5	5	7
650	29	9	5	5	10

[a]One mouse in this group died.

4.2.1 Assessment of nutritional status

At weekly intervals each mouse was weighed to monitor weight changes. Mouse food intake under the various treatments was estimated by measuring the amount of diet eaten over the 24 hour period prior to each weekly mouse weighing by each of 5 individual mice, chosen at random each week from each group. The average of these weekly 24-hour food intakes per mouse was used to estimate the mean daily mouse food consumption per group over the entire duration of the study.

4.2.2 Dynamics of *T. muris* primary infections

T. muris establishment and survival in primary infections were assessed by serial sampling of groups of 5 to 10 infected mice from each infection group on days 14, 21, 28 and 49 post infection (p.i). (Table 4.1). The numbers of larvae recovered on day 14 p.i. were used to estimate the establishment rate of *T. muris* under the various treatments given in this study. Faecal egg counts to estimate *T. muris* fecundity under the various treatments were performed on each mouse that remained in the infection groups after day 28 worm assessment (see Table 4.1). Egg counts were carried out on each mouse every two days from day 32 p.i. onwards until the end of the experiment on day 49 p.i.

4.2.3 Serological analyses

CBA/Ca mouse humoral responses to primary infections with *T. muris* were studied by using both the ELISA and Western blot techniques. The ELISA was used to study both the kinetics and titres of anti-*T. muris* ES specific total immunoglobulins (total IgGAM), IgG1 and IgA responses. Towards this end, blood was collected from all mice at weekly intervals (at the times of weekly weighings) throughout the experimental period.

The aim of the immunoblot analysis carried out in this study was to compare the *T. muris* ES antigen recognition profiles between protein deficient and normally fed mice in relation to both time and infection intensity. For this purpose, 3 to 4 representative sera corresponding to periods of initial parasite establishment (d. 14 p.i.), worm expulsion (d. 21 p.i.) and chronic adult stages (d. 49 p.i.) were chosen for the analysis from each treatment group. Antigen recognition by antibodies of the subclass IgG1 were studied since the ELISA results suggested that this antibody may form the major component of the CBA/Ca mouse humoral response to *T. muris* (Else & Wakelin, 1989).

4.3 RESULTS

4.3.1 Host survival and nutritional status

The protein content in the diets used in the present study did not appear to influence the survival of either control or infected mice; only three of the 134 mice utilized died during the experiments (Table 4.1). There was no obvious morbidity in hosts fed either diet.

Figure 4.1 depicts the cumulative body weights of the control and infected mice fed either of the diets over the 9-week study period. The results show that both the 16% and 4% protein diets supported CBA/Ca mouse growth in the present experiments. Analysis of the weekly weight measurements of mice which survived until the end of the experiments on day 49 p.i. (Table 4.2) further indicated that the levels of dietary protein used in this study did not differentially influence the growth rate of CBA/Ca mice (Analysis of Covariance (ANCOVA), with mouse weight at infection as a covariate; $F = 2.15$, d.f. = 1; $P > .05$). However, within each dietary group, infection was found to significantly reduce mouse growth rate (Table 4.2) (ANCOVA : $F = 3.53$, d.f. = 2; $P < .05$). This negative effect of *T. muris* infection on the growth of CBA/Ca mice, however, was significant only for the higher rate of infection (Tukey/Kramer (TK) test: $q = 3.74$, d.f. = 2,46; $P < .05$), there being no significant difference in the growth rate between control and lightly infected (with 10 *T. muris* eggs) mice in either dietary group (TK test: $q = 1.68$, d.f. = 2,46; $P > .05$).

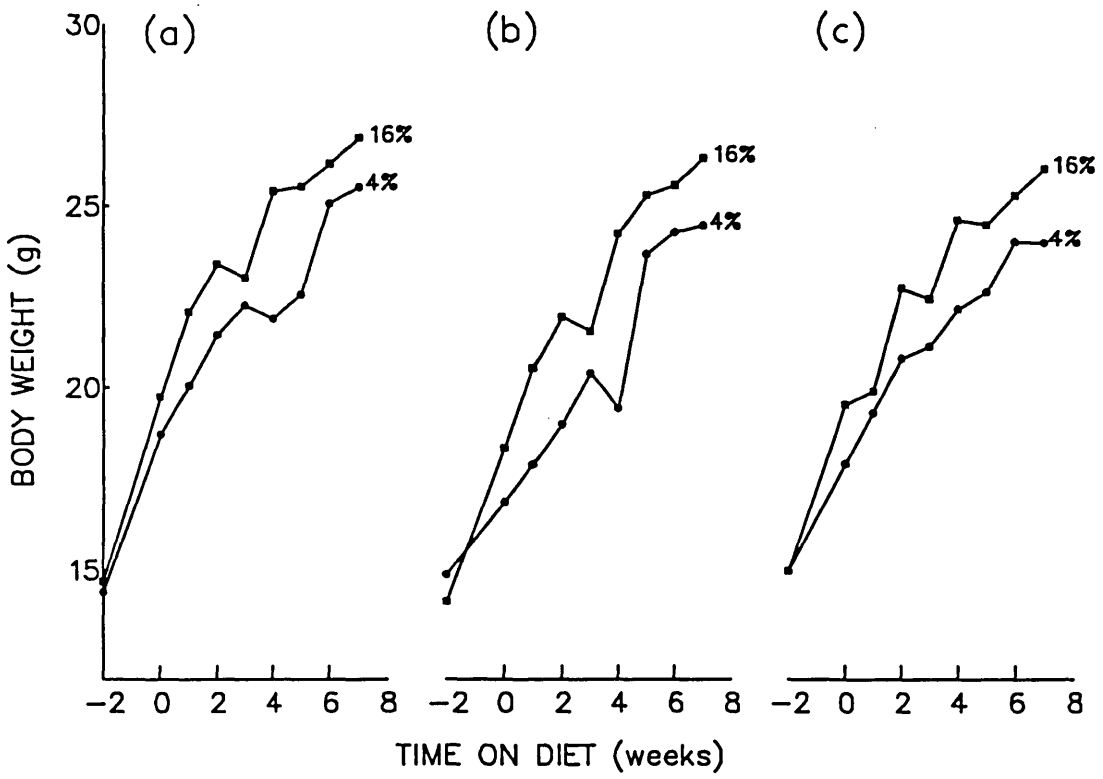

Figure4.1 *Change in mean mouse body weights with time : (a) control mice (b) mice infected with 10 eggs and (c) mice infected with 650 eggs.*

The overall mean daily food intake of CBA/Ca mice in the various treatment groups is displayed in Fig 4.2. Mice fed the 4% protein diet consumed significantly more food in comparison with those on the 16% diet (two-way Analysis of Variance (ANOVA) : F = 5.85, d.f. = 1; P< .05). Infection with *T. muris* further significantly increased CBA/Ca mouse food consumption in both the dietary groups (Fig 4.2) (two-way ANOVA : F = 20.20, d.f = 2; P< .001). Among infected mice, infection dose also appeared to influence mouse food intake, with animals given the lower dose of *T. muris* infection generally consuming slightly but not significantly more food in comparison to hosts infected with the higher dose (Fig. 4.2) (TK test : q = 2.54, d.f = 2,204; P> .05). Dietary protein content did not appear to affect this relationship between *T. muris* infection and CBA/Ca mouse food consumption.

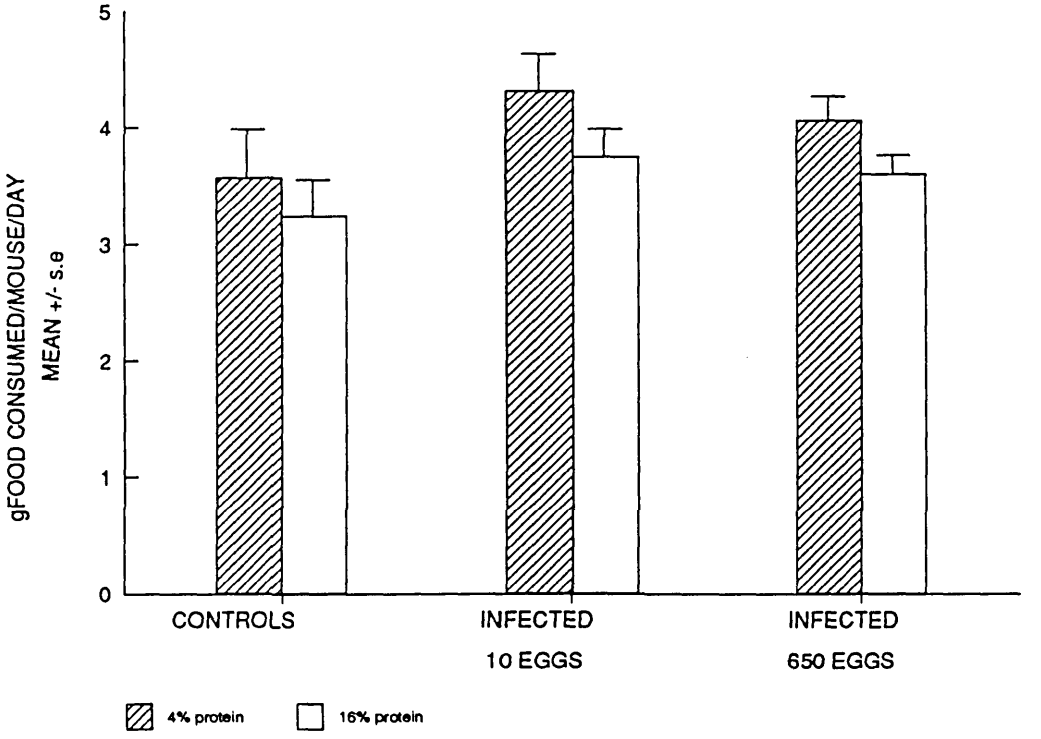

Figure4.2 *The effect of dietary protein and T. muris infection on the mean food consumption of CBA / Ca mice . The thin bars represent the standard errors of the means.*

It is apparent, further, from Table 4.2 that increased food consumption was insufficient to compensate for the lower protein content of the 4% diets. This is reflected both in the columns showing the change in mouse body weights during the period of study and in the estimated amount of protein consumed per day.

Table 4.2 *Food consumption and growth of mice fed either the 4% or 16% protein diet for the duration of the experiment.*

		weight on infection	weight on d.49 p.i	change in b.wt (g)	g food intake /mouse/day	g protein intake /mouse/day
diet gp.	n	Mean+/-s.e.	Mean+/-s.e	Mean+/-s.e.	Mean+/-s.e	Mean+/- s.e.
4% control	7	18.72+-0.31	25.51+- 0.17	6.80+-0.27	3.70+-0.13	0.148+-0.01
4% 10	10	18.22+-0.55	24.45+- 0.48	6.23+-0.24	4.46+-0.12	0.178+-0.01
4% 650	9	18.42+-0.55	23.96+- 0.38	5.54+-0.50	4.25+-0.10	0.170+-0.01
16% control	10	19.75+-0.59	26.87+- 0.42	7.12+-0.45	3.55+-0.10	0.569+-0.02
16% 10	7	20.07+-0.55	26.30+- 0.68	6.24+-0.23	4.20+-0.13	0.672+-0.02
16% 650	10	19.96+-0.27	26.00+- 0.65	6.04+-0.59	3.94+-0.10	0.630+-0.02

Table4.3 *The number of T. muris larvae recovered on d.14 p.i.*

Diet group	Infection dose[a]	No. of larvae recovered (Mean +/- s.e.)	Proportion establishment (Mean +/- s.e.)
4%	10	4.6+-1.3	0.46+-0.13
	650	134.7+-34.9	0.21+-0.05
16%	10	4.2+-1.2	0.42+-0.12
	650	123.0+-22.2	0.19+-0.03

[a] No. of *T. muris* eggs

4.3.2 Susceptibility to primary infection

Initial *T. muris* establishment in primary infection was estimated from d. 14 larval counts. The results given in Table 4.3. show that, for each infection rate, host protein status did not influence either the numbers of larvae recovered on d. 14 p.i. (two-way ANOVA : $F = 0.044$, d.f. = 1; $P > .05$) or the percentage establishment of *T. muris* in naive CBA/Ca mice(two-way ANOVA : $F = 0.024$, d.f. = 1; $P > .05$). Worm establishment rate, however, was significantly related to infection dose in both dietary groups (Table 4.3). While initial larval numbers were positively associated with infection dose (two-way ANOVA : $F = 128.16$, d.f. = 1; $P < .001$), the proportion of the infection dose which successfully established was significantly lower in mice given the higher infection dose (two-way ANOVA : $F = 6.52$, d.f. = 1; $P < .05$). This result thus supports earlier observations (chapter 3) that *T. muris* establishment in primary infection may be a density dependent function of infection dose.

4.3.3 Survival of primary infection

Mean numbers of *T. muris* recovered throughout the study for each of the various treatments are shown in Figs. 4.3 a,b,c,d. As each mouse was given only a single infecting dose, the dynamics of *T. muris* survival in primary infection may be examined under the assumption that it is as a pure death process, controlled by a single rate parameter (Anderson & Whitfield, 1975; Anderson & Michel, 1977). If this parameter, the instantaneous death rate u, defined per parasite per unit of

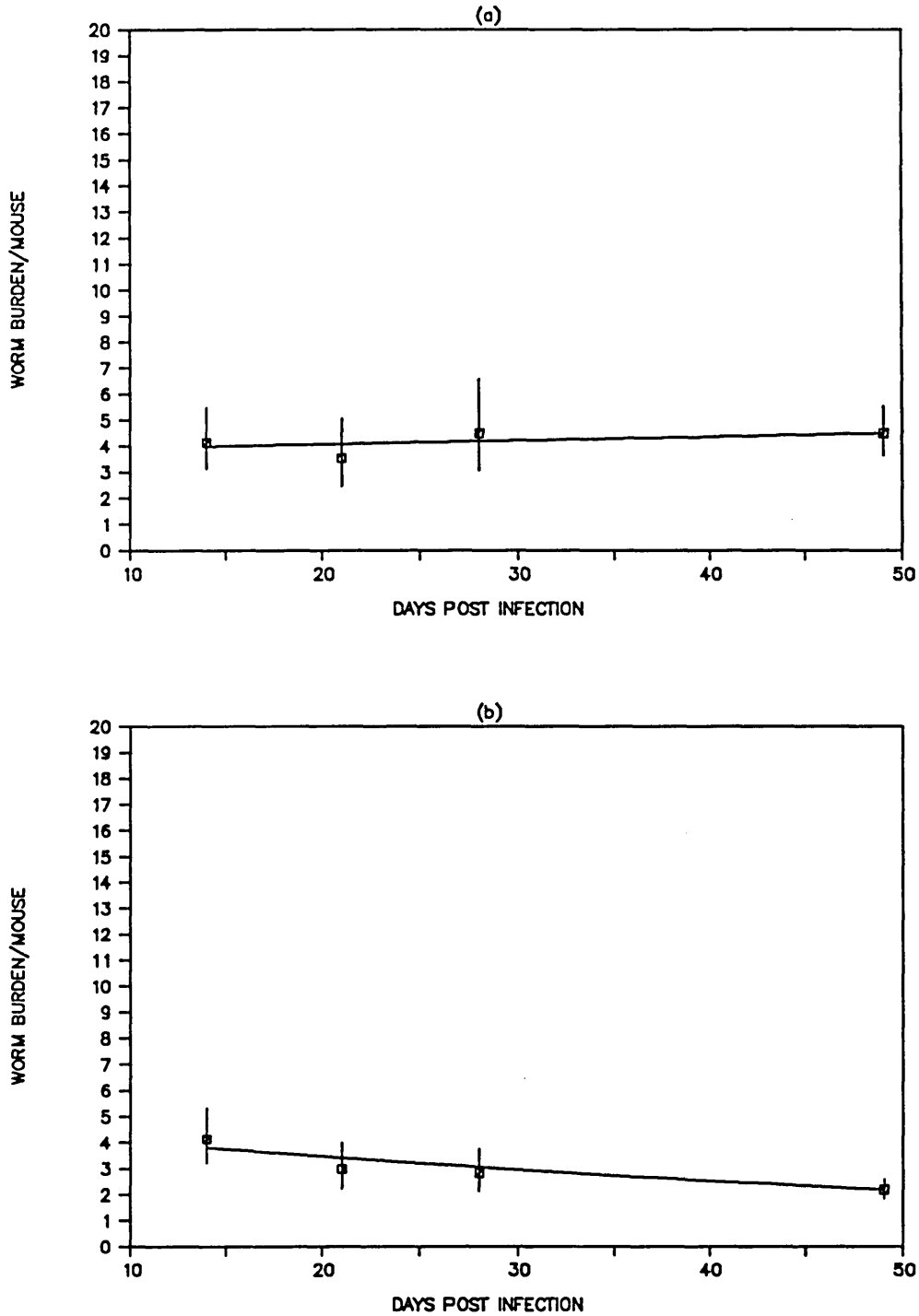

Figure4.3 *a,b. The effect of dietary protein on the survival of T. muris during primary infection in mice given the low infection dose of 10 eggs. (a) Mice fed 4% casein diet, (b) mice fed 16% casein diet. Open squares denote the mean number of parasites recovered per mouse at various times post infection; the vertical bars portray the standard errors of these means. Curves represent the predictions of the simple exponential survival model described in the text (Eqn 4.2). The values of the slopes are given in Table 4.4.*

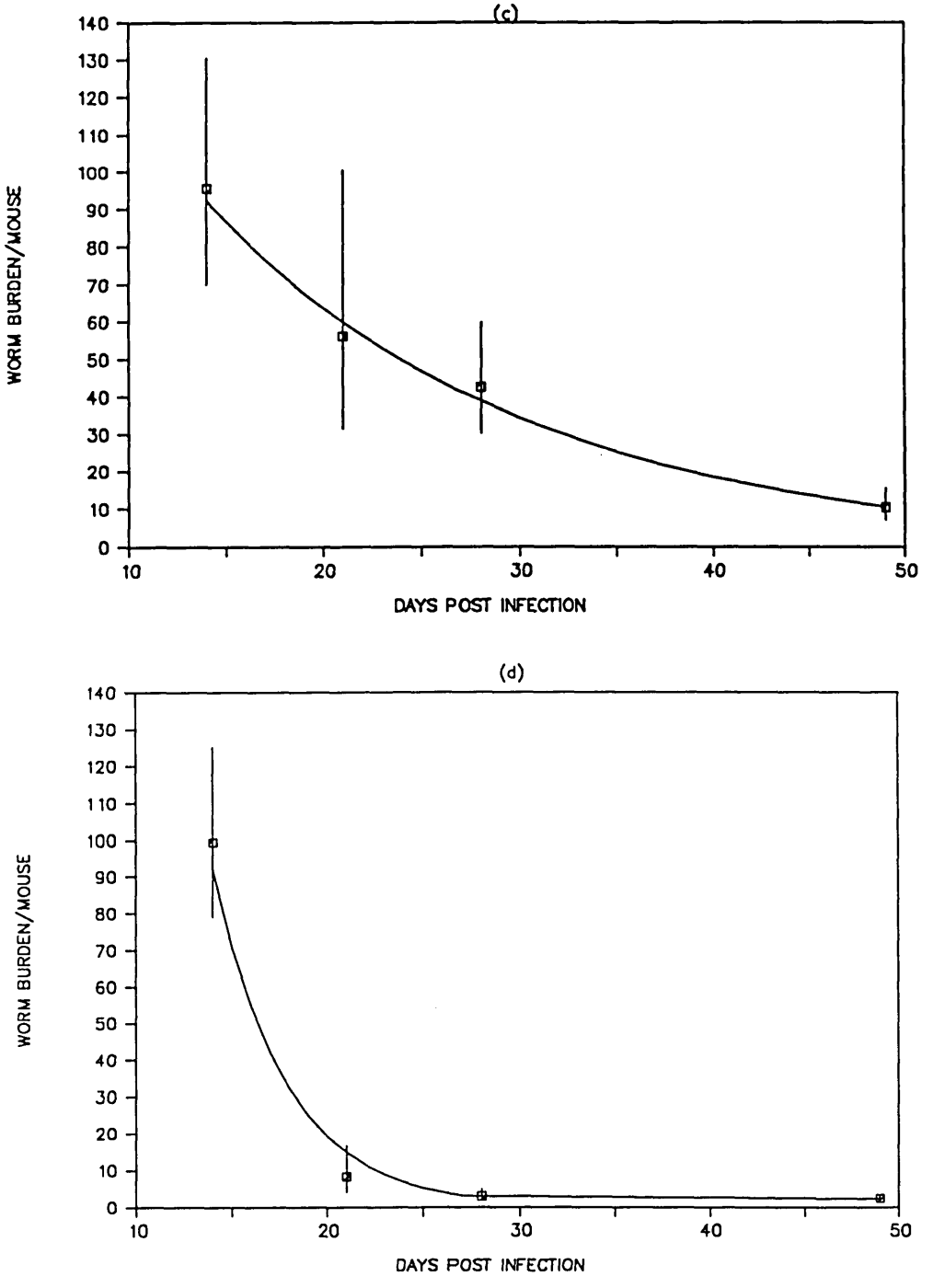

Figure4.3 *c,d . The effect of dietary protein on the survival of T. muris during primary infection in mice given the high infection dose of 650 eggs. (c) Mice fed the 4% protein (d) mice fed the 16% protein. Explanations of the squares, vertical bars and curves in the graphs are as given in* ***Figure 4.3*** *a,b.*

time, is constant throughout the period of experiment, the change in the number of worms N_t, surviving at time t can be described by the simple differential equation (Anderson & Michel, 1977):

$$dN_t/dt = -uN_t \qquad (4.1)$$

If the number of infective stages administered to a mouse at time $t = 0$ is N_0, then equation has the solution :

$$N_t = N_0 \exp(-ut) \qquad (4.2)$$

a model which predicts simple exponential decline of worm numbers through time.

A linear regression of $\ln N_t$ on t will yield estimates of u and N_0 by the slope and intercept, respectively. The predictions of the simple exponential model of parasite survival may then be generated by substitution of the estimates of u and N_0 in equation 4.2.

The estimates of u, the *per capita* worm death rate, obtained in the various mouse groups are given in Table 4.4. The results clearly indicate that a population model in which the rate of change of parasite numbers over time is considered to be constant may adequately describe *T. muris* survival in both the well-nourished and protein deficient mice given the low infections and in deficient CBA/Ca mice given the high infection dose. The predictions of the simple exponential parasite survival model (eqn 4.2) incorporating the *T. muris* death rates estimated in the above hosts are shown in Figs. 4.3 a,b,c. It is clear that the model closely approximated the observed change in worm numbers in these three treatment groups.

However, a model depicting a constant loss of parasite numbers through time does not appear to describe the observed change in worm burdens in mice given the large *T. muris* primary infection and fed the 16% protein diet (Table 4.4). Worm numbers in these mice declined non-linearly with time (Polynomial regression : time^2, c = 0.007, s.e = 0.0017, t = 4.38; P< .001); a result which implies that worm mortality varied significantly with time. The estimated larval (from d.14 to d.28 p.i.) and adult *T. muris per capita* death rates (from d.28 to d.49 p.i.) in these mice are shown in Table 4.4. The results show that the death rate was high initially but very low (not significantly different from zero) in later stages of infection. This support the contention that a primary spontaneous cure expulsion of larvae occurred from days

14 to 28 p.i., resulting in markedly higher mortality among larval parasites compared to adult worms. As can be seen from figure 4.3 d, substitution of these separately estimated time-dependent larval and adult worm *per capita* death rates into equation 4.2, gave an adequate empirical prediction of the survival of *T. muris* in the well-nourished, immunologically intact CBA/Ca mice given the high suprathreshold infective dose.

The estimates of *per capita* worm death rates given in Table 4.4 suggest that the survival of *T. muris* may be dependent on infection dose. Although *T. muris* death rates in malnourished mice were constant and independent of time, the death rate of worms was significantly higher in mice hosts given the higher infection dose (F test to compare *b* values : $F = 60.09$, $v1=2$, $v2=55$; $P < .05$) (Figs. 4.3 a,c).

In well-nourished CBA/Ca mice, on the other hand, not only was *T. muris* survival dose dependent but the functional form of parasite survivorship also differed markedly between the infective doses. Worm death rate was constant and independent of time at the low subthreshold infective dose, and was higher and significantly time dependent at the high infection dose. Thus, while primary infection was chronic in normally nourished mice given the low *T. muris* infection dose (Fig 4.3 b), it was typically acute in mice given the larger infective dose (Fig. 4.3 d).

Table 4.4 further indicatesthat among mice given the low infection dose, the death rate of *T. muris* may be marginally higher in well nourished CBA/Ca mice than in corresponding mice fed the 4% diet.

Table4.4 *Regression slope u for the linear relationship between worm burden and time d.14 - 49 p.i..*

Diet group	infection dose [a]			u +- s.e.
4% protein	10	Total period		0.003+-009[ns]
	650	Total period		-0.062+-013
16% protein	10	Total period		-0.016+-0.009[ns]
	650	d.14 - d.28		-0.26+-0.045
		d.28 - d.49	-	0.011 +-0.028[ns]

[a]No. of *T. muris* eggs
[ns] slope not significantly different from zero

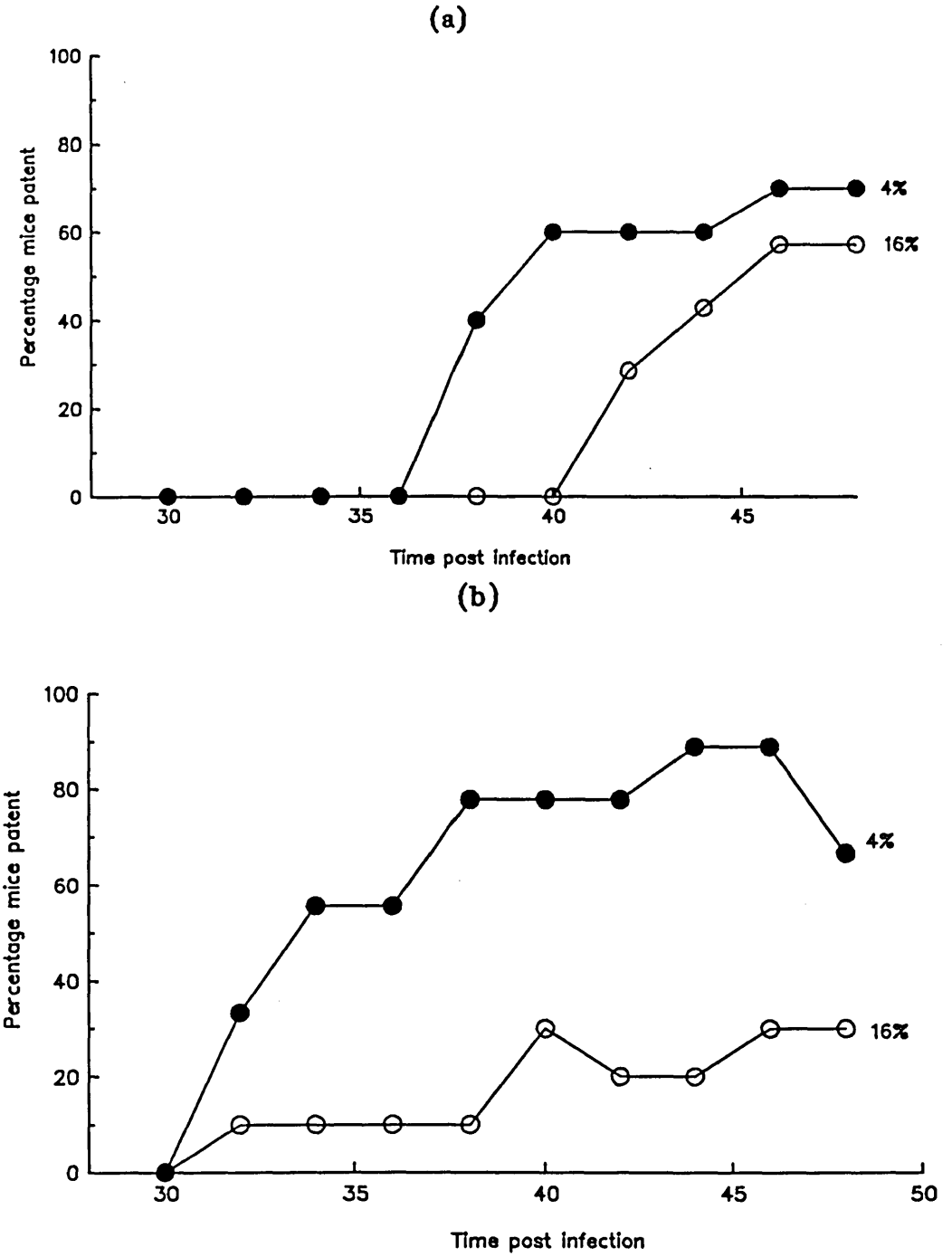

Figure4.4 *Relationship between dietary protein, infection rate and the pre-patent period of T. muris. The percentage of mice in either dietary group excreting eggs in faeces at various time periods post infection are shown in (a) for the low infection dose of 10 eggs and (b) for the high infection dose of 650 eggs.*

4.3.4 Parasite fecundity

The time to onset of fecundity of *T. muris* appears to be influenced by both host protein nutrition and infection rate (Figs. 4.4 a,b). Host protein deficiency significantly shortened the pre-patent period at both the infection levels studied (two-way ANOVA : F = 4.62, d.f. = 1; P = .045). Within each dietary group, however,

T. muris pre-patency was inversely related to infection dose; being significantly longer in mice given the lower rate of infection (two-way ANOVA : F = 6.01, d.f. = 1; P< .05). The number of patent mice per group was also found to be influenced by host nutrition; there being more patent hosts among mouse groups fed the 4% protein diet than in the corresponding well-fed mice at both infection levels (Figs. 4.4 a,b). Examination of Figs. 4.3 a,b,c,d and Table 4.5 suggests that this may be related to the significantly higher average adult worm intensities harboured by deficient mice in comparison to their well-nourished counterparts.

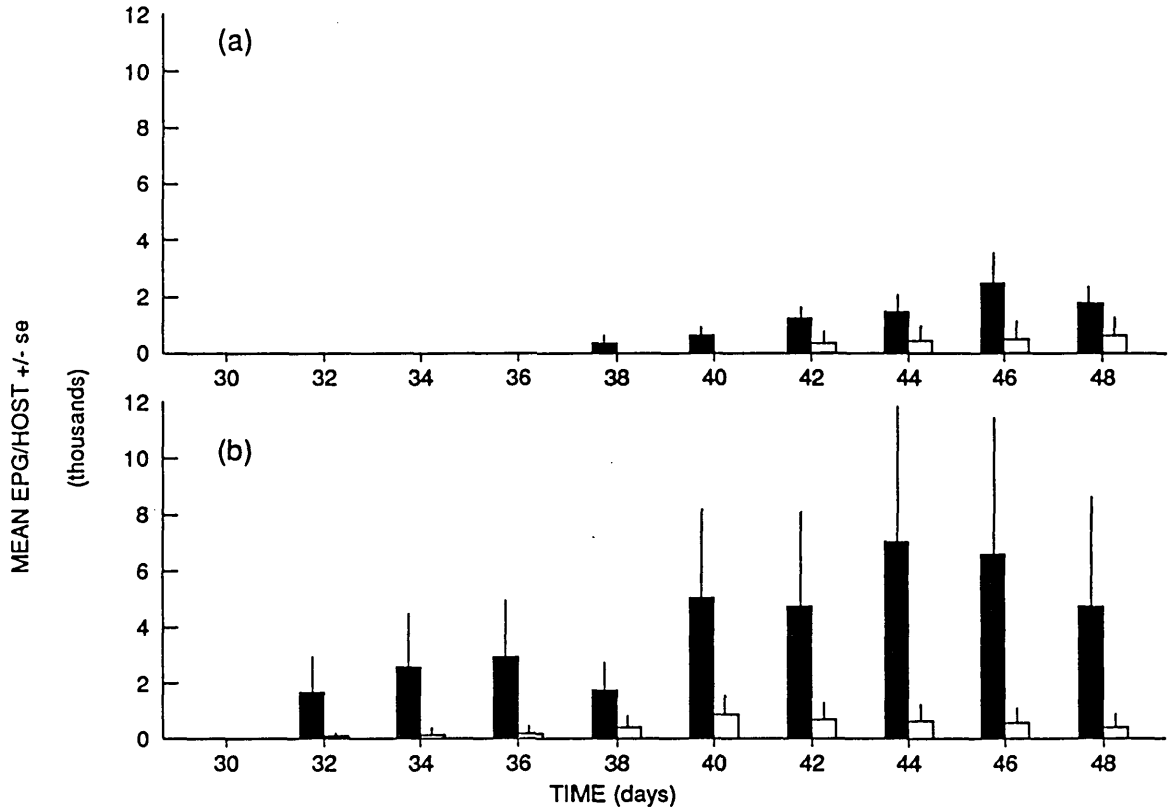

Figure4.5 *Comparisions of net egg output over time in mice fed the 4% (closed bars) or the 16% protein diet (open bars). Graph (a) shows the mean faecal egg output of hosts given the low infection dose of 10 eggs while graph (b) depicts the egg output of hosts given the high infection dose of 650 eggs. Thin bars denote the standard errors of the means.*

The estimated mean number of *T. muris* eggs in mouse faeces (eggs/gram faeces) was significantly higher in deficient hosts than in mice fed on the 16% protein diet independent of infection dose (Table 4.5 and Fig. 4.5 a,b). (two-way ANOVA : F = 57.48, d.f. = 1; P< .001). Furthermore, *T. muris* egg excretion in the faeces of deficient mice was positively and significantly related to infection dose (TK test : q = 6.695, d.f. = 2, 320; P< .05). By contrast, the estimated faecal egg output in well-nourished mice was unrelated to infection dose (TK test : q = 0.226, d.f. = 2,320; P> .05).

The mean numbers of adult worms recovered on d.49 p.i., the sex ratio of the burdens, and the *per capita* fecundity of female *T. muris*, estimated from d.48 p.i. faecal egg counts, are shown in Table 4.5. Dietary protein did not influence worm fecundity at either rate of infection (two-way ANOVA : F = 1.86, d.f. = 1; P> .05). However, within each dietary group, *T. muris per capita* fecundity was significantly lower at the higher rate of infection (two-way ANOVA : F = 8.67, d.f. = 1; P< .01)(Table 4.5).

4.3.5 Antibody responses to T. muris E/S antigen

The weekly change in the levels of specific total immunoglobulins (IgGAM), IgG1 and IgA estimated against *T. muris* E/S antigen, in serum samples of the various groups of CBA/Ca mice is shown in Figs. 4.6 (IgGAM), 4.7 (IgG1) and 4.8 (IgA). The means of the ELISA OD values obtained from individual mice in each mouse group are plotted against the days p.i. The results demonstrate that primary infection with *T. muris* may elicit substantial, time-dependent parasite specific antibody responses in CBA/Ca mice. The intensity and kinetics of the specific antibody response, however, was found to be influenced by both mouse dietary protein status and infection rate.

As shown, mouse infection rate significantly influenced the timing of the onset of specific antibody production; all 3 antibody types appearing earlier (generally just after d.14 p.i.), irrespective of host diet, in the sera of mice given the higher rate of infection (Figs. 4.6 a,b; 4.7 a,b; 4.8 a,b). This relationship was most dramatic for the specific IgA response; with detectable antibodies appearing in the sera of both deficient and well nourished mice given the low infection only at the very end of the experiment (Fig. 4.8 a) compared with the much earlier rise observed in mice given the higher rate of infection (Fig 4.8 b). It is significant, in the context of *T. muris*

Table 4.5 *Egg output during primary infection.*

Host diet	Infection dose	No. worms recovered (d.49p.i.) (Mean +- s.e.)	Sex ratio females/tot. worms) (Mean +- s.e.)	eggs per gram (Mean +- s.e.)	eggs per gram per female (Mean +/- s.e)
4% protein	10	4.30 +- 0.9	0.55 +- 0.09	879.27 +- 168.42	772.23 +- 150.22
16% protein	10	1.43 +- 0.3*	0.50 +- 0.13	216.48 +- 58.06*	1097.32 +- 301.31
4% protein	650	15.9 +- 5.02	0.48 +- 0.04	4119.3 +- 1027.3	355.02 +- 183.67
16% protein	650	3.70 +- 2.53*	0.52 +- 0.17	458.50 +- 154.91*	382.23 +- 101.7

* Parameter significantly lower in 16% hosts.

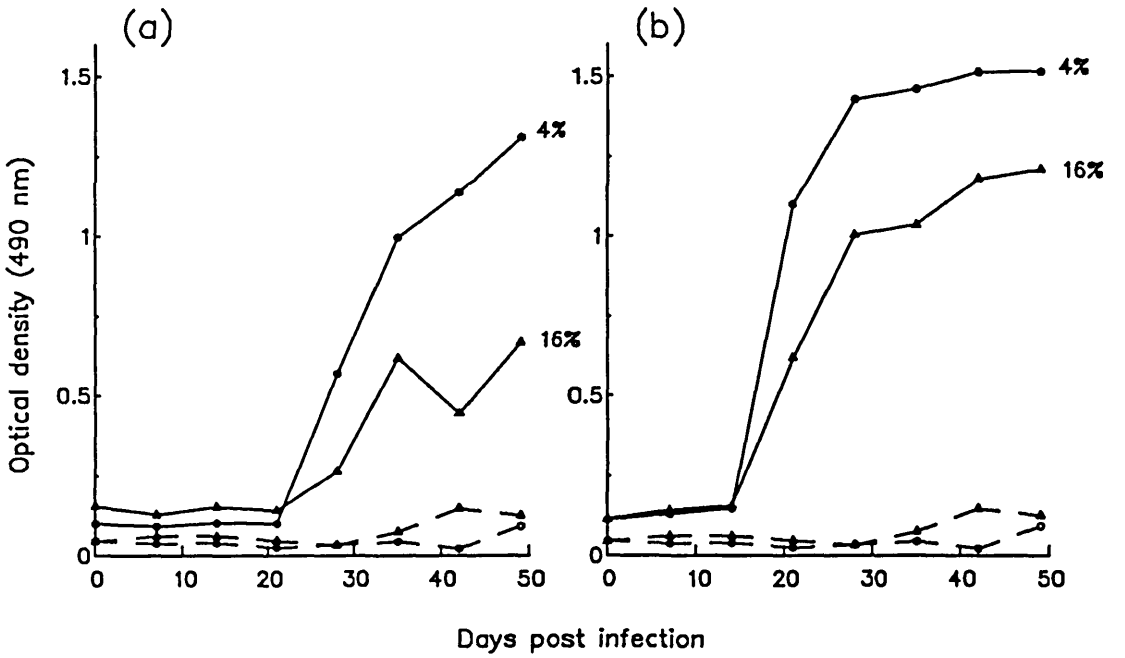

Figure4.6 *The effect of dietary protein on the kinetics of the specific total immunoglobulin response to T. muris E/S antigen in CBA/Ca mice given primary infections of either (a) 10 or (b) 650 infective eggs. Closed symbols connected by solid lines represent the mean ELISA OD values of infected hosts at various time days post infection while open symbols connected by disjointed lines denote the background values of uninfected controls.*

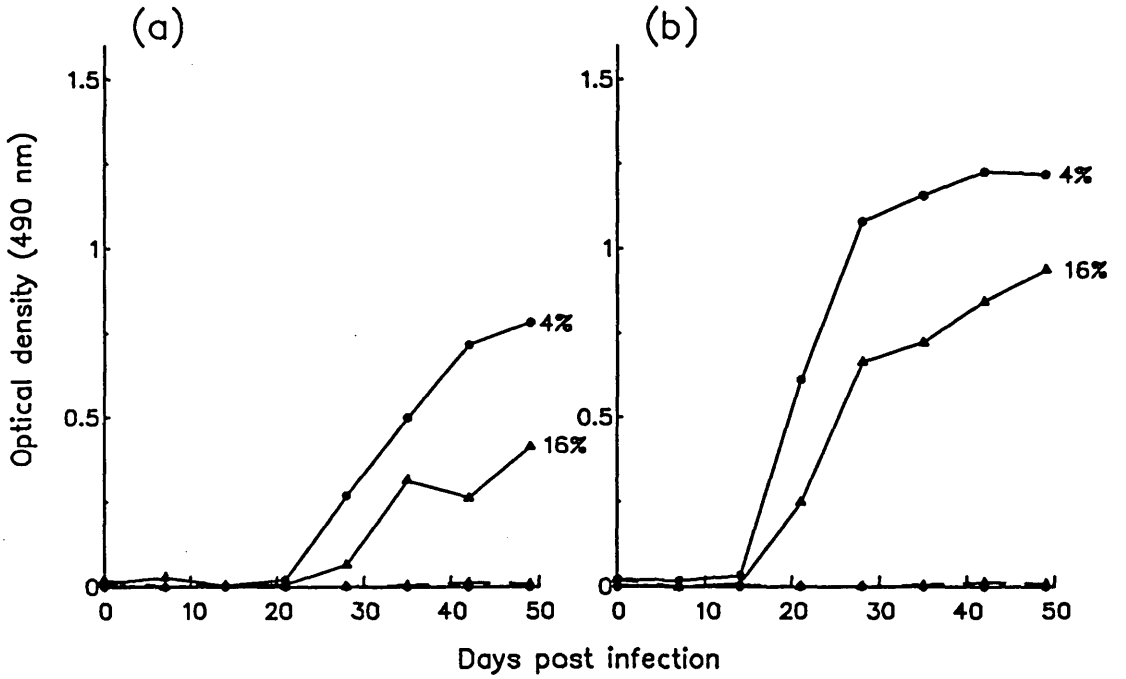

Figure4.7 *The effect of dietary protein on the kinetics of CBA/Ca mouse specific IgG1 response to T. muris E/S antigen during primary infection with either (a) 10 or (b) 650 infective eggs. Explanations for the symbols and lines depicted in the graphs are as given in* ***Figure 4.6*** *a,b.*

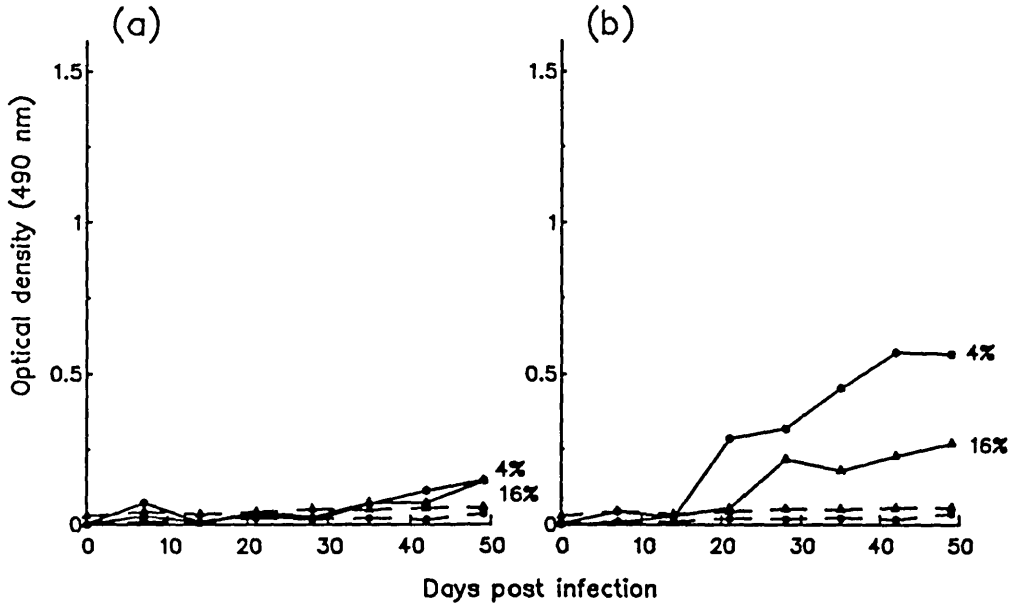

Figure4.8 *The effect of dietary protein on the kinetics of the specific IgA response to T. muris E/S antigen in singly-infected CBA/Ca mice given either (a) 10 or (b) 650 infective eggs. Explanations for the symbols and lines shown in the graphs are as given in* ***Figure 4.6*** *a,b.*

survival, that the parasite-specific antibodies arose before the worm expulsion period of 21 days in mice given the high infective dose, but was delayed until after this period in mice given the lower subthreshold infection (Figs. 4.6 a,b; 4.7 a,b; 4.8 a,b). Dietary protein does not appear to influence this relationship between infection rate and the onset of significant antibody production for any of the immunoglobulin types.

Although serum levels of all three antibody types increased significantly with time, the temporal pattern of antibody rise differed both in relation to the rate of host infection and the type of antibody (Figs. 4.6 a,b; 4.7 a,b; 4.8 a,b). In mice given the higher rate of infection, irrespective of dietary protein status, the specific total immunoglobulins and IgG1 responses against *T. muris* E/S antigen were found to increase non-linearly in relation to time (Figs. 4.6 b; 4.7 b). The rate of increase in antibody production was linear and most rapid during the early part of infection (from d.14 to 28 p.i.), *i.e.* during the larval stages; but appeared to plateau at a constant but high level during the later stages of infection (from d.28 onwards). The specific primary IgA response against *T. muris* E/S antigen shows a similar response, though with less tendency to plateau.

In mice given the low subthreshold infection dose, irrespective of dietary status, all three antibody types appeared later during primary infection and IgGAM and IgG1 rose steadily until the end of the experiment on d.49 p.i. (Figs. 4.6 a; 4.7 a; 4.8 a). Levels of IgA were on the margins of detectability by d.49 p.i.

Table4.6 *Mean T. muris-specific antibody titres (O.D) detected in mouse sera (weekly mean estimates).*

		Diet	
Antibody	Infection dose	4% protein	16% protein
		(Mean +- s.e)	(Meam +- s.e)
IgGAM	control	0.110 +- 0.01	0.118 +- 0.01
	10	0.830 +- 0.07	0.518 +- 0.04
	650	1.764 +- 0.09	1.259 +- 0.06
IgG1	control	0.000 +- 0.00	0.010 +- 0.01
	10	0.489 +- 0.06	0.215 +- 0.04
	650	1.665 +- 0.08	0.948 +- 0.07
IgA	control	0.040 +- 0.007	0.111 +- 0.006
	10	0.111 +- 0.015	0.094 +- 0.020
	650	0.655 +- 0.050	0.262 +- 0.030

Table 4.6 gives the means of the weekly estimates of *T. muris*-specific antibody titres obtained in serum samples of control and infected CBA/Ca mice fed either of the two protein diets. The results clearly show that, with the exception of the specific IgA response in mice given the low infection dose, primary infection with *T. muris* elicited significantly greater amounts of parasite-specific antibody in protein deficient CBA/Ca mice than in similarly infected well-nourished mice (two-way ANOVA : IgGAM : $F = 7.018$, d.f. =1; $P < .01$; IgG1 : $F = 16.56$, d.f. =1; $P < .001$; IgA : $F = 16.86$, d.f. = 1; $P < .001$). The estimated mean IgA levels in mice given the low infection dose, however, did not differ significantly in relation to host dietary status (TK test : $q = 0.56$, d.f. = 2,465; $P > .05$) (Table 4.6). Within each dietary group, infection dose was clearly associated with higher mean parasite-specific antibody titres in sera (two-way ANOVA : IgGAM : $F = 69.43$, d.f. = 2; $P < .001$; IgG1 : $F = 93.48$, d.f. = 2; $P < .001$; IgA : $F = 69.02$, d.f. =2; $P < .001$); although the mean specific IgA titre estimated in well-nourished mice given the lower rate of infection did not differ significantly from background levels (TK test : $q = 0.57$, d.f. =2,465; $P > .05$) (Table 4.6). Among infected mice, specific antibody production was further related to infection dose (Table 4.6). As can be seen, mice given the higher rate of *T. muris*

infection invariably produced significantly greater amounts of specific antibody in sera compared with the levels secreted by similarly fed counterparts given the low subthreshold infection; this relationship held for all three antibody types, irrespective of nutritional status, in the present study (Table 4.6).

4.3.6 Immunoblot analysis

The results of the Western blot analysis of E/S antigen recognition by specific IgG1 in representative mouse sera obtained on days 14, 21 and 49 p.i., are shown in Figs 4.9 and 4.10. *T. muris* E/S antigen recognition by IgG1 appears to be time-dependent, generally increasing with time or age of the animals. Temporal antigen recognition, however, was modified by both the effects of infection dose and mouse nutritional status (Figs. 4.9a,b; 4.10a,b). Thus, in both dietary groups, mice given the low subthreshold infection appeared to detect parasite antigens only during the adult stages of infection, *i.e.,* in the d.49 p.i. serum samples (Figs. 4.9a, 4.10a), although malnourished mice appear to recognise a broader range of antigens at this time compared to their well-nourished counterparts (Figs. 4.9a, 4.10a). By contrast, the immunodetection of antigens occurred much earlier during infection, at least by d.21 p.i., in mice given the high infection dose (Figs. 4.9b, 4.10b). Interestingly, both malnourished and well-nourished CBA/Ca mice appeared to recognize similar antigens at this time point, particularly the 4 major antigens of mol. wt. 116kD, 94kD, 67kD and 43kD (Figs. 4.9b; 4.10b). However, while the pattern of antigen recognition further intensified, both quantitatively and qualitatively, in malnourished mice by d.49 p.i., *ie.*, during the adult stages of infection (4.10,b), the immunodetection observed in correspondingly infected well-nourished mice continued to remain restricted despite the increase in duration of infection (Figs. 4.9b).

A feature of the immunoblot analysis carried out in the present study concerns the variability observed in the individual patterns of antigen recognition among mice given similar treatments. Variations in the number of worms harboured by individual mice may partly explain this heterogeneity in antigen recognition among the members of the present genetically inbred mouse strain (Figs. 4.9a,b; 4.10a,b).

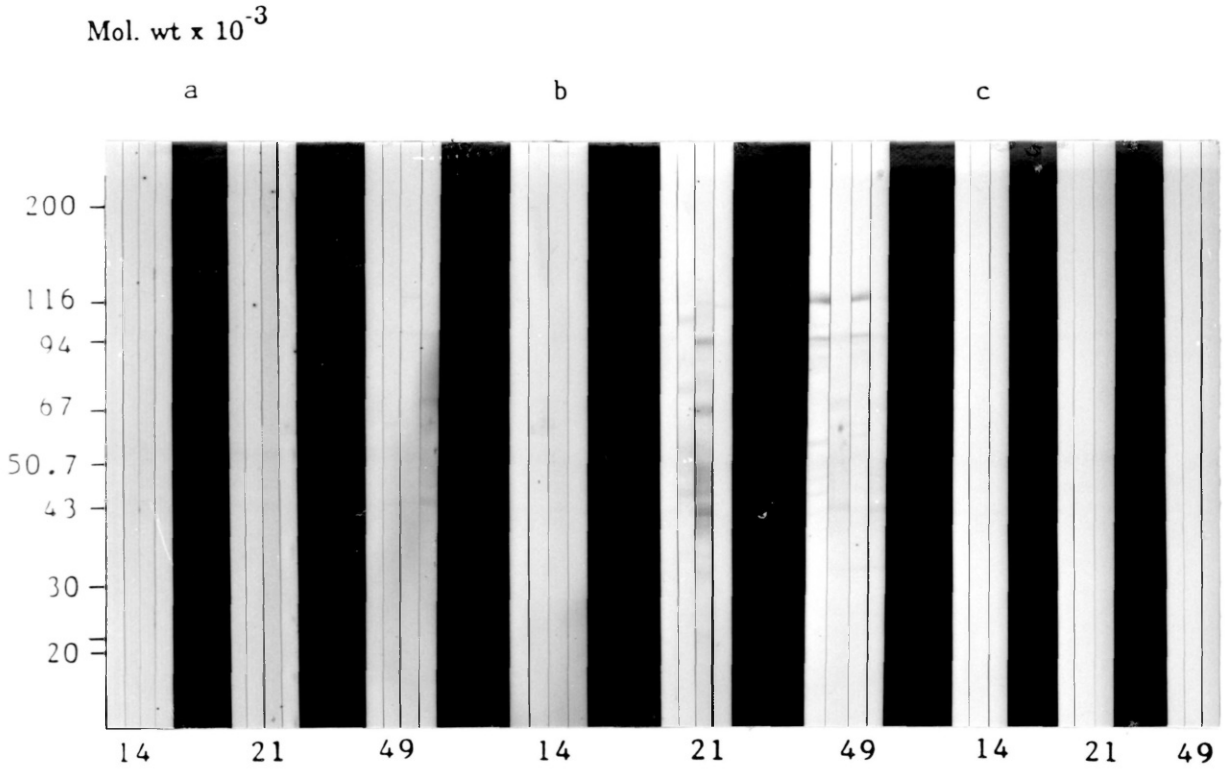

Figure4.9 *Western blot of T. muris E/S antigen-recognition by well-nourished mice during primary infection. (a) mice given 10 eggs, (b) mice given 650 eggs, and (c) uninfected controls. The figures at the bottom denote the days post infection.*

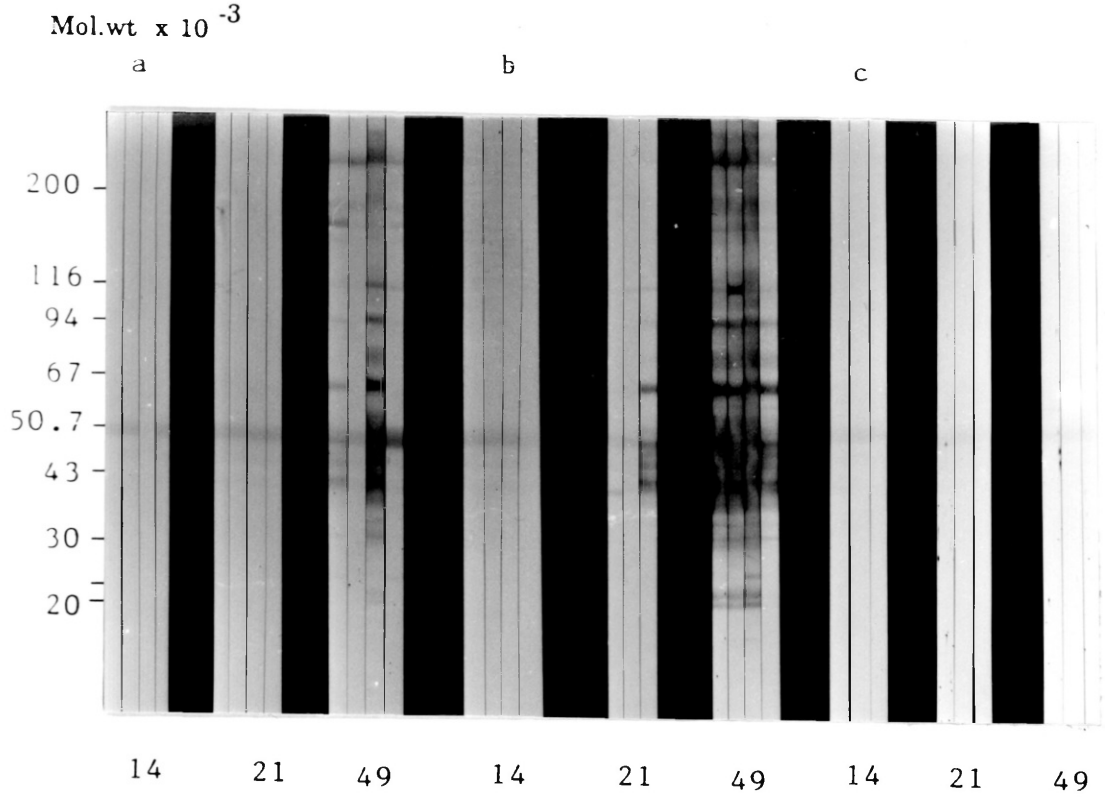

Figure4.10 *Western blot of T. muris E/S antigen-recognition by malnourished mice during primary infection. (a) mice given 10 eggs, (b) mice given 650 eggs and (c) uninfected controls. The figures at the bottom denote the days post infection.*

4.4 DISCUSSION

4.4.1 The effects of dietary protein and infection on CBA/Ca mouse nutritional status

The low protein diet used in the present study did not induce overt or severe malnutrition in CBA/Ca mice. Mice fed on the 4% (by mass) protein diet grew as well as mice fed the higher 16% diet, although the latter animals did grow slightly faster (Fig. 4.1). Despite this insignificant effect on mouse weight gain, the levels of dietary protein used in this study were found to exert a differential influence on the population dynamics of *T. muris*, largely as a result of effects on immunocompetence to infection. This finding suggests that murine functional responses, including immunological function, may become significantly compromised at levels of protein deprivation which have minimal effects on host body weight. The results of this study therefore highlight the importance of moderate or subclinical malnutrition in the transmission of parasitic infections.

In common with the observation of Pike (1972), infection with *T. muris*, irrespective of the diets fed, significantly depressed CBA/Ca mouse growth rate, although this effect was found to be significant only at the higher infective dose of 650 eggs (Fig. 4.1). It is noteworthy that this relationship held for the well-nourished mice given the high infection dose even though these mice had expelled almost the entire primary larval input early during infection. Since these mice continued to produce significantly higher antibody titres in sera when compared with mice given the low infective dose (Figs. 4.6, 4.7, 4.8), this finding of retarded growth in resistant mice may be suggestive that the initiation and maintenance of an effective immunity against parasitic infections may compete with host weight gain for limited physiological resources during infection. In malnourished mice, the presence of larger worm burdens throughout the infection period may further compound this relationship as suggested by the steeper fall in weight gain in these mice (Table 4.2).

Although the mice fed the 4% protein diet consumed significantly greater amounts of food compared with mice fed the adequate protein diet, it is clear that this response did not compensate for the low protein content of the 4% diets. On average, mice fed the 16% diets consumed 4 times more protein than corresponding mice fed the 4% protein diet (Table 4.2). Despite this difference in protein intake, mice belonging to either dietary group grew similarly. This appears to suggest the possibility that when faced with a moderate deprivation of protein, the deprived animal may retain and use nitrogen more effectively in comparison to

well-nourished counterparts given free access to adequate amounts of protein (Golden, 1985).

Infection with *T. muris* increased mouse food consumption in both dietary groups (Fig 4.2, Table 4.2). This finding differs from the results of numerous experimental investigations which demonstrate that helminth infections reduce host food consumption (see Crompton, 1984; Symons, 1985). Since the physiological mechanisms controlling normal food intake are unclear (see Mogenson & Calarasu, 1978; Hall,1975) and parasite-induced reduction in food intake or anorexia may itself be dependent on various factors such as the rate, duration and stage of infection, location of the parasite in infected hosts, as well as the effect of infection upon the physiology, structure and motility of the gastrointestinal tract (see Rosenberg & Bowman, 1984; Crompton, 1984; Symons, 1985), it is conceivable that variations in any one of the above infection parameters may explain the present findings.

4.4.2 The effect of dietary protein on parasite population parameters

The establishment of *T. muris* larvae in the large intestine of CBA/Ca mice at primary infection was not influenced by dietary protein (Table 4.3) This result therefore accords with the majority of the previous findings obtained on this aspect of the protein nutrition-intestinal nematode interaction (see Table 1.3).

Adaptive immunity is unlikely to influence *T. muris* larval establishment in the naive mouse large intestine, given the demonstration that protective immunity against *T. muris* primary infection develops later during infection, normally by 2-4 wks p.i. depending on the mouse strain (Wakelin, 1975a; Wakelin & Lee, 1987). On the other hand, gut physiology and structure, as shown by the importance of caecal content composition to the hatching of embryonated eggs (Panesar & Croll, 1980) and the requirement of initial larval entry into caecal glands in the establishment process (Panesar, 1981), are more likely to affect *T. muris* establishment rate in the naive mouse. Studies on the impact of protein malnutrition on the mammalian gastrointestinal tract have shown that protein deficiency may significantly alter the host intestinal environment, with effects ranging from reduced production and activity of pancreatic enzymes and bile salts to modifications of mucosal histology and gut motility (see Alleyne *et. al.*, 1977). The insignificant effects of protein deficiency on the establishment of *T. muris* in the present study, and of other nematodes in previous studies, most of which investigated the consequences of severe malnutrition, may therefore imply that the potential deleterious effects of protein deprivation, whether severe or moderate, on the gastrointestinal tract may

not be sufficiently disruptive to alter the rate at which intestinal helminths, at least those that establish directly in the intestine, may infect malnourished hosts.

As in the case of the immunocompromised mice studied in chapter 3, the present results also show that the proportion of *T. muris* establishing, irrespective of host nutritional status, was infection-dose dependent, declining significantly at the high infection dose (Table 4.3). This finding further supports the conclusion that *T. muris* establishment in naive hosts is uninfluenced by innate immunity but, may be regulated as suggested in chapter 3, by the availability of establishment sites within the large intestine.

A number of previous studies have, however, reported that protein deficiency may potentiate intestinal nematode establishment in malnourished hosts. This may reflect, on the one hand, the difficulty of interpreting nutrition studies, and one the other, real differences in the biology of the parasite. The first type of problem is illustrated by the study of Bawden (1969) who reported an increase in *H. polygyrus* establishment in mice fed a "low plane" diet. However, an examination of the diets used in that study showed that not only was the protein content of the diets relatively high (12% and 25% crude protein in the "low" and "high" plane diets respectively) but the two diets also varied markedly in their fibre content, being 12 times lower in the "low plane" diet (see Table 1 in Bawden, 1969). The importance of dietary fibre in increasing the establishment rate of intestinal helminths, including *H. polygyrus*, has been demonstrated by Dewitt & Weinstein (1964). In view of this, it is possible that the observed increase in larval establishment reported by Bawden (1969) in the mice fed the "low plane" diet was more a consequence of the difference in the fibre content of the two diets used than protein deficiency *per se*.

An example of the effects of parasite biology is given by the work of Donaldson & Otto (1947) who observed that protein deficiency (8.8% vegetable protein) may increase *N. brasiliensis* establishment rate in naive rats. Recently, Ash *et al.*, (1985), comparing the effects of two levels (2% versus 16% casein) of a semi-synthetic diet, similar to the one used in the present study, on *N. brasiliensis* burdens also found a similar enhancing effect of malnutrition on worm establishment. It should be borne in mind that *N. brasiliensis* larvae, unlike the larvae of the present *T. muris*, undergo extraintestinal migration as part of the life-cycle, before establishing in the small intestine. It has been shown that this migration, especially pulmonary migration, may evoke a potent range of specific and nonspecific host immune reactions, including lung inflammation and eosinophilia (see Coles, 1985). Given this, it is possible as concluded by Donaldson & Otto (1946), that the observed

increase in *N. brasiliensis* establishment in the naive malnourished rat intestine is a result of protein deficiency-induced suppression of host innate immunity acting against migratory larvae. This conclusion implies that the interaction between host nutritional status and the initial establishment of adult intestinal helminths may be modified by the peculiarities of the parasite life cycle. Thus, it is possible that while protein malnutrition may not influence the establishment rate of intestinal nematodes, such as *T. muris* and *T. trichiura*, which hatch and establish directly in the host intestine, it may, on the other hand, significantly potentiate the establishment of helminths, such as *N. brasiliensis* and *A. lumbricoides*, which undertake extensive extraintestinal larval migration.

The pattern of *T. muris* mortality differed in hosts fed either the high or moderately low protein diets (Figs. 4.3 a,b,c,d). However, this effect of dietary protein on worm survival appeared to be modified by rate of infection. Thus in both the well-nourished and malnourished mice given the low infection dose, the worm burdens were not found to change significantly over the 49 day study period, although more *T. muris* mortality was observed in the well-nourished mice (Figs. 4.3 a,b; Table 4.4). These results are consistent with previous work by Wakelin (1973) which established that *T. muris* survival was infection dose dependent, with only low-level infections, below an antigenic threshold of approximately 10-20 infective eggs, normally surviving to patency in the murine host.

It has been previously shown that *T. muris* primary infections, above a threshold dose of 10 eggs, evoke a strong immune expulsion response in CBA/Ca mice around d. 21 p.i., which may almost completely reject such suprathreshold infections from the murine large intestine (Wakelin, 1975a; Lee *et al.*, 1983). The present results suggest that protein deficiency, even at moderate levels, may impair this "spontaneous cure" response, with significant implications for *T. muris* transmission rate. Thus, *T. muris* death rate in well-fed mice given the high infection dose changed significantly with the length of time post infection. The highest rate of worm mortality in these mice occurred during the larval stages (Table 4.4), indicating that the spontaneous cure expulsion of larvae occurred, as normal, around d.21 p.i. On the other hand, in correspondingly infected malnourished mice, the worm death rate was found to be constant and independent of time, implying that the time-dependent spontaneous cure response in these mice may have been either impaired or ablated by protein deficiency (Table 4.4; Figs. 4.3c). As shown in Figs. 4.3c,d , the consequence of this was that while murine trichuriasis is typically acute and transient, in the well-nourished hosts, in malnourished mice,

parasite transmission may be enhanced by the establishment and survival of significant, chronic, patent infections (Figs. 4.3 c).

Studies, of other intestinal nematodes, have invariably shown that dietary protein deficiency increases the survival of intestinal helminths during primary infections (Table 1.3). The ubiquitousness of this feature in malnutrition, especially among the acute nematode infections, suggests that the enhanced survival of intestinal nematodes in the malnourished host is due to protein deficiency-induced impairment in host immunity (both innate and adaptive) rather than due to favourable alterations in the intestinal environment.

The present studies of parasite fecundity suggest that the pre-patent period of *T. muris* may be influenced by both infection dose and host protein nutrition. The time to onset of patency was significantly shortened, irrespective of mouse nutritional status, at the higher infection dose (Figs. 4.4a,b). This inverse effect may be related to the increased probability of mating of dioecious helminths at high densities (Macdonald, 1965; May, 1977). Protein deficiency was also observed to shorten the pre-patent period of *T. muris* and increase the proportion of patent hosts (Figs. 4.4a,b). Since the malnourished mice in the present study harboured significantly greater number of adult worms compared to well-fed mice (Table 4.5), it is likely that an increase in helminth mating probability may also underlie this observation.

An important finding in this study concerns the effect of host nutritional status on net parasite egg production (eggs/gram faeces). Net *T. muris* egg output per mouse was found to be significantly higher in malnourished mice than in hosts fed an adequate protein diet (Table 4.5; Fig. 4.5 a,b). Furthermore, the estimated egg output in the faeces of deficient mice was related to exposure, increasing significantly at the higher rate of infection. By contrast, parasite faecal egg output in well-nourished mice was not only low but also unrelated to exposure (Figs. 4.5a,b). Since the *per capita* fecundity of *T. muris* was uninfluenced by the effects of protein deficiency (Table 4.5), it would appear that the increased parasite egg excretion observed in the malnourished mice was a direct result of the heavier adult worm burdens harboured by these mice (Table 4.5). This finding suggests that helminth transmission rate during primary infection may be enhanced mainly as a consequence of the prolonged survival of parasites in malnourished hosts. The practical importance of this finding is that malnourished hosts may not only constitute the group at increased risk of infection but may also form a significant source of infection to the host community.

The finding that the *per capita* fecundity of female *T. muris* was uninfluenced by the low protein diet suggests that moderate protein malnutrition does not exert a depressive effect on helminth fecundity. Previous studies (Table 1.4) have shown that protein malnutrition may either have no effect on helminth fecundity (Abbott *et al.*, 1985,1986; Slater, 1987) or actually cause an increase in egg production (Dobson & Bawden, 1974; Wagland *et a.*, 1984; Lucker & Neumayer, 1947).

4.4.3 Nutrition-immunity-infection interactions

The aim of the serological analyses carried out in this study was to examine the possibility that protein nutrition associated differences in mouse immunity may underlie the observed differential transmission of *T. muris* between well- and malnourished CBA/Ca mice. Antibody responses were chosen for this investigation because they represent the initial component in resistance to *T. muris* (Wakelin, 1975a; Wakelin & Lee, 1987). It has also been suggested that differences in the time of worm expulsion between mouse strains reflect variation in the level and timing of antibody production during infection (Wakelin, 1975a). Humoral antibody activity was ascertained, in this study, using adult parasite excretory/secretory (E/S) products, which have been shown to be highly immunogenic and relevant to protection in *T. muris* (Wakelin & Lee, 1987). Indeed, it has been shown that immunization with as little as 10*ug* of E/S antigen may induce a significant level of host protection (upto 79% reduction in challenge burdens). Components of the adult E/S antigen complex have also been demonstrated to cross-react with both adult and d.14 larval homogenates as well as antigens derived from embryonated eggs (Wakelin & Selby, 1973; Jenkins & Wakelin, 1977,1983; Wakelin & Lee, 1987), making such antigenic preparations particularly suitable for the present time-course experiments, although the possibility exists that there may be stage-specificity in parasite E/S antigen release (see Ey, 1988).

The present results indicate that primary infection with *T. muris* may elicit, irrespective of CBA/Ca mouse nutritional status, significant time-dependent parasite specific responses with all the 3 antibody types studied (Figs. 4.6, 4.7, 4.8). IgA gave the lowest response and was extremely infection dose-dependent, rising significantly above control values only in the mice given the higher infective dose (650 eggs) (Fig. 4.8b). A previous study (Else & Wakelin, 1989) failed to detect specific IgA in sera (against E/S antigen), perhaps because no infection dose exceeded 400 *T. muris* eggs. These results do not necessarily imply that serum IgA

is unimportant in the protective immune response to *T. muris*. Indeed, IgA monoclonal antibodies have been shown to passively transfer immunity to this parasite (Roach, 1986).

The similarities observed in the response patterns of specific total immunoglobulins and IgG1 (Figs. 4.6, 4.7) provide support for the suggestion that the primary antibody responses to *T. muris* E/S antigen may predominantly comprise an IgG1 response (Else & Wakelin, 1989). Increased production of specific IgG1 during infection has also been reported for other nematodes, notably *H. polygyrus* (Chapman *et al.*, 1979; Williams & Behnke, 1983; Langford, 1989).

Infection dose, irrespective of murine nutritional status, significantly influences both the levels and kinetics of the specific antibody response. Thus, for all 3 antibody types, mice given the higher infection dose produced significantly greater levels of antibodies in sera (Figs. 4.6, 4.7. 4.8). However, antibody production appeared to be dependent upon the stimulation supplied both by initial larval burdens as well as by the number of later stage parasites retained by the mouse. Malnourished mice, perhaps due to their longer retention of parasites, invariably produced higher antibody levels in sera compared with well-nourished mice given a similar rate of infection. The effect of initial larval establishment on antibody production was more clearly seen among well-nourished mice. In these mice, animals given the higher infection dose continued to produce greater amounts of specific antibody in sera throughout the infection in comparison with lightly infected hosts, even though they had expelled the majority of their larval populations early during infection and harboured similar adult burdens as their lightly infected counterparts at the end of the experiment (Table 4.5). The only difference, therefore, lay in the size of the larval populations which established initially during infection, with significantly greater larval burdens establishing in the mice given the higher infection dose (Table 4.3). Else & Wakelin (1989) also showed that specific IgG levels against E/S antigens may continue to rise in the murine host irrespective of whether the parasite has been expelled from the gut. These findings suggest that past larval history, perhaps manifested in the form of residual circulating antigens, plays a role in specific antibody production against *T. muris* (see Behnke, 1987).

A significant finding made in the present study was that infection dose may inversely influence the time of onset of specific antibody production in mouse sera (Figs. 4.6, 4.7, 4.8). Thus, while specific antibodies (all 3 types) appeared during the 3rd week of infection in mice given the high infection dose, significant antibody

production was delayed until either the 4th week (specific total immunoglobulin and IgG1) or later (specific IgA) in mice given the low subthreshold infection. Since immune expulsion of *T. muris* occurs around d.21 p.i. in CBA/Ca mice, it is conceivable that this inverse relationship between infection dose and the onset of specific antibody production may play an important role in the dose-dependent immune regulation of worm numbers in murine trichuriasis (Wakelin, 1987). Mice given suprathreshold infections (above 10 eggs) may mount a protective response against *T.muris*, largely because of the generation of substantial amounts of potentially protective specific antibody in sera by d.21 p.i., *i.e.,* at the time of worm expulsion (Figs. 4.7b, 4.8b). By contrast, in mice given the low subthreshold infection, specific antibody rose significantly after the normal time of expulsion (Figs. 4.6a, 4.7a, 4.8a).

This may be too late to be effective since it has been suggested that *T. muris* stages surviving beyond d.21 p.i. may be unaffected by any subsequently developed immunity, either as a result of increased size or due to possible immunomodulatory activities of such stages (Wakelin & Lee, 1987; Else & Wakelin, 1988).

Moderately protein malnourished CBA/Ca mice were found to produce significantly greater amounts of specific antibodies (all 3 types) in sera when compared to their well-nourished counterparts (Table 4.5). This finding is in accordance with numerous other studies in protein malnutrition (see section 1), that mammalian B-cell function may be unimpaired in moderate-severe protein malnutrition.

Several factors may underlie the increased antibody response in the malnourished mice. Malnourished mice may produce greater levels of parasite-specific antibody because of their higher worm burdens. This may reflect a need for more antibodies to react against the greater amounts of parasite antigens encountered in these hosts. (Else & Wakelin, 1989). However, other mechanisms primary to protein deficiency *per se*, may also underlie the increased antibody response observed in the malnourished host. For example, Pocino & Malave (1981) demonstrated that *in vitro* antibody response may be enhanced during dietary protein restriction as a result of defects in the normal regulation of antibody synthesis. The authors showed that the mouse primary antibody response may be enhanced in moderate protein malnutrition (8% casein) by a selective depletion of suppressor inducer T-cells during the first weeks of dietary restriction. Thus, it possible that down- regulation of antibody synthesis in response to antigen may be impaired in moderately malnourished animals.

Alternatively, the increased specific antibody response may reflect a greater systemic spread of parasite antigens. Several mechanisms have been suggested to contribute to the increased uptake of antigen in protein malnutrition: increased permeability of the gut wall (Rothman *et al.*, 1982a,b); reduced secretory IgA clearance of antigen at the mucosal surface (Watson *et al.*, 1985); and impaired removal of antigen by the hepatic reticuloendothelial system (Chandra, 1977).

The principal finding in this study was that although the specific antibody response appeared to be intact in both the well-nourished and malnourished CBA/Ca mice, the increase in antibody was found to be associated with worm expulsion only in the case of the well-fed mice. The expulsion of *T. muris* involves at least two immunologically mediated components, an initial antibody-mediated phase and a subsequent lymphoid cell-mediated phase (Wakelin, 1975a; Wakelin & Lee, 1987). The lack of effectiveness of the intact antibody response in malnourished mice in the present study may be due to protein nutrition associated changes in mouse cellular immunity. This conclusion is suggested by the general finding that moderate-severe protein malnutrition principally disrupts cell-mediated immunity (CMI) as opposed to B-cell function (including antibody production) (Narayanan *et al.*, 1977; see section 1). Although numerous aspects of CMI may be adversely affected by protein malnutrition, the findings of depletion in the numbers of intraepithelial lymphocytes and mucosal T-cells (Allardyce & Bienenstock, 1984; see section 1), and impaired mesenteric lymphoblast localization in the intestinal mucosa (McDermott *et al.*, 1982) may be particularly relevant to mouse protective immunity to *T. muris*, given the suggestion that the immune expulsion of *T. muris* may be effected directly by the activated mesenteric lymph node derived cytotoxic T-cell subpopulation of the intraepithelial lymphocytes (see Wakelin & Lee, 1987).

Although impaired cellular immunity may constitute the most likely candidate, it is possible that other factors, more intrinsic to antibody function, may also partially contribute to the abrogation of the normal worm expulsion response observed in the malnourished mice. Thus, it is possible that differential antigen recognition may underlie the functional difference in the humoral immunity observed between well-nourished and protein deficient mice. For example, it is recognized that cell-surface glycoproteins may be altered in protein-energy malnutrition (PEM) (Chandra, 1983a). However, the results of the Western blot analysis carried out in this study suggest that this may not be relevant in the present situation since the IgG1-antigen recognition profiles obtained for both the highly infected well- and malnourished mice are broadly similar at the expected time of worm expulsion (Figs. 4.9 & 4.10). This may, however, be too simplistic an interpretation as it has also

been shown that antibody affinity may be reduced significantly in PEM (Passwell *et al.*, 1974; Chandra *et al.*, 1984), and that protein deficiency may facilitate the induction of antibody tolerance in deficient animals (Stokes *et al.*, 1983; Lamont *et al.*, 1987a,b), possibly as a result of defective intravascular clearance of antigen from the general circulation (Gershwin *et al.*, 1985).

The Western blot analysis carried out in this study highlighted further points concerning the effects of infection dose and dietary protein on antigen recognition by specific IgG1, although the immunoblots did not support the suggestion that differential antigen recognition is a factor in the altered anti-*T. muris* immunity observed in the malnourished animals.

The time course of immunodetection of antigen by mouse sera was found to be infection dose dependent (Figs. 4.9, 4.10). In both dietary groups, mice given the low infection dose detected antigens only at the chronic stage of infection (*i.e.*, in the d.49 serum samples), while antigen detection by the highly infected mice occurred much earlier, at least by d.21 p.i. The pattern of antigen recognition, therefore, mirrored the quantitative estimates of the specific IgG1 temporal response shown in Fig. 4.7. Together, these findings strongly suggest that mouse specific antibody production during *T. muris* primary infection may be directly driven by the amount of parasite antigens encountered or detected. An important consequence of this is that mice given the low infection dose fail to detect parasite antigens at the expected time of worm expulsion, *i.e.*, on d.21 p.i. (Figs. 4.9a, 4.10a). Subthreshold *T. muris* infections may, therefore, survive in the mouse host because they fail to produce sufficient antigenic material to evoke a protective response at the appropriate time. This mechanism may underlie the demonstrated infection threshold-dependent immune expulsion and hence regulation of worm numbers in murine trichuriasis (see Wakelin, 1987).

The immunoblot analysis suggests that antigen recognition by IgG1 at the expected time of worm expulsion (d.21 p.i.), appears to involve 4 main antigenic components, of mol. wt. 116kD, 94kD, 67kD and 43kD (Figs. 4.9b, 4.10b). This finding, obtained in both malnourished and well-nourished CBA/Ca mice given the high infection dose, may suggest a protective role for these antigens. Indeed, preparations containing the 43kD antigen have been successfully used to induce significant immunity against *T. muris* challenge infections (see Wakelin & Lee, 1987; Jenkins & Wakelin, 1983).

The Western blot results also show that during the chronic stages of infection (*i.e.*, on d.49 p.i.), malnourished mice, at both infection levels, detect a wider range of antigens in sera in comparison with similarly infected well-nourished mice (Figs. 4.9, 4.10). Since the range of antigens recognized on d.49 p.i. by the deficient mice given the high infective dose, also appeared to increase over that detected by d.21 p.i. serum samples (4.1 0b), it is possible that a large portion of the increased specific antibody production in the protein deficient mice was directed against irrelevant antigens. Such a response could form one of the mechanisms by which the suggested immunomodulatory activity of the later stages of *T. muris* may operate (Else & Wakelin, 1988).

The time-dependence of antigen recognition may be suggestive of stage-specificity in *T. muris* antigen release. The finding that the well-nourished mice given the high infection dose (in which worm expulsion occurred) detected similar antigens during both the larval (d.21p.i.) and chronic stages (d.49 p.i.) (4.9b), provides additional support to the suggestion that part of the specific antibody response in primary murine trichuriasis may be against residual larval antigens.

In conclusion, this study has shown that marginal protein malnutrition may enhance the survival but not the establishment and *per capita* fecundity of *T. muris* during primary infection. The enhanced survival appears to be related to the suppression of cell-mediated immunity and unrelated to antibody, either serum levels or immunorecognition, both of which are elevated in the malnourished animals.

CHAPTER 5 THE EFFECT OF DIETARY PROTEIN ON THE IMMUNOLOGY AND POPULATION DYNAMICS OF *TRICHURIS MURIS* REPEATED INFECTIONS

5.1 INTRODUCTION

Although primary infection studies, such as those documented in Chapter 3 and 4; may provide important information concerning the innate rate parameters governing the abundance of a given parasite population in a naive host, they may not necessarily reflect the natural situation in the field where the mammalian host, including humans, is more likely to be continually exposed to infection over time (Anderson & May, 1985; Behnke, 1987). Under the latter infection situation, the parasite burden within an individual host at a given time point may be considered to result from a dynamic interplay between the rates of exposure, recruitment (establishment) and mortality of the parasite (Anderson & May, 1985; Crombie & Anderson, 1985). It is thus possible that the rates of the parasite population parameters (initial parasite establishment, survival and fecundity) estimated during primary infection may be modified under the conditions of repeated infection (Keymer & Hiorns, 1986a; Anderson & Crombie, 1985). As such, it is important that laboratory experiments aimed at investigating the factors which may affect the dynamics of parasite populations within a particular host community should recognise this aspect of natural exposure to infection (Crombie & Anderson, 1985; Anderson & May, 1985; Keymer, 1985; Barger, 1987).

In the laboratory, the continuous infection phenomenon may be best studied by employing what is often referred to in the literature as the repeated or trickle infection technique, in which hosts are repeatedly infected with constant numbers of parasite infective stages over regular intervals of time (Anderson, 1987). The inclusion of serial sampling under such infection conditions would allow the elucidation of the dynamical aspects of parasite abundance. Such methods may, in addition, also facilitate a more "realistic" but controlled testing of theoretical predictions concerning the manner in which the mean parasite burden may change with host age during continual infection (Anderson & Crombie, 1985; Anderson, 1987; Keymer & Hiorns, 1986a).

A number of studies have attempted to investigate the population dynamics of nematode parasites during repeated infections. In the field, Michel (1963,1969) showed that parasite burdens may rise to, and remain roughly constant at, levels which were positively related to infection rate in calves infected daily with *Ostertagia ostertagi*. The results were perceived principally as being indicative of worm population regulation during repeated infection by a density-dependent turnover of adult worms, although a gradual decline in the proportion of incoming larval establishment was also noted (Michel, 1970). This conclusion is further supported by a recent mathematical re-analysis of the data which showed clearly that the observed changes in the mean worm burdens in the calves may be explained by a decline in the proportional establishment of ingested L3 larvae, and a rise in the death rate of the 5th-stage worms as the duration of exposure to infection increased (Grenfell *et al.*, 1987). Barger *et al.* (1985) revealed that a broadly similar, though more complex, regulatory mechanism may also operate in the case of *Haemonchus contortus* population growth in sheep repeatedly exposed to infective larvae. In this study, peak parasite burdens per host were reached between 6 & 9 weeks of infection, the magnitude of which was found to be related to infection rate. Subsequently, worm burdens fluctuated around this maximal level in sheep given low rates of infection, but declined rapidly in corresponding hosts given high rates of repeated infection (Barger *et al.*, 1985). Using radiolabelling techniques, the authors further showed that, apart from adult worm loss, the establishment of incoming larvae may also decline markedly during repeated infection. The proportion of incoming larvae arrested in their development may also increase as the infection progressed. It was concluded that *H. contortus* numbers may be regulated by both the development of resistance to infection and by a loss of established worms which was related to current rate of larval intake as well as the host's previous experience of infection (Barger *et al.*, 1985). Donald & Walker (1982), on the other hand, provided evidence for a different regulatory pattern in the case of *Trichostrongylus vitrinus* and *T. colubriformis* repeated infections in sheep. In these infections, it was observed that adult worms may accumulate during repeated infection until further increase in numbers was prevented by an increasing failure of incoming larvae to establish. At this stage, previously established worms persisted for a variable period without apparent loss but were ultimately expelled. In general, therefore, these field studies have tended to emphasize the role played by regulatory mechanisms in preventing the unrestricted growth of intestinal nematode populations in the face of continual exposure to infection. Although most authors have considered host acquired immunity to reinfection (acting either to reduce parasite establishment and/or increase parasite mortality) to be the major regulatory mechanism mediating parasite abundance during repeated infection,

other factors unrelated to immunity may also play equally important roles. Thus, it is possible that *Trichostrongylus* spp. may continue to accumulate with time during repeated infection mainly as a result of a long adult life span. On the other hand, *H. contortus* may exhibit a more rapid population turnover during continual infection owing to the parasite's shorter adult life expectancy (Courtney *et al.*, 1983).

In the laboratory, Kerboeuf (1982) and Keymer & Hiorns (1986a) have studied repeated infections of *H. polygyrus* in mice. In each case, the mean worm burden per mouse increased to a peak during the first few weeks of a repeated infection, but while worm numbers declined later during infection in male CD1 Swiss mice given increasing doses of larvae (Kerboeuf, 1982), the average worm burden plateaued at peak levels in the case of repeated infection with constant larval doses in outbred MF1 mice (Keymer & Hiorns, 1986a). The latter authors also showed a concomitant increase in the variance to mean ratio of the parasites recovered from infected mice over the period of trickle infection. This result was interpreted as being indicative of a genetically-based heterogeneity in host immunocompetence to reinfection among individuals of the outbred MF1 mouse strain used. Keymer & Hiorns (1986a) further analyzed their data using a mathematical model that simulated repeated infection on the basis of the dynamics of primary infection. The comparison of observed data with predictions of the model yielded evidence for the density-dependent regulation of parasite population growth during repeated infection. It was suggested that acquired immunity to reinfection may be the most likely causative factor underlying the observed regulation of *H. polygyrus* numbers during repeated infection.

Using the trematode parasite, *Schistosoma mansoni*, Crombie & Anderson (1985) showed that changes in parasite load with duration of host (mouse) infection may depend on the intensity of exposure. It was found that age- intensity profiles of mean adult worm recovery may vary from a monotonic rise to a stable plateau at low weekly infection rates, to a highly convex form at the highest infection level.

The most dramatic difference in nematode population dynamics between primary and repeated infection regimes may be observed in the case of *Nippostrongylus brasiliensis* infections in rats (Jarrett *et al.*, 1968; Jenkins & Phillipson, 1970). Heavy primary infections with *N. brasiliensis* normally elicit an acute spontaneous cure response in the rat host which may expel most of the adult worms on or soon after the 10th day of infection. Jenkins & Phillipson (1970), however, showed that this acute immune response may be circumvented and significant worm burdens accumulated by trickling rats with low numbers of infective larvae (5

larvae/weekday). This accumulation of *N. brasiliensis* burdens during repeated infection, however, may be modified by infection dose, higher rates of infection resulting in lower recovery of worm numbers towards the later stages of infection (Jenkins & Phillipson, 1970). Partial resistance to reinfection may also occur during repeated infection since stunted worms were recovered and the percentage "take" or establishment of the parasite appeared to be lower than that found in typical primary infections. These findings suggest that the population dynamics of *N. brasiliensis* may differ considerably between primary and repeated infections principally as a result of changes induced by the type of infection exposure, whether single or repeated, in the host immune response.

Some direct evidence for altered function of host immunity during repeated infections may be derived from the observations of Moqbel (1980), which suggest that the immune response of adult rats (as assessed by both parasitological and histopathological parameters) against *Strongyloides ratti* may be retrophasic. In this, immunity was found to be aimed solely against intestinal phase during primary infection, both pulmonary and intestinal phase during secondary infection, and the skin migratory phase following repeated infection, thus clearly indicating the dynamical nature of the developing mammalian immunity during repeated infections. Further it is also possible in the case of nematode infections, as in rodent schistosomiasis, that the immunity elicited in the host by adult parasites during repeated infection may be directed towards incoming larval stages (see Behnke, 1987). Such a possibility may explain the frequently reported increased resistance of the repeatedly infected mammalian host against incoming larvae in most of the nematode infestations discussed above. Several studies with the nematode, *H. contortus* have shown that this resistance to reinfection may be crucially dependent on the prolonged and uninterrupted presence of parasitic infection (Dineen & Wagland, 1966; Wagland & Dineen, 1967; Donald *et al.,* 1969). The early curtailment of repeated infection by drug treatment may result in a relatively rapid loss of resistance. By contrast, Kerboeuf & Jolivet (1984) demonstrated that in mice given increasing doses of *H. polygyrus* larvae, drug treatment administered early during infection produced marked resistance to subsequent infection, whereas a similar effect was not observed for the late treatment. These findings indicate not only the complex dynamical nature of the developing immunity during repeated

nematode infections, but also the complications which may be induced in the host immune response by the immunomodulatory activities of the adult parasites themselves.

To date, relatively little work has been done on repeated infections of *T. muris*. Available work suggests that repeated infections with *T. muris*, unlike that of *N. brasiliensis*, may not cause stable worm burdens in responder mouse strains such as TFI or CFLP (Wakelin, 1973; Behnke & Wakelin, 1973). As in the case of primary infection, it appears that once the immune threshold level is exceeded during repeated infection, strong immune expulsion responses are triggered which may remove the worm population in its entirety. Subsequent work by Behnke *et al.*, (1984), however, established that the survival of *T. muris* repeated infections may be enhanced in either chemically immunocompromised hosts or in mice infected concurrently with the immunomodulatory nematode, *H. polygyrus*. Behnke *et al.* (1984) also demonstrated that some worms may mature successfully during trickle infection in the low responder $C57BL_{10}$ mice. These results therefore appear to suggest that repeated infections of *T. muris* may survive in hosts which are immunocompromised, whether genetically based, induced chemically or due to the immunomodulatory effects of other infections. Behnke *et al.* (1984) further noted that the immunity elicited by adult worms during repeated infection may be directed largely against incoming larvae while the initial worms to establish may remain relatively unaffected.

A number of studies have investigated the effects of protein deficiency on the population dynamics of nematode parasites during repeated infection. Thus, Foster & Cort (1932,1935) showed that malnourished dogs may accumulate considerably higher *Ancylostoma caninum* burdens during repeated infection than correspondingly infected well-nourished counterparts. The authors attributed this to defects in resistance to reinfection in the dogs fed the deficient diet, which was restored rapidly when such hosts were replaced on balanced diets. A similar finding was also obtained by Donaldson & Otto (1947) with regard to the effects of protein deficiency on repeated *N. brasiliensis* infections in rats. Their results showed that while hosts fed the balanced diet had developed an highly protective immunity manifested by low worm recoveries, those on the deficient diet appeared highly susceptible and contained considerably higher worm burdens at necropsy. The authors also demonstrated that restoration of rats fed on deficient diets onto the balanced diet before the administration of infection resulted in the development of a very pronounced immunity.

The most complete investigation to date into the effect of protein malnutrition on the dynamics of nematode parasites is that of Slater & Keymer (1986a,b) on chronic *H. polygyrus* infections in outbred CD1 mice. Their results clearly indicated that while *H. polygyrus* abundance in malnourished mice (2% casein) accumulated in direct relation to the rate of host infection, in well-nourished hosts, parasite population growth was checked by the effects of an effective acquired immunity, acting principally to reduce the survival of adult worms. The authors also found that the *per capita* fecundity of the parasites was significantly depressed in hosts fed the 8% protein diet in comparison with estimates obtained in their malnourished counterparts.

The main aim of the present study was to investigate the impact of host protein nutrition on the dynamics of *T. muris* during repeated infections, in order to obtain a more realistic picture of the role of nutrition in the transmission of *T. muris*. As in the case of the primary infection study (Chapter 4), serological analysis, both quantitative (levels of antibody) and qualitative (antigen-recognition), was performed with a view to ascertain the role of specific antibody responses in the differential transmission of *T. muris* in well- and malnourished CBA/Ca mice. The study also examines the interaction between the degree of mouse protein nutriture (normal nutrition, moderate and severe malnutrition) and *T. muris* population dynamics. Preliminary growth trials (chapter 2) suggested that it may be possible to model the above states of protein nutriture in the CBA/Ca mouse host by feeding mice with 16%, 4% and 2% casein diets respectively.

5.2 METHODS AND EXPERIMENTAL DESIGN

Male CBA/Ca mice, at 3-4 weeks of age, were used in all experiments described in this chapter. The techniques employed to feed and infect mice, recover larval and adult *T. muris* and count parasite eggs are described in chapter 2. The nutritional composition of each of the 3 diets (16%, 4% and 2% casein) used in the present study is given in table 2.1. The immunological techniques used to collect sera, obtain *T. muris* E/S antigen and determine both the amount of specific antibody in the mouse sera (ELISA) as well as antigen recognition by specific IgG1 (Western blot analysis) are as described in chapter 2. The mice were normally housed 5 to 10 in cages fitted with wire bottoms and given the diets and water *ad libitum* at all times.

All mice were weighed on receipt and randomly assigned to each of 3 dietary groups containing either 16%, 4% or 2% (w/w) casein. Mice were acclimatized onto the artificial diets for a period of 2 weeks before infection. On the day of infection (day 0), the mice in each dietary group were further subdivided into 3 treatment groups- one uninfected control group, and 2 experimental infected groups, each receiving either a low infection rate of 5 eggs/mouse/10 days or a high infection rate of 50 eggs/mouse/10 days (Table 5.1). 10 days later, cohorts of mice chosen at random from each treatment group (usually 5 experimental and 3 control) were examined for parasite numbers, while the remaining experimental animals in each dietary group were reinfected with the appropriate number of eggs. This 10- day regime of reinfection and *post mortem* examination was continued until the end of the experiment on d. 60 post initial infection (p.i.i.). Prior to their *post mortem* examination, each mouse was weighed to monitor nutritional status and blood was collected via the tail vein for immunological analysis.

Faecal egg counts to estimate *T. muris* fecundity under the various treatments were performed on grouped faecal samples collected from 5 hosts chosen at random from the mice that remained in each infection group following each 10- day worm assessments after d. 30 p.i.i. Egg counts were carried out on mouse groups, thus selected, every two days from d. 30 p.i.i. onwards until the end of the experiment on d. 60 p.i.i.

Table5.1 *Summary of the experimental design.*

group[a]	No. of mice per group
2% diet	
control	18
5	34
50	34
4% diet	
control	18
5	30
50	30
16% diet	
control	18
5	30
50	30

a approximate numbers of eggs per 10 days

The kinetics of parasite E/S antigen specific total immunoglobulins (IgGAM), IgG1 and IgA production was examined by determining the titres of antibody contained in each mouse blood sample obtained during infection. E/S antigen recognition by specific IgG1 was analyzed in representative sera (3-4) obtained from each mouse group corresponding to the periods of early (d.20 p.i.i.), mid (d.40 p.i.i.) and late (d.60 p.i.i.) phase of repeated infection.

The experimental design followed and the numbers of mice used are summarized in Table 5.1.

5.3 RESULTS

5.3.1 Host survival and nutritional status

The concentration of protein in the diets used in the present study did not influence the survival of control or repeatedly infected CBA/Ca mice. Fig. 5.1 shows the cumulative body weights of the control and infected mice fed each of the 3 diets during the course of the present study. As expected, mice fed either the normal (16%) or 4% casein diet gained weight steadily over the entire period studied. By contrast, control mice fed the 2% protein diet gained little weight during the course of the experiment, indicating a severely malnourished state. The growth rates of mice fed either the 16% or 4% protein were not found to differ significantly (ANOVA: $F= 0.60$, $d.f = 1$; $P >.05$). Repeated infection with constant numbers of *T. muris* eggs also did not appear to affect the growth of these mice at either rate of infection studied (2- way ANOVA: $F= 0.07$, $d.f = 2$; $P >.05$).

In contrast, as shown in Fig. 5.1, repeatedly infected severely malnourished mice lost weight significantly, in comparison with control animals, at both the levels of infection used (low infection: TK test: $q = 4.50$, $d.f. = 2,186$; $P <.01$, high infection: TK test: $q = 5.43$, $d.f. = 3,186$; $P <.001$). There was, however, no significant difference in weight loss among severely malnourished hosts given either the low or high trickle infection dose (TK test: $q = 1.17$, $d.f. = 2,186$; $P >.05$).

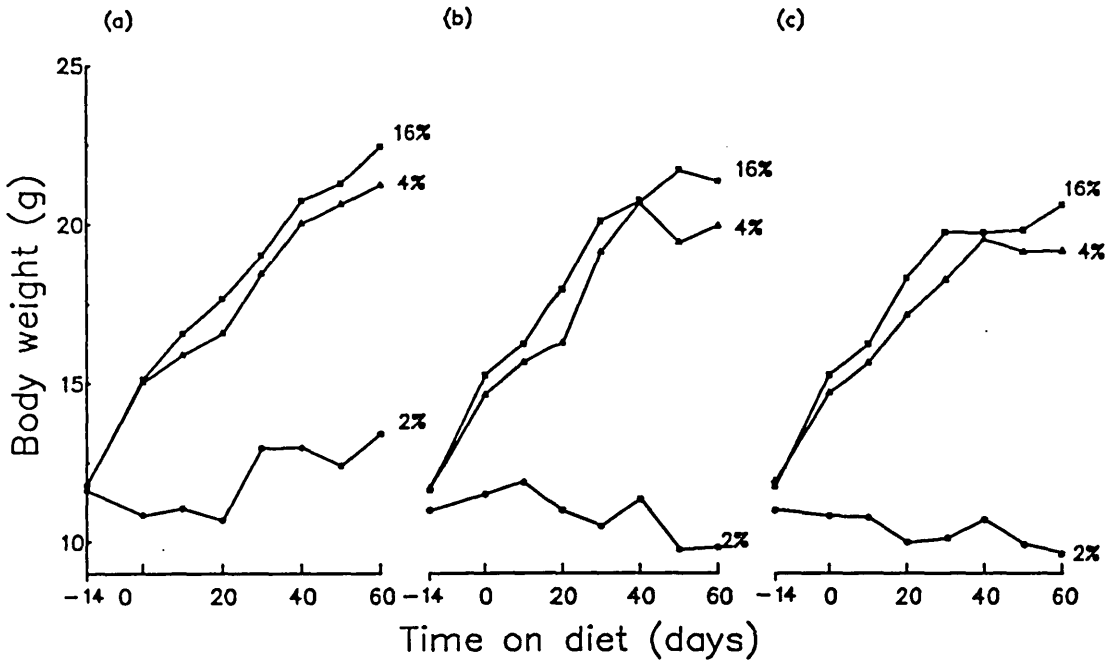

Figure5.1 *The effect of dietary protein content on the cumulative body weights of mice given either (a) no infection or (b) 5 and (c) 50 T. muris infective eggs per 10 days. Closed symbols represent the mean body weights (g) of the various mouse groups.*

5.3.2 Recovery of adult and larval parasites

The mean numbers of adult parasites recovered from mice fed each of the protein diets are depicted in Figs. 5.2a,b,c (low infection dose) and 5.3a,b,c (high infection dose). The results clearly demonstrate that mouse protein nutriture may play an important role in the establishment of *T. muris* repeated infections. As shown, despite being repeatedly infected, and irrespective of exposure rate, very few adult worms were recovered from the well- nourished CBA/Ca mice fed the 16% protein diet (Figs. 5.2a,5.3a). Furthermore, the number of adult worms recovered appeared to decrease with time post initial infection at both levels of repeated infection, such that by d.50 p.i.i. the mean number of parasites recovered did not differ significantly from zero (low dose: $t = 1.59$, $v=8$; $P > 0.05$, high dose: $t = 0$, $v = 8$; $P > 0.05$)(Figs 5.2a, 5.3a) (Table 5.2). There was also no significant difference in the mean number of adult worms recovered in the well- nourished mice with regard to the levels of repeated infection studied (ANOVA: $F = 2.19$, d.f. = 1; $P > 0.05$).

In contrast, both groups of malnourished mice accumulated adult parasites at a rate proportional to the rate of exposure from d.30 p.i.i onwards (the time delay corresponding approximately to the mean maturation period of *T. muris* (chapter 1)) (Figs. 5.2b,c, 5.3b,c) (Table 5.2). The small increase in adult worms observed in both

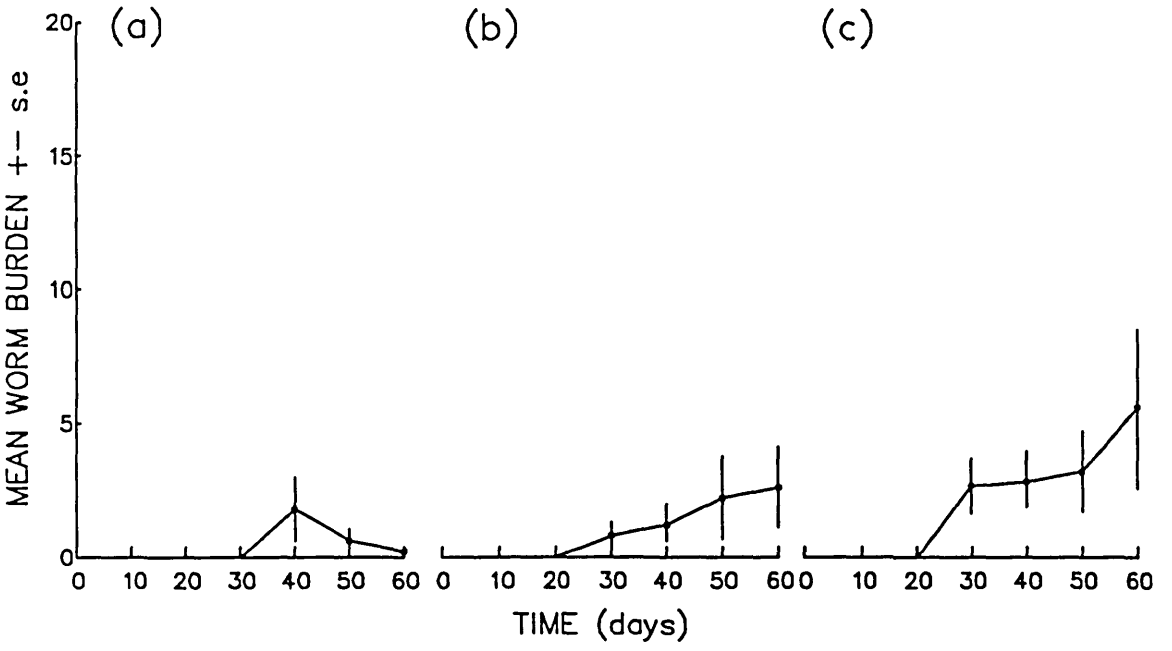

Figure5.2 *Recovery of adult worms over time from mice fed on (a) 16%, (b) 4% and (c) 2% protein diets. Mice were repeatedly infected with 5 eggs every 10 days. Bars represent standard errors of the means.*

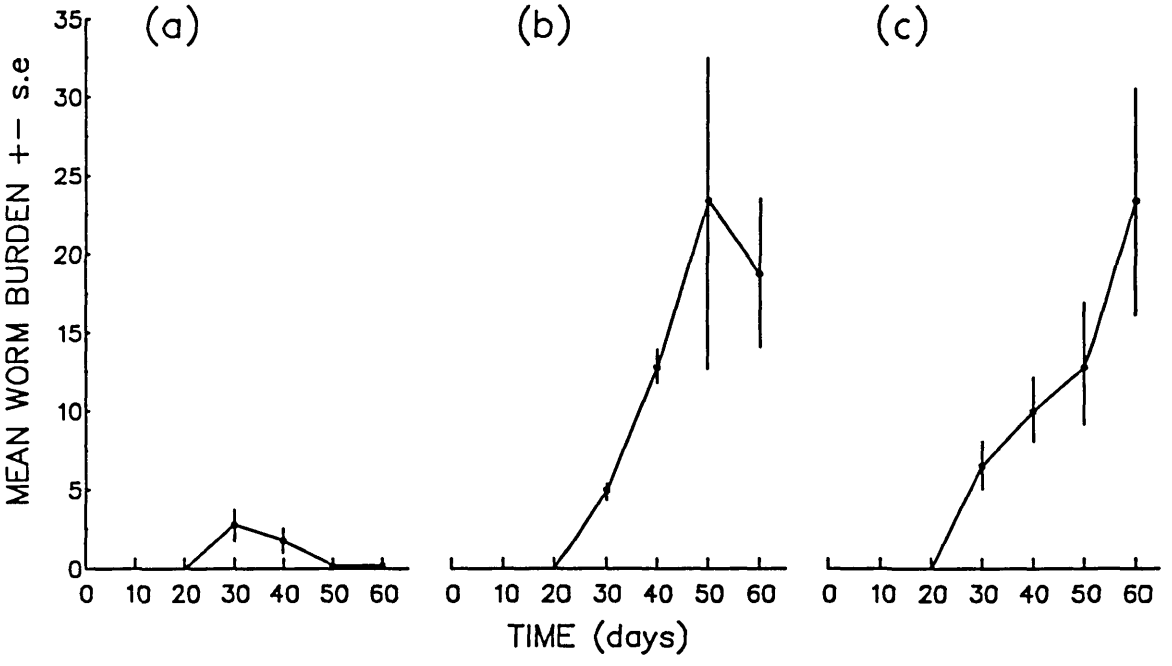

Figure5.3 *Recovery of adult worms from mice repeatedly infected with 50 eggs every 10 days and fed diets containing (a) 16%, (b) 4% or (c) 2% protein diets. Bars show standard errors of the means.*

Table5.2 *Regression coefficients (b +- s.e$_b$) for the relation between time (d. 30 to 60 p.i.i.) and adult worm burden during repeated infection with T. muris in mice fed different levels of protein and given varying rates of infection.*

Group	b +- s.e$_b$
2% diet	
5	0.009 +- 0.013$^{n.s}$
50	0.031 +- 0.011*
4% diet	
5	0.021 +- 0.012$^{n.s}$
50	0.031 +- 0.013*
16% diet	
5	-0.025 +- 0.018$^{n.s}$
50	-0.040 +- 0.009**

$^{n.s}$ slope not significantly different from zero

* $p<0.05$ ** $p<0.01$

groups of malnourished mice given the low infection intensity, however, was not statistically significant, perhaps because of the high rate of variability (Figs. 5.2b, 5.3b) (Table 5.2). Lowly infected mice fed the 2% diet were observed to harbour significantly more mean worm burdens compared to similarly infected counterparts fed the 4% casein diet (TK test: q = 3.21, d.f. = 2,79; P< 0.025). By contrast, worm burdens appeared to increase linearly until the end of the experiment in either group of malnourished mice given the high infection dose (Table 5.2) (Figs. 5.3b,c), although there was a suggestion of a slight turnover in worm numbers during repeated infection in the hosts fed the marginal 4% protein diet (Polynomial regression: c = -.003, s.e. = .001, t = -2.01; P = 0.06) (Fig. 5.3b).

Fig. 5.4a,b portrays the mean numbers of *T. muris* larvae (at varying stages of development) recovered from CBA/Ca mice fed on each of the 3 protein diets during repeated infection. As can be seen, in each mouse dietary group and at both rates of infection, larval burdens rose from d.10 to 20 p.i.i., following which there appeared to be a tendency towards a decrease. The pattern of change of mean larval numbers through time, however, varied according to both infection dose and mouse nutritional status. At the low infection exposure of 5 eggs/mouse/10 days (Fig. 5.4a) regression analysis showed that larval recovery did not change significantly with time in the case of malnourished mice fed either the 4% or 2% protein diet (4% diet: F = 2.93, d.f. = 1; p.05, 2% diet: F = 4.00, d.f. = 1; P >.05). By contrast, larval

recovery from correspondingly infected well-nourished mice appeared to decline significantly with respect to time, the rate of decline varying with time (Cubic polynomial regression: F = 15.46, d.f. = 3; P< .001).

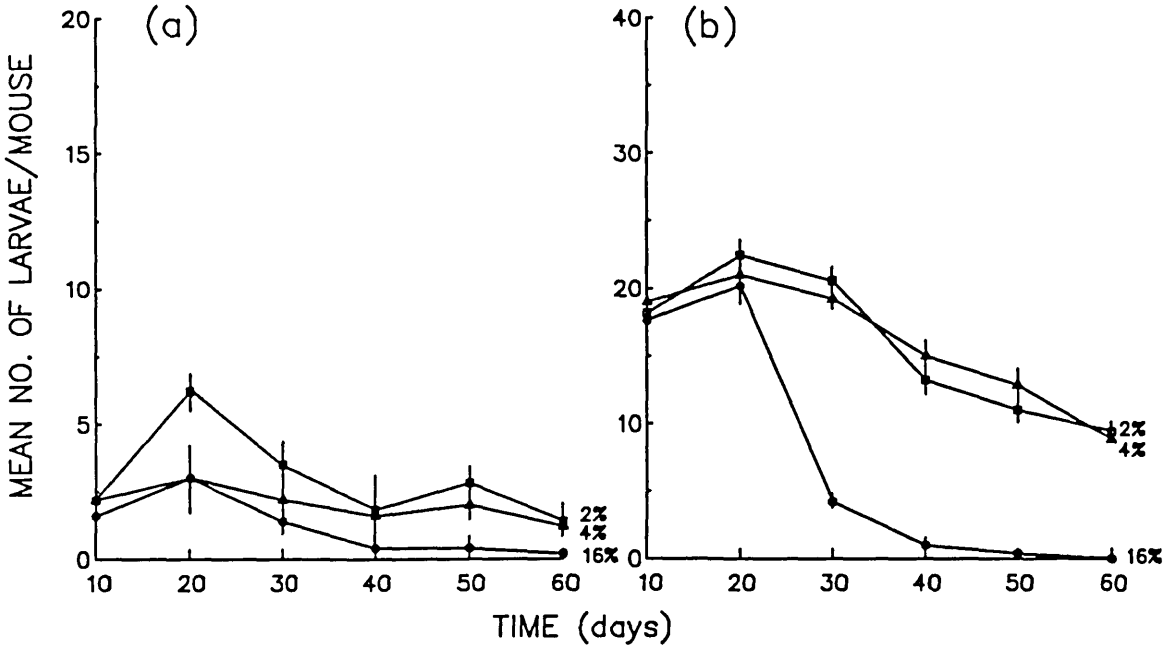

Figure5.4 *Recovery of larval worms over time in mice fed varying levels of dietary protein. Mice were repeatedly infected with either (a) 5 or (b) 50 T. muris eggs every 10 days. Bars represent standard errors of the means.*

In hosts given the high infection dose, mean larval recovery declined significantly for each of the 3 dietary treatments studied (Fig. 5.4b). The mean larval burdens obtained from both groups of malnourished mice appeared to decrease at a gradual, constant rate, however the rate of decline in larval numbers in well- nourished mice was significantly time- dependent (Fig. 5.4a,b) (Table 5.3). The cubic polynomial once again was found to adequately describe the temporal change in larval burdens in highly infected well- nourished CBA/Ca mice (Cubic polynomial regression: F = 67.02, d.f. = 3; P< .001).

It therefore appears that the temporal pattern of larval recovery during *T. muris* repeated infection in well- nourished, 'normal' CBA/Ca mice was similar at both the levels of infection presently studied. Thus, at both rates of infection, *T. muris* larval establishment initially increased during repeated infection (from d.10 to 20 p.i.i.) and then declined at a rapid rate from d.20 to 40 p.i.i., following which the well-

Table 5.3 *Regression coefficients (b +- $s.e_b$) for the relationship between time (d.10 to d.60 p.i.i.) and larval recovery during repeated infection in hosts fed different levels of protein and given varying rates of infection.*

Group	b +- $s.e_b$	b_1 +- $s.e_{b1}$	b_2 +- $s.e_{b2}$
2% diet			
5	-0.009 +- 0.005$^{n.s}$	-	-
50	-0.015 +- 0.006*	-	-
4% diet			
5	-0.007 +- 0.004$^{n.s}$	-	-
50	-0.026 +- 0.008**	-	-
16% diet			
5	0.178 +- 0.065*	-0.006 +- 0.002**	$0.61*10^{-4}$ +- $0.20*10^{-4}$***
50	0.161 +- 0.081	-0.008 +- 0.003**	$0.83*10^{-4}$ +- $0.25*10^{-4}$***

$^{n.s}$ slope not significantly different from zero

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

nourished mice appear to be highly refractory to any further larval re-establishment (Fig. 5.4a,b). This suggests that normal CBA/Ca mice may acquire a time dependent resistance against *T. muris* larval invasion during repeated infections, with significant resistance appearing only after d.20 p.i.i.(Fig. 5.4b).

Although larval burdens declined significantly over time in both groupsof highly infected malnourished mice, the recovery of high numbers of larvae at all the sampling points indicates that parasite recruitment occurred in these mice throughout the period of reinfection (Fig 5.4b). There also appeared to be no significant difference in the rate of decline in larval recoveries between both groups of malnourished mice studied at either rates of infection (low infection dose: see Table 5.3) (high infection dose: F test to compare b values: $F = 0.68$, d.f. = 2,58; $P > .05$).

5.3.3 Parasite fecundity during repeated infection

T. muris eggs were absent from the faeces of well- nourished mice at both of the two rates of infection studied (Fig. 5.5 a,b), but eggs were recovered from the faeces of mice fed the 2% or 4% protein diet (Fig. 5.5a,b). In these mice, there appeared to be no significant difference in the density of eggs in faeces with respect to diet (2-way ANOVA: $F = 3.32$, d.f. = 1; $P > .05$), although at both infection rates, hosts fed the 2% protein produced slightly higher numbers of eggs (Fig. 5.5 a,b). Mice given the higher infection dose produced significantly greater number of eggs in faeces (2-way ANOVA: $F = 26.84$, d.f. = 1; $P < .001$) (Fig. 5.5a,b).

The sex ratios of the adult parasites (number of females/total number of worms) recovered from both the moderately and severely protein deficient mice during repeated infection are shown in Fig. 5.6a,b. The mean sex ratio of *T. muris* did not differ from equality in both groups of malnourished mice at either level of repeated infection studied (t-tests: 2% diet:low infection: $t = 0.74$, v=19; $P > .05$, high infection: $t = 1.48$, v=21; $P > .05$, 4% diet: low infection: $t = 1.61$, v=14; $P > .05$, high infection: $t = -0.42$, v=19; $P > .05$). There was also no significant change observed in the sex ratio either with respect to the degree of dietary deficiency, infection dose or time post initial infection (3- way ANOVA: $F = 0.46$, d.f. = 1; $P > .05$; $F = 2.11$, d.f. = 1; $P > .05$; $F = 0.93$, d.f. = 3; $P > .05$ in each case with none of the interactions significant).

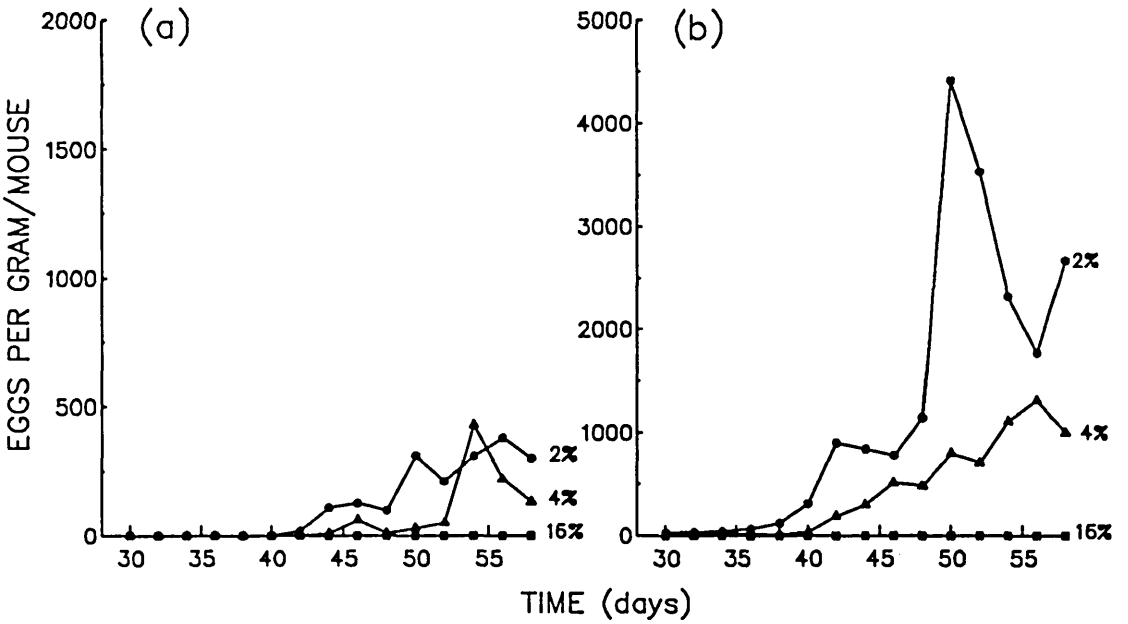

Figure5.5 *Change in T. muris egg output over time during repeated infection in mice fed varying levels of dietary protein. Mice were repeatedly infected with (a) 5 or (b) 50 eggs per 10 days. No parasite eggs were recovered from the faeces of well-nourished mice at any point during repeated infection.*

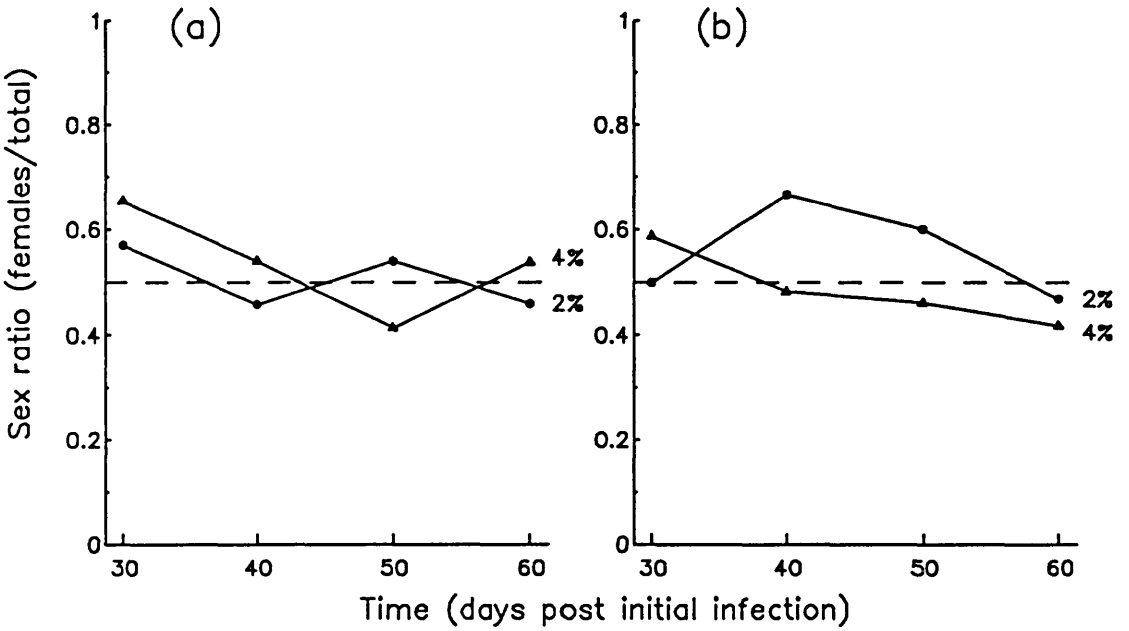

Figure5.6 *Change in the sex ratio of the adult parasites over time during repeated infection in malnourished mice given either (a) 5 or (b) 50 eggs every 10 days.*

5.3.4 Antibody responses to T. muris E/S antigen during repeated infection

The temporal change in the mean titres (OD values) of specific total immunoglobulins (IgGAM), IgG1 and IgA estimated by means of the ELISA against *T. muris* E/S antigen, in serum samples from the various mouse treatment groups is depicted in Figs. 5.7, 5.8 and 5.9 respectively. The results demonstrate that, as in the case of primary infection (see Figs. 4.6a,b; 4.7a,b; 4.8a,b), repeated infection with *T. muris* may evoke a significant, time-dependent increase in parasite specific antibody production, with both the intensity and kinetics of the response influenced by diet and infection rate.

The time of onset of antibody production was influenced by infection dose. Irrespective of host diet, all 3 antibody types appeared earlier in infection (just after d.20 p.i.i.),in the sera of mice given the high infection rate (Figs. 5.7a,b; 5.8a,b; 5.9a,b). Infection dose also appeared to influence the intensity of the specific antibody response. As shown in Table 5.4, independent of protein diet, mice exposed

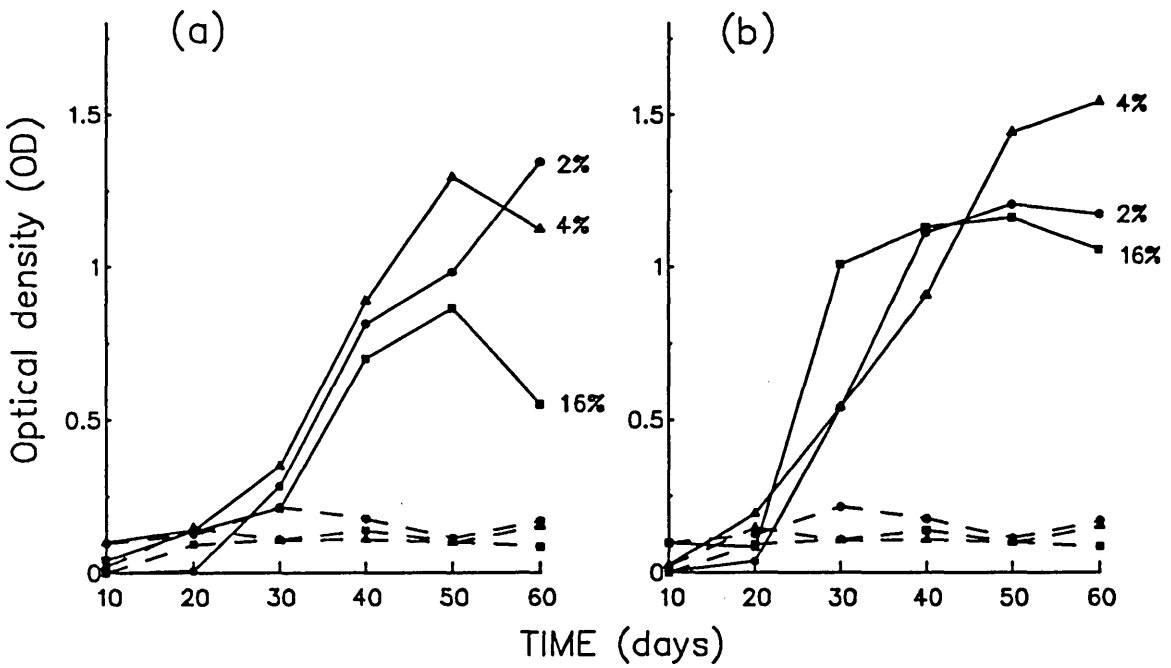

Figure5.7 *The effect of dietary protein on the time course of CBA/Ca mouse specific total immunoglobulin response to T. muris E/S antigen during repeated infection. Mice were repeatedly infected with (a) 5 or (b) 50 eggs per 10 days. Symbols joined by solid lines represent the mean OD values estimated in infected mice; symbols connected by disjointed lines show the background levels in uninfected dietary controls.*

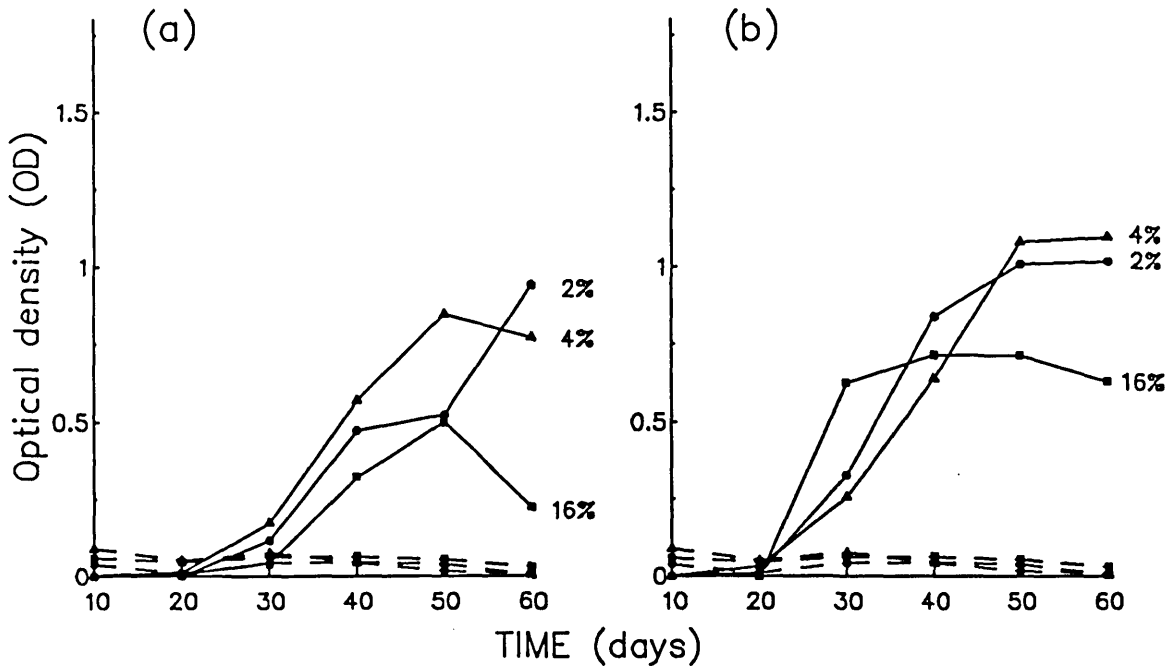

Figure5.8 *The effect of dietary protein on the time course of the specific IgG1 reponse to T. muris E/S antigen during repeated infections. (a) Mice given 5 eggs per 10 days, (b) mice given 50 eggs per 10 days. Symbols as for figure* ***Figure 5.7.***

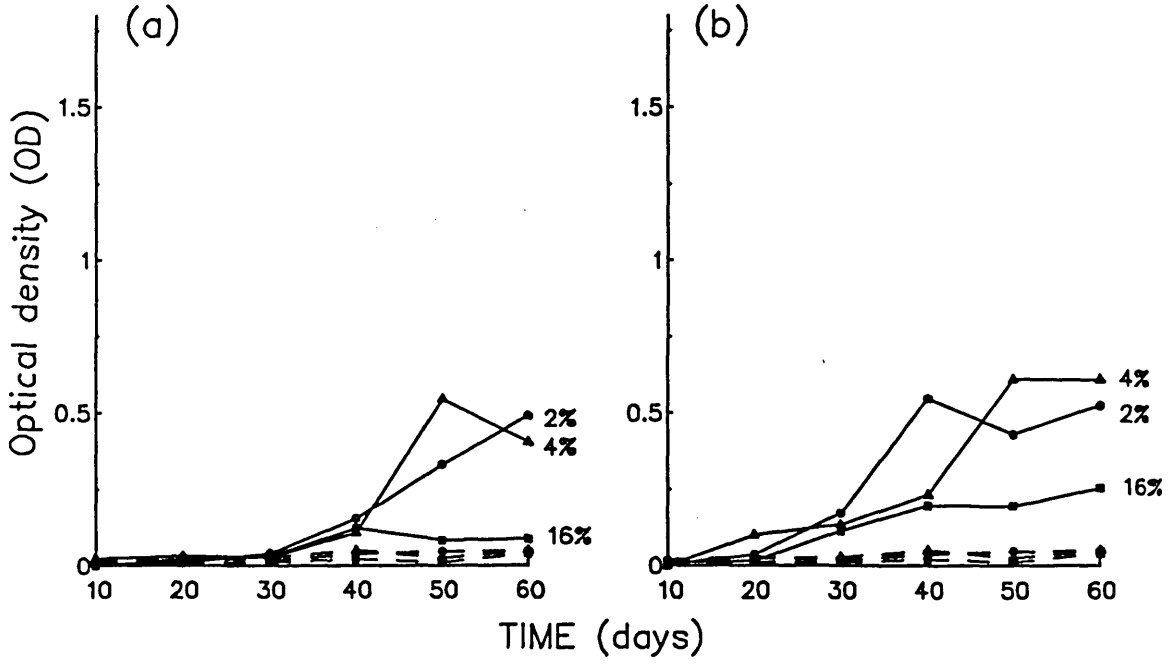

Figure5.9 *The effect of dietary protein on the time course of the specific IgA response to T. muris E/S antigen during repeated infections. (a) mice given 5 eggs per 10 days, (b) mice given 50 eggs per 10 days. Symbols as for* ***Figure 5.7.***

Table 5.4 *Rate of increase of specific antibody levels during repeated infection.*

Ab	Group	2% diet		4% diet		16% diet	
		b	b_1	b	b_1	b_1	b_2
IgGAM	control	0.008	-	0.001	-	0.001	-
	5	0.018	-	0.016	-	0.009	-
	50	0.037	-.0003	0.020	-	0.047	-.0004
IgG1	control	-.003	-	-.001	-	-.001	-
	5	0.013	-	0.014	-	0.005	-
	50	0.016	-	0.017	-	0.039	-.0004
IgA	control	0.0006	-	0.0006	-	0.0006	-
	5	0.008	-	0.008	-	0.002	-
	50	0.009	-	0.010	-	0.005	-

to the high trickle infection produced antibodies at a higher rate in sera. This relationship held for all 3 antibody types examined (Table 5.4). As illustrated in Figs. 5.7a,b; 5.8a,b & 5.9a,b the rate of antibody production also appeared to be related to mouse nutritional status. In general, protein malnourished mice on both the 2% and 4% casein diets, exhibited a higher rate of antibody production than their well-nourished counterparts (Table 5.4). Infection rate, in general, did not appear to alter this relationship for any of the three antibody types studied (Figs. 5.7a,b; 5.8a,b; 5.9a,b) (Table 5.4). The rates of antibody production given in Table 5.4 further show that severely and moderately protein deficient mice did not differ significantly in their ability to produce serum antibodies during repeated infection at either of the two infection doses studied, although at the higher infection dose, mice fed on the 4% protein diet tended to produce greater titres of all 3 antibody types (Figs. 5.7b, 5.8b, 5.9b).

As with primary infections, the pattern of antibody rise during repeated infection was also related to infection dose, mouse dietary status, and antibody type (Figs. 5.7 a,b; 5.8a,b; 5.9a,b). with low infection rates, irrespective of dietary status, all 3 antibody types rose linearly from the time of first appearance until d.50 p.i.i. In some cases , there appeared to a drop in antibody production in the well- nourished mice at d.60 p.i.i for each of the 3 immunoglobulin types examined (Figs. 5.7a, 5.8a, 5.9a). At the higher infection dose, the pattern of antibody production varied with diet. Total immunoglobulins increased steadily until the end of the experiment in mice fed the 4% diet, but significant non-linearity in the rise of this antibody was observed in both the severely malnourished and well- nourished mice (Fig. 5.7b) (Table 5.4). In these mice, antibody levels rose rapidly to a stable plateau (Fig. 5.7b). In well- nourished mice, the rate of increase in specific total immunoglobulins appeared most rapid from d.20 to 30p.i.i., while in severely malnourished mice, this extended from d.20 to 40 p.i.i. (Fig. 5.7b).

In contrast, whereas specific IgG1 titres increased in a linear fashion during infection in both groups of malnourished mice given the high infection dose, the pattern of antibody rise was distinctly non-linear in similarly infected well-nourished CBA/Ca mice (Fig. 5.8b) (Table 5.4). In the latter mice, as with the total immunoglobulin response, specific IgG1 titres rose linearly and rapidly between d.20 to 30 p.i.i. following which the production of antibody appeared to decline (Fig. 5.8b).

Both rates of infection elicited significant amounts of parasite specific serum IgA (Fig. 4.9a,b). This finding is in contrast to the results obtained during primary infection (Chapter4) when IgA levels were at the margins of detectability in mice given the low single infection dose of 10 eggs. Irrespective of mouse diet or infection rate, the specific IgA response appeared to increase linearly until the end of the experiment (Fig. 5.9a,b), although in the well- nourished animals given the low infections, mean IgA titres tended to decrease slightly towards the end of the experiment on d.60 p.i.i. (Fig. 5.9a).

5.3.5 Immunoblot analysis

Recognition of E/S antigen by specific IgG1 in representative sera obtained from each mouse group corresponding to the periods of early (d.20 p.i.i.), mid (d.40 p.i.i.) and late (d.60 p.i.i.) phase of repeated infection was analyzed using the Western blotting technique. The results are shown in Figs. 5.10 (16% diet), 5.11 (4% diet) & 5.12 (2% diet). *T. muris* E/S antigen recognition by IgG1 during repeated infection was clearly time-dependent. In all mouse groups, irrespective of diet and infection rate, first detection of parasite antigens occurred on 40 p.i.i., the earlier recognition of the 50.7kD antigen at d.20 p.i.i. by malnourished mice probably representing a non specific response (Else & Wakelin, 1989) (Figs. 5.11a,b,c; 5.12a,b,c).

The results show that malnourished mice, irrespective of infection dose, may detect a broader range of parasite antigens than well-fed counterparts at d.40 p.i.i., although this was beset by much individual variability. By d.60 p.i.i., these mice appeared to more consistently recognize a broader and more intense range of antigens, although this effect was less apparent in mice given the low infection dose (Figs. 5.11a,b; 5.12a,b). A very slight response was observed in well-nourished mice at this time point (Fig. 5.10a,b).

The antigen components recognized by well-nourished mice also appear to be qualitatively similar to those detected in singly-infected counterparts (see Chapter 4). By contrast, the molecular weight of the antigens recognized by both groups of malnourished mice varied from 20-23kD to 200kD (Figs. 5.11a,b; 5.12a,b).

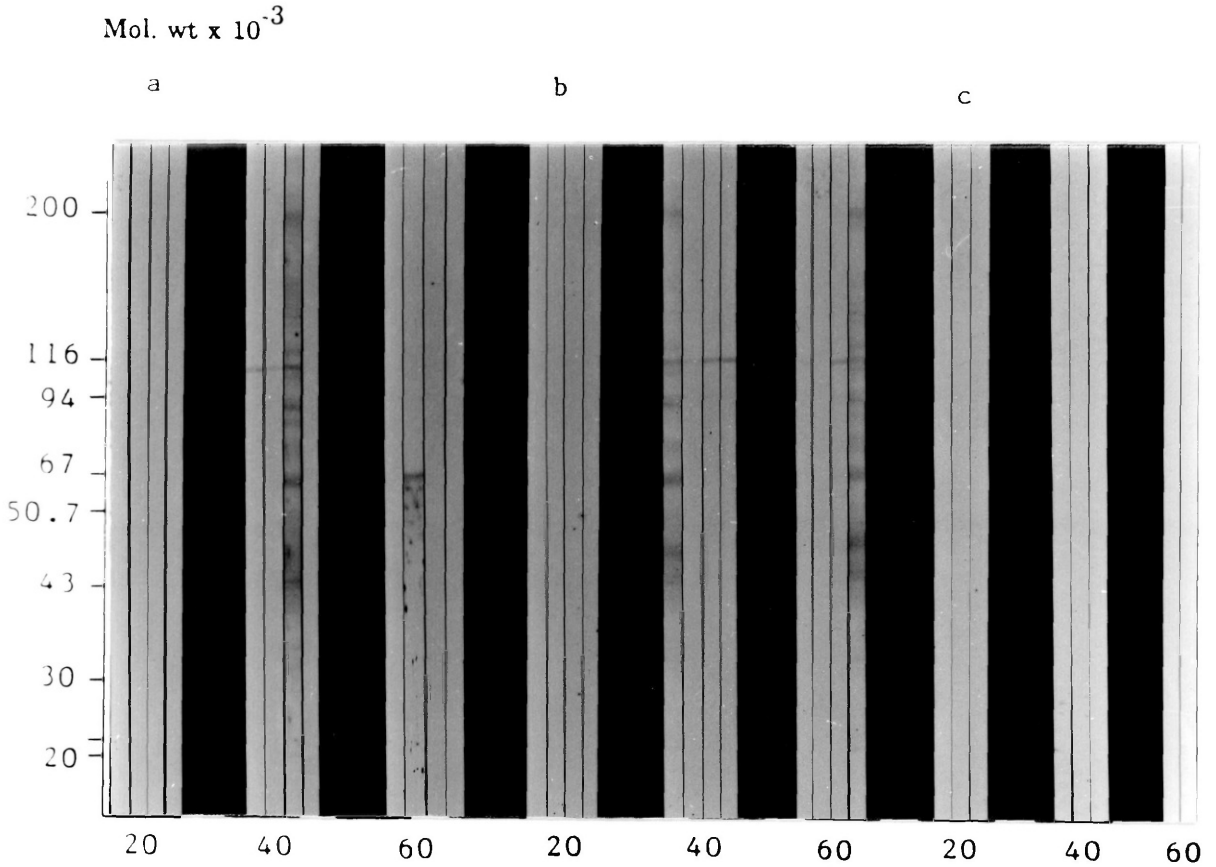

Figure5.10 *Western blot of T. muris E/s antigen-recognition during repeated infection by mice fed the 16% protein diet. (a) hosts given 5 eggs/10 days, (b) hosts given 50 eggs/10 days and (c) control mice. The figures at the bottom denote the days post initial infection.*

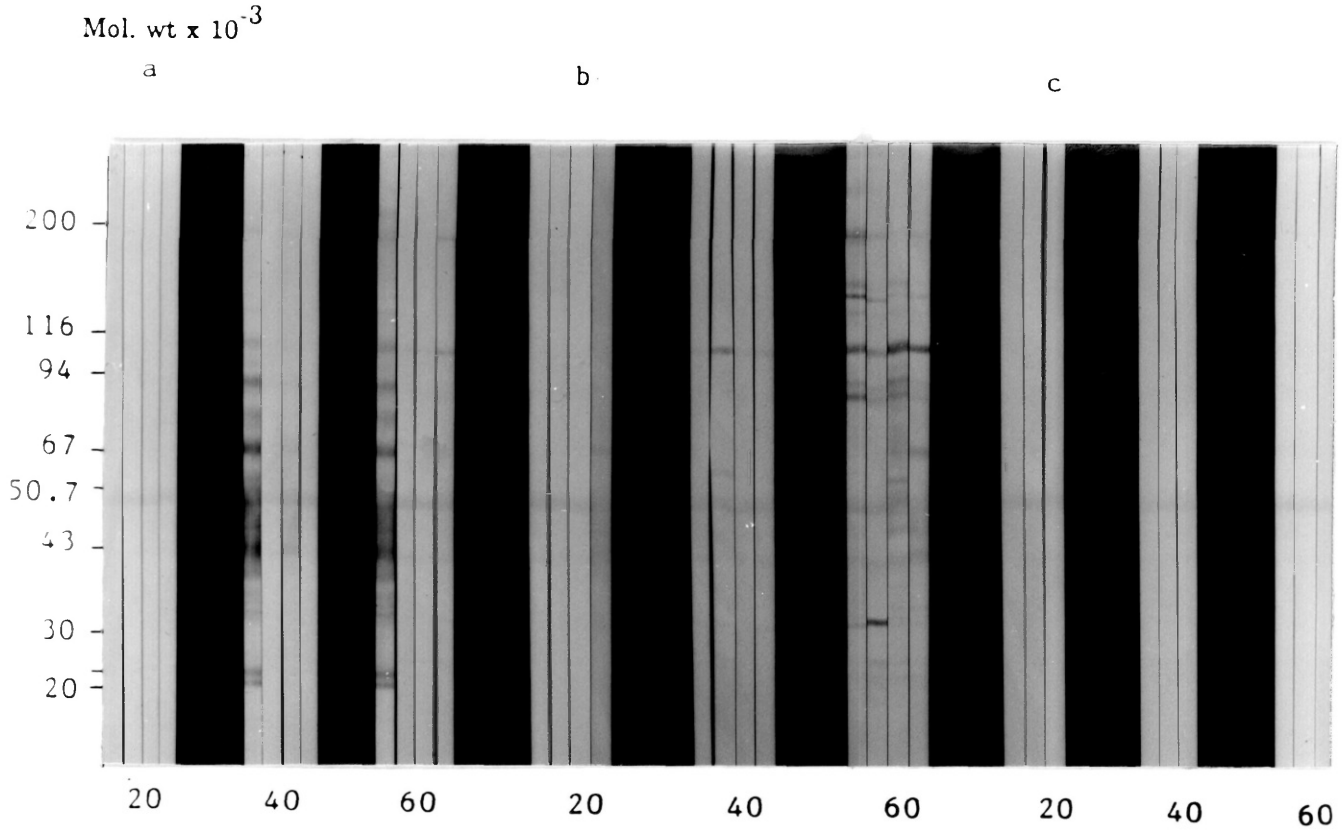

Figure5.11 *Western blot of T. muris E/s antigen recognition during repeated infection by mice fed the 4% protein diet. (a) hosts given 5eggs/10 days, (b) hosts given 50eggs/10 days and (c) control mice. The figures at the bottom denote the days post initial infection.*

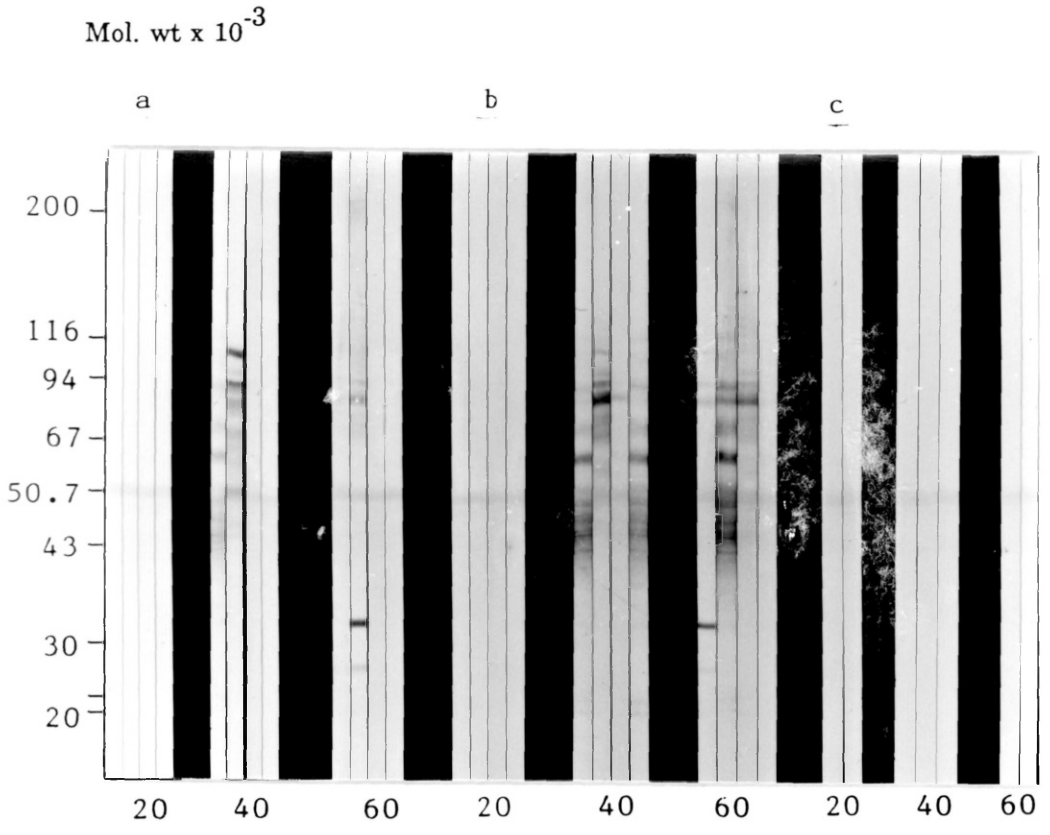

Figure5.12 *Western blot of T. muris E/S antigen recognition during repeated infection by mice fed the 2% protein diet. Labels and figures as for* ***Figure 5.10.***

5.3.6 Correlation between specific antibody response and T. muris adult and larval burdens

The relationship between antibody levels (specific total immunoglobulin & IgG1) and either adult or larval burdens recovered from individual mice belonging to the various treatment groups was examined using the non-parametric Spearman's Rank correlation statistic. The results are shown in Table 5.5. The results indicate that at low rates of infection, the antibody titres (both specific total immunoglobulin and IgG1) elicited are positively associated with adult worm burdens, irrespective of dietary level (Table 5.5). On the other hand, in mice given the high rates of infection, the total immunoglobulin and IgG1 responses were positively correlated with worm burden in malnourished animals, but not in well-nourished mice (Table 5.5).

The levels of total immunoglobulins and IgG1 did not show any significant correlation with larval burdens in malnourished mice at either infection level. In contrast, antibody titres were found to be significantly negatively associated with larval recoveries from well-nourished mice at both infection rates (Table 5.5)

5.4 DISCUSSION

5.4.1 The effects of protein nutrition and T. muris repeated infection on CBA/Ca mouse growth rate

The results obtained on CBA/Ca mouse growth in this study confirm previous observations that semi- synthetic diets containing 2% casein may induce obvious or severe protein malnutrition as characterized by insignificant growth (Slater, 1987,1988; Slater & Keymer, 1986a). On the other hand, in common with findings recorded in chapter 2 and chapter 3, the results indicate that diets containing either 4% or 16% casein may support near- normal growth (Fig. 5.1a). Although host food consumption was not measured in the present study, by analogy with the results obtained during primary infection (chapter 4), it is likely that the retarded growth of mice fed the 2% casein diet relative to the growth of counterparts fed the higher 4% or 16% diet was mainly due to the insufficient intake of protein. As such, the sharp difference in growth observed between mice fed the 2% or the 4% casein diet

Table 5.5 *Spearman rank correlations between specific antibody levels and either T. muris adult or larval burdens in CBA/Ca mice fed various levels of protein at either of the two infection rates studied.*

	Diet					
	2%		4%		16%	
Ab	larvae	adults	larvae	adults	larvae	adults
a) Infection dose of 5 eggs/mouse/10 days						
IgGAM	$-0.28^{n.s}$	0.41^{*}	$-0.12^{n.s}$	0.66^{**}	-0.43^{*}	0.56^{*}
IgG1	$-0.30^{n.s}$	$0.24^{n.s}$	$-0.05^{n.s}$	0.56^{*}	-0.52^{**}	0.60^{*}
b) Infection dose of 50 eggs/mouse/10 days						
IgGAM	$-0.29^{n.s}$	0.66^{**}	$-0.28^{n.s}$	0.69^{**}	-0.74^{***}	$0.08^{n.s}$
IgG1	$-0.30^{n.s}$	0.61^{**}	-0.44^{*}	0.41^{*}	-0.73^{***}	0.12n.s

$^{n.s}$ no significant correlation
$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

may indicate the existence of a threshold in dietary protein intake, probably occurring between 2 and 4% dietary casein, below which protein resources may become limiting for the support of normal mouse growth.

Repeated infection with *T. muris* at both the present rates of 5 eggs/mouse/10 days and 50 eggs/mouse/10 days was not observed to significantly depress the growth rate of CBA/Ca mice fed either the 16% or the 4% casein diet (Fig. 5.1b,c). This finding would appear to contradict the observation recorded in chapter 4 that, irrespective of the level of dietary protein, heavy primary infections of *T. muris* may significantly retard CBA/Ca mouse growth. It is likely, however, that this discrepancy is due to the considerably lower rates of infection used in the present study. Highly infected mice in this study received approximately 300 eggs over the 60- day trickle infection period, whereas the corresponding heavy infections used in the primary infections comprised a much higher 650 eggs. It is also possible that this difference may partly reflect the ability of hosts given continuous low or moderate levels of infection to accommodate parasitic challenge with relatively little adverse effects, when compared with the often debilitating effects observed in the host exposed to large single infections (see Sykes, 1982).

In contrast with the above, repeated infections of *T. muris* were found to adversely affect the growth of severely malnourished CBA/Ca mice fed the 2% protein diet (Fig. 5.1a,b,c). This effect held for both of the trickle infection doses studied. It thus appears that *T. muris* may negatively effect the growth of severely malnourished hosts at levels of infection which may not produce similar detrimental effects in either moderately or normally nourished hosts. This finding may be indicative of a possible interaction between the pathogenic effects of murine trichuriasis and the degree of host protein deprivation. A similar synergistic relationship between severe malnutrition and the pathogenic effects of helminth infection on mammalian growth rate has been shown for other nematode infections, particularly *N. brasiliensis* in the rat (Crompton *et al.*, 1981; Keymer *et al.*, 1983b; Ash *et al.*, 1985), *H. polygyrus* in the mouse (Brailsford & Mapes, 1987) and *A. suum* in the pig (Forsum *et al.*, 1981). These findings emphasize the role of host nutrition as a factor influencing the severity or pathogenicity of nematode infections.

5.4.2 The effect of dietary protein deficiency on the dynamics of T. muris repeated infection

The adult worm recoveries depicted in Figs. 5.2 & 5.3 demonstrate unequivocally that host protein nutrition may significantly influence the transmission of *T. muris* in CBA/Ca mice. Both groups of malnourished mice fed on either the severely

deprived 2% or marginal 4% protein diet, continued to accumulate adult parasites for the duration of each repeated infection (Fig. 5.2b,c; 5.3b,c). Furthermore, the rate of gain of adult worms during repeated infection in these mice appeared to be related to infection dose, with hosts given the higher infection dose tending to accumulate significantly greater parasite loads over time. This relationship has also been shown previously for *H. polygyrus* trickle infections in severely malnourished (2% casein diet) CD1 mice (Slater & Keymer, 1986a). The authors considered the linear gain of adult *H. polygyrus* under repeated infection in the malnourished hosts to be principally a consequence of increased adult worm survival, largely due to protein deficiency- induced defects in host acquired resistance (Slater & Keymer, 1986a). In the present study, the accumulation of adult *T. muris* through time probably reflects both increased adult worm survival and continued establishment and development of invading larvae throughout the period of repeated infection. Continuous recruitment of *T. muris* larval populations during repeated infection in the malnourished mice is clearly indicated by the significant numbers of larvae recovered from these mice at each of the time points sampled (Fig. 5.4a,b). That adult worm survival may also be enhanced by protein malnutrition is suggested indirectly by the linearity observed in the rise of worm burdens during trickle infection in both groups of malnourished mice, particularly at the high infection dose (Fig. 5.3b,c) (Table 5.2). If worm survival in these mice was as low as that observed in well- nourished counterparts (see Figs. 5.2a, 5.3a & Table 5.2), a convex pattern of mean worm burden rise with time would be expected rather than a simple linear increase (see Anderson, 1987b).

It has been shown previously that *T. muris* repeated infections in normal, immunologically intact hosts typically results in a rapid immune expulsion of both invading larvae and resident worms at the time of each reinfection (Wakelin, 1973; Behnke & Wakelin, 1973). This implies that the accumulation of *T. muris* burdens within malnourished mice during repeated infection is due to impairment in acquired immunity rather than the direct effects of changes in host nutrition on parasite establishment or survival. This conclusion is supported in this study by the differences observed in the patterns of larval recoveries from the well- and malnourished mouse groups (Fig 5.4a,b). As shown in the figures and indicated in table 5.3, mean larval burdens declined rapidly and non- linearly with increasing duration of infection in well- nourished mice, whereas the mean larval recovery from malnourished mice appeared to decrease at a steady, constant rate with time. Severe non- linearity in the rate of decline of larval recovery may be taken to be indicative of the operation of the rapid immune expulsion process acting to abruptly remove invading larval burdens.

The rate of gain of *T. muris* burdens during repeated infection was similar for both severely and moderately malnourished mice (Fig. 5.2b,c; 5.3b,c; Table 5.2). This observation suggests that, despite near- normal growth (Fig. 5.1), the mice fed the marginally deficient 4% casein diet may be as immunologically susceptible to infection with *T. muris* as severely malnourished hosts fed the 2% protein diet. The results support the hypothesis that moderate or subclinical protein malnutrition may be as important as clinical malnutrition to the transmission of parasitic infections (McMurray *et al.*, 1981; Bundy & Golden, 1987).

Parasite survival, however, may be slightly higher in severely malnourished mice in comparison with survival in moderately malnourished hosts. Mice fed on the 2% casein diet and given the low infection rate were found to harbour a significantly greater mean number of adult worms when compared with the worm burdens obtained during infection in correspondingly infected counterparts fed on the 4% diet. At the higher infection rate, while there was evidence for a slight turnover in mean worm burden during repeated infection in moderately malnourished mice, the rate of gain of adult parasites during the same time period in severely malnourished hosts appeared to be linear (Fig. 5.3b,c). Since the rates of decline of larval parasites in both these groups of highly infected malnourished mice were found to be similar, it is possible that this difference may signify changes in adult parasite survival, with *T. muris* survival being slightly greater in severely malnourished hosts. These findings do not support the view that severe protein malnutrition may adversely affect the growth of intestinal helminth populations as a direct result of change in mammalian gut physiology and structure (see Bundy & Golden, 1987). The slightly greater mortality of *T. muris* in the moderately malnourished CBA/Ca mice may reflect a higher degree of immunocompetence in comparison with hosts fed the 2% casein diet.

Although both groups of malnourished mice continued to recruit significant numbers of *T. muris* larvae throughout the duration of each trickle infection, the results also showed that the mean larval recovery at the higher dose declined significantly at a constant rate with time (Fig. 5.4a,b). A similar gradual decline in the temporal rate of larval recovery during continuous infection has been reported (and considered an important regulatory feature) for various other intestinal nematodes, including *O. ostergagia* (Michel, 1970; Grenfell *et al.*, 1987), *H. contortus* (Barger *et al.*, 1985) and *Trichostrongylus* spp (Donald & Walker, 1982). Most authors have regarded the limiting effects of host acquired immunity on incoming larval establishment to be the main mechanism underlying the decline in the rate of nematode larval recovery during repeated infection. The decline observed in the

present study, however, is unlikely to be mediated by host acquired immunity since larval numbers were observed to decline at a constant rate (Fig. 5.4b). Murine acquired immunity against *T. muris* is manifest by rapid expulsion of incoming larval populations (Wakelin, 1973). Hence, an immunologically, intact host would be expected to cause significant non- linearity in the temporal pattern of larval recovery during repeated infection (similar to that obtained in well- nourished mice (Fig. 5.4a,b)). Moreover, the protein deficiencies used in this study would be expected to impair immunity against *T. muris* (see Wakelin & Lee, 1987). Thus, the decline in larval recovery in the malnourished mice may reflect the regulatory effects of non- immune mechanisms related to density rather than host defence mechanisms. For example, as suggested in chapter 3, it is conceivable that the reduction observed in *T. muris* larval recovery at increasing durations of repeated infection (= parasite densities) in the malnourished mice may arise merely as a result of a reduction in the availability of establishment sites within the repeatedly infected murine large intestine (see Panesar, 1981). These findings in the malnourished mice, and in the immunosuppressed animals in chapter 3, illustrate the general point that helminth establishment may be limited even in susceptible hosts when the infection densities are high. The density- dependent mechanisms underlying intestinal helminth establishment in susceptible hosts may be related to purely ecological processes, such as intraspecific parasite competition for limited host resources, rather than host immunity.

The present results support and extend the previous findings that *T. muris* repeated infections in normal, immunologically intact hosts are characterized by the rapid expulsion of incoming larvae (Fig. 5.4a,b) and the loss of established adult worms (Figs. 5.2a, 5.3a) (Wakelin, 1973; Behnke & Wakelin, 1973). This situation in murine trichuriasis contrasts with that found in *Nippostrongylus brasiliensis* infections, in which it has been demonstrated that the normal host (rat) primary response may be circumvented, and significant worm burdens established at both low (5 larvae per day) and moderate rates (50 larvae per day) of repeated infection (Jenkins & Phillipson, 1970). This difference may reflect variations in several facets of the host- parasite relationship; including relative parasite susceptibilities to host immune responses, relative potencies of host parasite expulsion mechanisms, and differences relating to infection site and habitat which may facilitate the easier expulsion of *T. muris* from the murine large intestine as opposed to the ejection of *N. brasiliensis* from the rat small intestine.

5.4.3 The effect of protein nutrition on parasite egg production during repeated infection

The results in the present study clearly support the conclusion (Chapter 4) that the significant numbers of *T. muris* eggs in the faeces of malnourished CBA/Ca mice is a result of the greater survival of adult worms in such hosts (Figs. 5.2, 5.3 & 5.5.). No parasite eggs were recovered at any time during infection from the faeces of well-nourished mice (Fig. 5.5).

Although the mean net *T. muris* egg output (eggs/gram faeces) of either group of malnourished mice did not appear to differ significantly, inspection of the data suggest that parasite egg production may be slightly higher in severely malnourished mice (Fig. 5.5). Since the sex ratios of the adult parasites recovered from both the moderately and severely protein deficient mice did not differ both in relation to infection dose or time post infection (Fig. 5.6), it is possible, as suggested by Slater & Keymer (1986a) that the slightly higher rate of helminth egg output observed in the severely malnourished host may reflect either or both the establishment of heavier worm burdens (see above) or increased *per capita* fecundity of female worms in such mice.

5.4.4 Nutrition-immunity-infection interactions during T. muris repeated infections

As was seen with primary infections (chapter 4), the malnourished mice produced greater amounts of specific antibody during repeated infection than their well- fed counterparts (Figs. 5.7, 5.8, 5.9 & Table 5.4). The results of the rank correlation analysis indicating an association between murine specific antibody production (total immunoglobulin and IgG1) and worm burdens (Table 5.5), further suggests that the higher antibody response of malnourished mice may be due to their heavier worm loads. This conclusion is supported by the results of the Western blot analysis (Figs. 5.10a,b, 5.11a,b & 5.12a,b), which show that malnourished mice (those fed the 4% and 2% casein diet), may recognize a broader and more intense band of parasite E/S antigens (by specific IgG1) than similarly infected well- nourished mice. As discussed in chapter 4, a broader host E/S antigen recognition profile in murine trichuriasis may reflect the establishment of chronic, adult infections in susceptible mice. A more intense recognition of antigens, on the other hand, may be suggestive of the higher host antigenaemia which may be associated with heavy *T. muris* infections.

Specific antibody production by both the severely and moderately malnourished mice during repeated infection appeared to be infection dose dependent. Animals given the the higher dose tended to produce greater amounts of antibody in sera. The dose- dependent increase in antibody production in the protein deficient mouse groups is probably related to the greater number of adult worms accumulating during infection in those mice given the heavier trickle infections (Figs. 5.2b,c & 5.3b,c).

Other mechanisms primary to protein deficiency *per se* may also underlie the enhanced antibody response of the malnourished host. It is conceivable that the increased antibody response may be due to regulatory defects in antibody synthesis (Pocino & Malave, 1981, see chapter 4); or the result of a greater systemic spread of parasite antigens, due to increased leakage through the malnourished gut wall (Rothman *et al.*, 1982a,b), defects in secretory IgA clearance of antigen at the mucosal surface (Watson *et al.*, 1985) or impaired removal of antigen by the hepatic reticuloendothelial system (Chandra, 1977).

Specific antibody production in well- nourished mice, irrespective of infection dose and antibody type, appeared to increase rapidly during the initial stages of repeated infection (between d.20 to 40 p.i.i.) before reaching a plateau or declining slightly at the later stages of infection (Figs. 5.7a,b, 5.8a,b, 5.9a,b). As with primary infections, it is evident that the period of rapid rise in antibody (Fig 5.7a,b; 5.8a,b) coincided with the initiation of immunity against *T. muris*, which was manifested by the rapid expulsion of both incoming larvae and established larval and adult burdens (Figs. 5.2a, 5.3a, 5.4a,b). This observation supports the conclusion (Chapter 4) that the production of significant amounts of parasite- specific antibody (perhaps above a threshold level) was required for the initiation of worm expulsion in murine trichuriasis.

The results further show that antibody levels in well-nourished mice may be correlated with worm burden only for mice given the low, but not high infection dose (Table 5.5). This may indicate the influence of infection exposure in stimulating the mammalian antibody response to nematode infections. Such an immunogenic role (perhaps mediated by cross- reactive egg antigens (Jenkins & Wakelin, 1977)) may underlie the persistence of the antibody response against *T. muris* E/S antigens observed during repeated infection, despite the loss of worms early during infection (Figs. 5.7a,b, 5.8a,b, 5.9a,b).

Despite an intact antibody response in both the well-nourished and malnourished mice, *T. muris* were expelled only from the well- fed hosts (Table 5.5). This result supports the previous conclusion (Chapter 4) that malnutrition associated susceptibility to *T. muris* is due to defects in cellular immunity rather than the antibody response. However, as discussed in Chapter 4, several direct effects of protein malnutrition on antibody, such as the induction of tolerance (Stokes *et al.*, 1983; Lamont *et al.*, 1987a,b) and lowered affinity (Passwell *et al.*, 1974; Chandra *et al.*, 1984), may also significantly impair the effectiveness of antibody in initiating worm expulsion mechanisms in malnourished mice.

The development and intensity of antibody produced in the present trickle- infected CBA/Ca mice presented some contrasts with those in the singly- infected mice reported in chapter 4. This may be most clearly seen in the case of the IgA response depicted in Fig. 5.9a,b), which unequivocally show that, irrespective of mouse diet, repeated infection with *T. muris* may evoke the secretion of significant amounts of this antibody in sera against E/S antigen even at low infection rates. This finding contrasts with the IgA response in singly- infected hosts (Chapter 4), which achieved a similar level of antibody production only at the high dose of 650 eggs. This result may indicate the boosting effect of repeated infection on serum antibody responses against parasitic infections (Roitt *et al.*, 1985). A similar enhancing effect of repeated infection was also observed in the case of IgGAM and IgG1 response. Thus, for example, while mice fed the 4% or 16% protein diet and given the single high infection of 650 eggs showed mean OD values of 1.51 and 1.21 respectively for total specific immunoglobulins on d. 49 p.i.(chapter 4), the mean values obtained for the same immunoglobulin in correspondingly- fed mice given the high repeated infection dose of 50 eggs/10 days on d.50 p.i.i. was almost similar at 1.44 (4% diet) and 1.16 (16% diet), even though the latter mice received only 250 eggs.

The timing of onset of specific antibody production appeared, by comparison with primary infection (chapter 4), to occur later during repeated infections for all 3 antibody types at either of the infection levels studied. This may be related to differences in the initial level of infection experienced, being lower in the present trickle infections, rather than due to any intrinsic effect of repeated infection on murine antibody production.

The similarities observed in the response patterns of specific total immunoglobulins and IgG1, but not IgA, in the present study (Figs. 5.7, 5.8, 5.9) and in the previous primary infection study (chapter 4), provide support for the suggestion that the murine antibody responses to *T. muris* E/S antigen may predominantly comprise an

IgG1 response (Else & Wakelin, 1989). A similar increased production of specific IgG1 during infection has also been reported for other nematodes, notably *H. polygyrus* (Chapman *et al.*, 1979; Williams & Behnke, 1983; Langford, 1989).

The results of the Western blot analysis carried out in this study (Figs. 5.10, 5.11, 5.12) support the suggestion (chapter 4) that malnourished CBA/Ca mice may recognize a broader and more intense range of E/S antigens mainly as a result of their larger worm burdens. Antigen- recognition by well- nourished mice during repeated infection, on the other hand, appeared limited to antigens associated with *T. muris* larval stages (Fig. 5.12a,b). Since the limited recognition of these antigens by well- fed hosts appears to be coupled with resistance to *T muris* trickle infections (as indicated by the correspondence between the time of decline of adult and larval burdens (Figs. 5.2a, 5.3a, 5.4a,b) and detection of antigen (after d. 20p.i.i) (Fig 5.12a,b)), it is possible that the broader antigen range detected by a large proportion of these antibodies may be directed against antigens unrelated to protection. The finding that antigen recognition by IgG1 in the resistant, well- nourished CBA/Ca mice during *T. muris* repeated infection may continue to be restricted to mainly 4 antigens, *viz.*, antigens of mol. wt. 116kD, 94kD, 67kD and 43kD (Figs. 5.12a,b), furthermore supports the suggestion (chapter 4) that these antigens may be host protective in murine trichuriasis.

In conclusion, the results of this study demonstrate that the transmission of *T. muris* may be enhanced to a similar degree in both moderately or severely protein malnourished CBA/Ca mice, probably due to impairment in cell mediated immunity rather than antibody responses. Worm burdens may accumulate during repeated infection in malnourished mice as a result of both continued recruitment of larval parasites and the enhanced survival of adult worms. On the other hand, in well-nourished mice, worm burdens were transient, characterized by the time-dependent rapid expulsion of both incoming larvae and established larval and adult parasites.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

The principal aim of this project was to use the *T. muris*- CBA/Ca mouse model to examine the effect of protein malnutrition on the population dynamics of intestinal nematode infections.

The initial establishment of *T. muris* larvae in naive, previously uninfected mice was found to be uninfluenced by the levels of host dietary protein; whether 16%, 4% or 2% casein (Chapter 4; d.10 post infection larval recoveries in Chapter 5 (Figs. 5.4a,b)). This result conforms with the majority of previous findings (see Table 1.3). However, parasites such as *Nippostrongylus brasiliensis*, which undergo extraintestinal larval migration, may establish at greater levels in malnourished hosts, possibly as a result of protein deficiency-induced suppression of host innate immunity acting against the migratory larvae (Donaldson & Otto, 1947) (see Chapter 4). The negligible effect of host nutrition on the establishment of *T. muris* and other nematodes which hatch and establish directly in the host intestine (Table 1.5), may therefore indicate that immunity against such larval parasites is not effective immediately but develops over the course of infection. In the case of *T. muris,* it has been demonstrated that host protective immunity normally develops only by 2-4 wks p.i., depending on the mouse strain (Wakelin, 1975a; Wakelin & Lee, 1987).

Irrespective of nutritional status, the number of *T. muris* larvae initially establishing in CBA/Ca mice was dependent on infection dose, increasing significantly with infection rate (Chapter 4). These findings suggest that initial parasite establishment in murine trichuriasis is mainly governed by the rate of exposure to infection, and that this association is not modified by host nutrition.

In contrast to larval numbers, the proportion of larvae establishing in the mice fed either the 16% or 4% casein diet (Chapter 4), was found to decrease significantly at the higher infection rate. This finding supports the suggestion made in Chapter 3, that *T. muris* establishment in naive hosts is uninfluenced by innate immunity but may be regulated by the availability of establishment sites within the large intestine. In contrast to worm establishment rates with single infections, host protein nutrition had a significant influence on the rate at which invading larvae establish or survive in repeatedly infected hosts (Chapter 5). In well-nourished mice, after an initial increase, larval numbers decreased abruptly from d20 to

40p.i.i, following which the mice appeared solidly immune to further infections (Fig. 5.4a,b). This concurs with the observations of Wakelin (1973) and Behnke & Wakelin (1973) that repeated infections with *T. muris* induce an expulsion response once the mouse strain-specific immune threshold level (= approximately 10 worms) is exceeded in normally nourished mice. These findings suggest that the continuous larval recruitment observed during repeated infection in the malnourished mice is due to impaired resistance.

However, despite susceptibility to re-infection, the mean larval recovery in the malnourished mice declined steadily over time at a rate related to infection dose (Figs 5.4a,b). As suggested in Chapter 5, it is possible that this temporal decline in larval numbers is indicative of worm population regulation by non-immune density-dependent mechanisms, such as space limitations. This conclusion suggests that in the absence of immunity, non-immune mechanisms may play an important role in the regulation of parasite population growth (see Chapter 3).

In contrast to the effects on worm establishment, protein deficiency was found to potentiate *T. muris* survival irrespective of whether a single or trickle infection was used. In single infections, protein deficiency clearly facilitated the establishment and survival of significant, chronic, patent infections in moderately malnourished mice (Fig. 4.3c). In the case of repeated infection, it appears that enhanced parasite survival contributed in part to the steady rise in worm burdens observed in both the moderately and severely malnourished mouse groups (Figs. 5.2b,c; 5.3b,c).

T. muris infections resulting from single or repeated exposures were observed to be acute and transient in well-nourished CBA/Ca mice (Figs 4.3d, 5.2a,5.3a), although this effect appeared to be modified by infection dose in primary infections (Figs. 4.3a). The infection patterns indicate that the survival rate of *T. muris* is severely curtailed in normal, intact hosts mainly as a result of the development of strong immunity against infection; manifested either by the spontaneous rejection of larvae during primary infection (Fig. 4.3d) or the rapid expulsion of both incoming larvae and established worms during repeated infection (Figs. 5.2a, 5.3a, 5.4a,b). There, however, appeared to be some difference in the survival of *T. muris* between primary and trickle infections in well-nourished hosts. When given as a primary infection, a single infection of 10 eggs may result in the establishment of patent infection (Fig 4.3a). This is in accordance with the threshold hypothesis of Wakelin (1973).

On the other hand, repeated infection even at low levels (*i.e.,* 5 eggs/10 days) may fail to result in the establishment of stable worm infections (Figs. 5.2a, 5.3a), probably because such infections elicit a strong immune response once the antigenic threshold is exceeded (Wakelin, 1973; Behnke & Wakelin, 1973). Furthermore, while a small number of worms from a primary infection may survive to patency following the expulsion process (Fig. 4.3d), the response in repeated infection appears to be able to reject completely both established adult worms and incoming larvae (Figs. 5.2a, 5.3a, 5.4a,b).

These findings in normally-fed mice suggest that the increased survival of *T. muris* in malnourished mice may be due to impairment of the host immune response rather than deleterious changes in host gut environment induced by protein deficiency (see Bundy & Golden, 1987). The results on parasite establishment and survival (Figs. 4.3a,b,c,d; 5.2a,b,c; 5.3a,b,c; 5.4a,b) also indicate that the higher intensity of *T. muris* in malnourished mice is a result of an enhancement in both the recruitment and survival of the parasite in such hosts.

Net *T. muris* egg output per mouse during both single and trickle infections was found to be significantly higher in malnourished mice (Figs. 4.5a,b; 5.5a,b) and was related to exposure, increasing significantly at the higher rates of infection. By contrast, parasite faecal egg output in well-nourished mice was not only low but also unrelated to exposure during primary infections (Figs. 4.5a,b). In the case of repeated infections, no parasite eggs were recovered from the faeces of such mice presumably due to the insignificant number of adult worms surviving. These findings, together with the fact that the *per capita* fecundity of *T. muris* did not appear to be influenced by the effects of protein deficiency (Table 4.6), suggest that the increased parasite egg excretion observed in the malnourished mice was a direct result of their heavier adult worm loads. It appears, therefore, that nematode transmission rate may be enhanced in malnutrition mainly or partly as a consequence of the prolonged survival of parasites. The practical importance of this finding is that malnourished hosts may not only constitute the group at increased risk of infection but may also form a significant source of infection to the community.

An important result of the trickle experiments (Chapter 5) was that, moderately malnourished mice, which were apparently normal and exhibited near-normal growth rate, had similar susceptibility to *T. muris* infections as severely malnourished hosts which showed no weight gain. This result highlights the importance of moderate or subclinical malnutrition in the transmission of parasitic infections. This observation may be of relevance to human communities given that

the milder forms of malnutrition are the most prevalent in endemic populations (Goldsmith, 1974; Whitehead, 1980).

The results (Figs. 4.3a,b,c,d; 5.2a,b,c; 5.3a,b,c) clearly demonstrate that host nutrition may profoundly alter the normal host-parasite relationship, with protein deficiency facilitating the establishment of long-term, chronic, *T. muris* infections. This result obtained in a resistant CBA/Ca mouse strain (Lee & Wakelin, 1983), supports the view that host nutrition, by modifying a genetically determined factor (immune competence), may create heterogeneity in infection intensity between individuals (Bundy, 1988). Slater & Keymer (1986a,b) demonstrated that protein malnutrition, by suppressing the development of acquired immunity in outbred mice, may override the role of host genotypic variability in generating parasite overdispersion. Thus, while the transmission rate of *H. polygyrus* was found to be significantly raised in a semi-naturally infected community of protein malnourished outbred CD1 mice, a greater degree of parasite overdispersion and a concomitant lower rate of *H. polygyrus* transmission were observed in corresponding well-nourished mouse populations (Slater & Keymer, 1986b). These preliminary findings suggest that the interaction between nutrition host immunogenetics may have important implications for the transmission of parasitic infections. Further work in this area of nutrition-infection interaction is clearly needed in order to more fully understand the role of nutrition in the transmission and control of parasitic infections in genetically heterogeneous host populations, such as endemic human communities.

The immunological results obtained in the present nutrition experiments indicate that murine humoral immunity is unimpaired in moderate-severe protein malnutrition under both single and repeated *T. muris* infection protocols (Figs. 4.6a,b; 4.7a,b; 4.8a,b; 5.7a,b; 5.8a,b; 5.9a,b). Indeed, malnourished hosts produced higher levels of specific antibody in sera. The enhanced antibody production observed in nutrient deficient hosts was correlated with their heavier parasite burdens, although the responses obtained in well-fed animals indicate that exposure, even to unsuccessful infection, may also contribute to the levels of antibody produced. Such relationships may explain the hyper immunoglobulinaemia commonly observed in malnourished patients (Chandra, 1980,1984; Gerswhin *et al.*, 1985). However, as discussed in Chapters 4 & 5, it is also possible that the increased antibody levels in the malnourished hosts may be due to the effects of malnutrition *per se* rather than infection intensity. Thus, regulatory defects in antibody synthesis and a greater systemic spread of parasite antigens in nutrient deficient hosts may also enhance specific antibody response.

These findings suggest that mammalian specific antibody production may reflect not only the size of the worm burden but also host nutritional status and past history of exposure, with obvious negative implications for the use of antibody levels as markers of infection intensity.

The Western blot analyses indicate that E/S antigen-recognition by specific antibody (IgG1) is unimpaired in moderate-severe protein deficiency. In fact, malnourished mice generally recognized a broader and more intense band of antigens than did correspondingly infected well-fed counterparts (Figs. 4.9a,b; 4.10a,b; 5.10a,b; 5.11a,b; 5.12a,b). Recognition was most clearly demonstrated and consistent at the time of established adult infections (see d.49 p.i primary blots in Fig. 4.10a,b and d.60 p.i.i. trickle blots in Figs 5.11a,b; 5.12a,b). The increased specific antibody production observed in the malnourished mouse groups appears, therefore, to be largely related to their heavier worm loads.

By contrast, antigen-recognition by well-nourished mice during both primary and trickle infection appeared to be restricted to a small group of putatively "larval" antigens. These antigens were first detected at d.21 p.i. and remained the only antigens detected (Fig. 4.9a,b; 5.10a,b). However, despite this limitation in antigen recognition, such hosts are highly resistant to *T. muris* infection. This finding provides the basis of the suggestion made in Chapters 4 & 5 that a large proportion of the increased antibody response (both antigen-recognition and levels of antibody) observed in malnourished hosts may be directed against irrelevant parasite antigens. As concluded in Chapter 4, such a response could form one of the mechanisms by which the putative immunomodulatory activity of the adult *T. muris* may operate (Else & Wakelin, 1988).

These results on CBA/Ca mouse humoral immunity thus suggest that, although serum antibody responses appeared to be intact in both well- and malnourished hosts, antibody is associated with protection against *T. muris* only in the case of well-fed mice. As discussed in Chapters 4 & 5, this may indicate that the observed susceptibility of protein malnourished host is due to defects in cellular immunity rather than in the specific antibody response.

Alternatively, the several direct effects of protein malnutrition on antibody, such as the induction of tolerance and lowered affinity (see Chapter 4), may also act to impair the effectiveness of antibody in nutrient deficient hosts. It is also possible that the IgG1 hypergammaglobulinaemia observed in the present malnourished

mice may have a "blocking" or suppressive effect on host-protective responses (Chapman *et al.*, 1979b; Mitchell *et al.*, 1982).

Comparisons of the response patterns of the 3 antibody types (specific total immunoglobulins, IgG1 and IgA) examined in the experiments reported here, show that the murine antibody response to *T. muris* E/S antigens may predominantly comprise an IgG1 response. This result is consistent with the findings of Else & Wakelin (1989). Increased production of specific IgG1 during infection has also been reported for other intestinal helminths (Chapman *et al.*, 1979a; Williams & Behnke, 1983).

Overall, the results of this study indicate that even moderate protein malnutrition may potentiate the transmission of *T. muris*. The extent to which the results of this, and other animal studies (Table 1.5), may be extrapolated to the human situation is open to debate (see Chapter 1). Human data on this aspect of parasitism is still sparse, presumably owing to the difficulty of quantifying the nutrition-infection interaction (Biesel, 1982; Pawlowski, 1984). Nevertheless, it has been shown that an association may indeed exist between PEM and increased infection with the common human intestinal nematodes, *Ascaris lumbricoides* and *Trichuris trichiura* (Mata, 1972, 1978). New research in this area of human helminthology is now imperative, especially in view of the observed widespread co-occurrence of malnutrition and helminth infections in typical, endemic regions of the world (Stephenson, 1987).

The finding that protein deficiency may enhance susceptibility to parasitic infections, may have important implications for the control of human intestinal helminthiasis in endemic communities (which typically experience both malnutrition and infection). The intervention strategies normally proposed for such communities typically tend to focus only on one aspect of the relationship between malnutrition and infection, controlling either infection (mainly via chemotherapy) or malnutrition (usually by nutritional supplementation). Present knowledge does not indicate whether the treatment of one condition (either malnutrition or infection) will necessarily improve the other (Bundy & Golden, 1987). While it is possible that nutritional supplementation may boost host resistance, the combined effects of continued infection exposure, immunomodulatory and nutritional consequences of resident infections (Beisel, 1980; Chandra, 1984), and genetically determined differences in host susceptibility to infection (Wakelin, 1988) may still act to limit the effectiveness of nutrient supplements alone in controlling helminthic disease. Alternatively, helminth control programmes, whether by chemotherapy or

vaccination, may be hindered by the rapid community re-establishment of infection due to the continued susceptibility of the malnourished subpopulation (Anderson & Medley, 1985). These findings suggest that an integrated approach, combining both nutritional supplementation and the treatment of infection, may well be indicated for the control of helminth infections in typical, endemic areas (Gupta *et al.*, 1977; Duncombe *et al.*, 1979; Gopaldas *et al.*, 1983). An improved understanding of the role of the nutrition-immunity interaction in the transmission dynamics of parasitic infections is clearly pivotal towards the development of such integrated strategies. Simplified animal studies, in which infection exposure and host nutritional and immune status can be precisely controlled, could form an important starting point.

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Density dependence in establishment, growth and worm fecundity in intestinal helminthiasis: the population biology of *Trichuris muris* (Nematoda) infection in CBA/Ca mice

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SUMMARY

The results are presented of an experimental study of the population biology of chronic *Trichuris muris* (Nematoda) infection in cortisone-treated CBA/Ca mice. Attention is focused upon both the validity of the common use of faecal egg counts to demonstrate density dependence in helminth fecundity, and the identification of other possible density-dependent mechanisms that may regulate worm numbers in chronic trichuriasis. The results show that faecal egg counts, although demonstrating high daily variation, are not an artefact of host faecal output but a significant density-dependent function of worm burden. This finding contrasts with the observations on *Heligmosomoides polygyrus* infection in outbred MF1 mice, but accords with similar studies in a wide variety of host – helminth systems. Worm establishment in the murine host is found to be a density related function of infection dose. This is attributed to the probable existence of a physical gut-carrying capacity in the murine host for *T. muris*. Worm distribution in the gut is also shown to be density dependent, with worms being displaced from the caecum to the colon at increasing intensities of infection. The sex ratio of the adult parasites, however, is found to be both unitary and independent of worm burden. Evidence for a significant density-dependent decline in female *T. muris* growth or size is presented. The results also show a significant positive association between female *T. muris* weight and *per capita* fecundity. These findings indicate that the stunted growth of individual worms at high parasite densities may be a potential mechanism underlying density dependence in helminth fecundity.

Key words: *Trichuris muris*, density dependence, fecundity, intestinal helminthiasis.

INTRODUCTION

The characteristic temporal stability exhibited by most mammalian helminth parasite populations is thought to be generated by density-dependent mechanisms, particularly those acting on worm fecundity (Anderson, 1982; Croll, Anderson, Gyorkos & Ghadirian, 1982; Keymer, 1982; Anderson & May, 1985; Smith, Grenfell & Anderson, 1987). This assumption is based upon the substantial evidence for density-dependent helminth fecundity demonstrated for a wide variety of host–helminth systems (reviewed by Keymer, 1982; Anderson & May, 1985).

Recently, however, the widespread acceptance of the biological validity of density-dependent helminth fecundity has been questioned (Keymer & Hiorns, 1986; Keymer & Slater, 1987). The primary criticism here pertains to the validity of the common use of faecal egg counts to estimate helminth egg production. This is because, firstly, faecal egg counts are not only notorious for their inconsistency and variability (Brown, 1927; Scott & Headlee, 1938; Scott, 1946; Moriya, 1954; Hall, 1982; Sinniah, 1982; Anderson & Schad, 1985), but may also be affected by host faecal output (Hall, 1981). For example, faecal bulk could either dilute or concentrate the amount of eggs passed out in the faeces. In addition, Keymer & Hiorns (1986) recently demonstrated that there may be a causal, positive relationship between temporal helminth egg production and host faecal output. This relationship for primary infections of *Heligmosomoides polygyrus* in outbred MF1 mice, suggests that the observed density dependence in helminth fecundity may arise as a result of the reduced faecal egestion suffered by the more heavily infected hosts in the population. These studies indicate that faecal egg counts may not reflect actual helminth egg production. Consequently, the widely assumed density dependence in helminth fecundity (as assessed by faecal egg counts) may simply be an artefact of host faecal output.

This paper describes the population biology of chronic primary infection of *Trichuris muris* in cortisone-treated CBA/Ca mice, an animal model for human trichuriasis (Lee & Wakelin, 1983). The present study has two specific aims. The first is the assessment of the validity of the common use of faecal egg counts to enumerate helminth egg production. In this connection, attention is also focused

Table 1. Experimental protocol

Day post-infection	Experimental procedure
0	Infection by gavage.
7–15	5 × cortisone acetate injection (1·25 mg).
36	Acclimatization.
37–42	5 × 24 h faecal collection 5 × 24 h food intake.
44	Assessment of worm burden.

upon host food intake as a possible source that may generate the differences in faecal output between infected mice. The second aim of the study relates to the identification of possible density-dependent mechanisms in the host–parasite relationship that may regulate worm numbers in chronic trichuriasis.

MATERIALS AND METHODS

Mice

Inbred, male CBA/Ca mice aged approximately 6 weeks at commencement of the study were used throughout. Animals were housed 5–6 to a cage on wood shavings and fed water and a standard mouse chow diet *ad libitum*.

Experimental infections, faecal egg counts and worm burden estimates

Table 1 summarizes the experimental protocol followed in the present study.

The methods used for the maintenance, infection and recovery of *T. muris* were as described by Wakelin (1967). Between 10 and 1000 infective eggs were administered to individual animals to give 6 groups ($n \geqslant 5$) of mice, each group having a different intensity of infection (Table 2). On days 7, 9, 11, 13, and 15 post-infection (p.i.) all the mice were injected subcutaneously with 1·25 mg of cortisone acetate (Sigma Chemical Company Limited), following the procedure of Lee & Wakelin (1983).

Twenty-four hour (h) faecal specimens were collected daily for egg counts from individual mice from day 36 to day 42 p.i. (6 consecutive days) using the procedure described by Keymer & Hiorns (1986). The collections made on day 36 were not used for analysis, to allow for a period of acclimatization by the mice to the experimental procedure.

In order to determine egg numbers, each 24 h faecal specimen was weighed at the time of collection and enough 0·1 M solution of NaOH was added to give a 1 in 10 dilution of the faeces. After standing overnight, the suspension was shaken with 5 glass beads (3·5–4·5 mm diameter) until homogeneous; it was then passed through a 212 μm sieve, and resuspended to 10 ml with excess 0·1 M NaOH. After thorough shaking, 0·1 ml of this faecal suspension was then taken for egg counting under the microscope. Results are presented as eggs per gram (epg), and eggs per day (epd) of the 24 h faecal material collected from each animal.

The daily food consumption of each mouse was measured by weighing all the food placed in the hoppers at the beginning of each 24 h period, and weighing uneaten pellets as well as fragments in the collection tray at the end of the 24 h period.

On day 44 p.i. worms were carefully removed from the large intestine of each mouse post-mortem and were sexed, counted and stored in 10% formal saline before the wet weights of the females were measured.

RESULTS

Relationship between worm burden and infection dose

The number of worms recovered from the large intestine of the 37 mice on day 44 p.i. ranged from

Table 2. Summary of the numbers of worms recovered from the large intestines of the 6 groups of mice on day 44 p.i., with their associated mean 24 h faecal output, food intake and faecal egg counts on days 37–42

(All group figures are the average of the means of 5 values for each animal in the group ±S.E.)

Group	No. of mice	Infection dose	Mean no. of worms/24 h	Mean g food/24 h	Mean g faeces/24h	Mean epg/f* /($\times 10^{-3}$)	Mean epd/f† ($\times 10^{-3}$)
A	10	10	2·7±0·85	3·63±0·09	0·66±0·02	2·16±0·99	1·41±0·64
B	5	50	13·0±1·86	3·77±0·21	0·77±0·05	5·88±0·75	4·28±0·58
C	5	100	32·2±3·64	2·99±0·35	0·57±0·09	11·16±1·61	5·18±0·37
D	5	200	65·8±7·37	4·18±0·14	1·01±0·06	3·61±0·57	3·40±0·58
E	6	600	72·7±8·45	3·61±0·20	0·99±0·07	3·51±0·66	3·43±0·68
F	6	1000	170·3±42·50	4·35±0·13	1·09±0·03	1·72±0·69	1·66±0·62

* Estimated no. of eggs/g faeces/female worm.
† Estimated no. of eggs present in 24 h faecal sample/female worm.

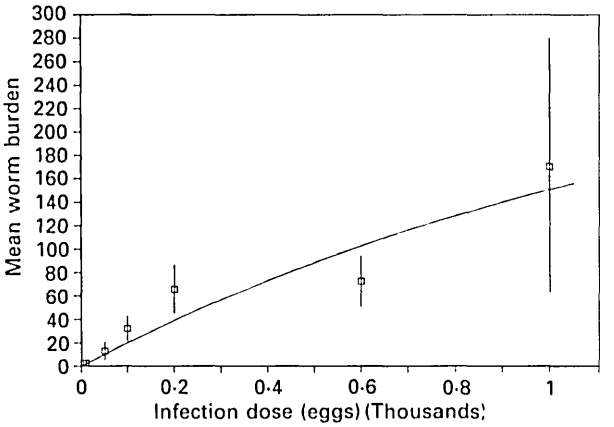

Fig. 1. The density-dependent relationship between the number of *Trichuris muris* established in a single host and the infection dose administered. (□) Observed mean number of worms established; the bars represent 95% confidence limits for these means. (—) Best-fit empirical saturation model described in the text (Eqn (1)) with parameter values $K = 300$ and $d = -0{\cdot}0007$.

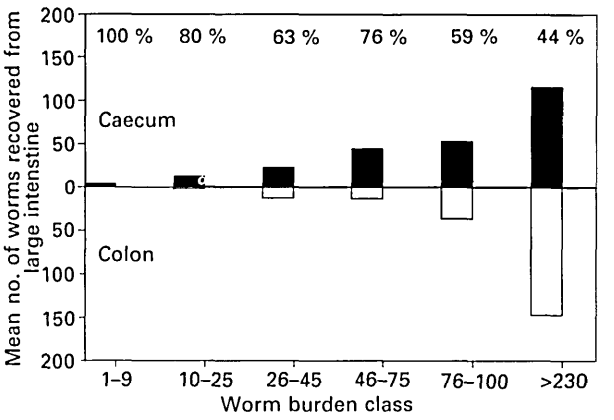

Fig. 2. The distribution of *Trichuris muris* in the murine large intestine as a function of worm intensity. (■) Mean number of worms found in the caeum; (□) mean worm number obtained in the colon for each of the 6 worm class described at the bottom of the graph. The figures at the top indicate the percentage of worms recovered from the caecum for each worm burden class.

0 to 280 (Table 2), and was found to be proportional to the infection dose administered to each mouse (Fig. 1). Regression of the logarithmic relationship between worm recovery and infection dose, however, resulted in a slope that was found to differ significantly from 1 ($b = 0{\cdot}85$, S.E. $= 0{\cdot}06$, $t = 2{\cdot}5$, 35 D.F., $P < 0{\cdot}02$), suggesting the probable presence of a threshold effect on *T. muris* establishment with increasing exposure rates. These results are better described by an empirical saturation model of the form:

$$P(L) = K(1-\exp(-dL)) \quad (1)$$

where P, is the mean number of parasites that establish in the host at each infection dose, L; K is

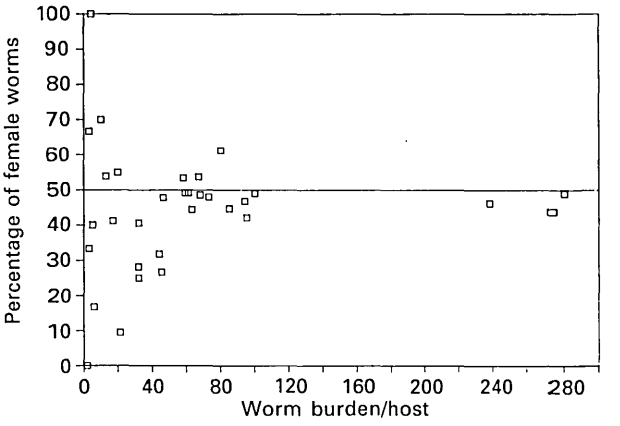

Fig. 3. The sex ratio of worms recovered from each of 37 mice, and the density-independent relationship between sex ratio and worm burden.

the maximum worm burden, which in this study was found to be approximately 300 for *T. muris* in CBA/Ca mice; and d, is a shape parameter (unit = 1/burden) which is related to the dose at half-maximum burden (say $L = q$, and $P = K/2$) by the simple relation $d = \ln(2)/q$ (Fig. 1).

Distribution of T. muris *in the large intestine*

Fig. 2 illustrates the spatial location of *T. muris* recovered from the murine large intestine in relation to worm density. The data clearly suggest the existence of a parasite density effect on the distribution of worms in the large intestine: at increasing intensities of infection, an increasing proportion of the worms colonizes the colon as well as the more normal location in the caecum ($t = 7{\cdot}36$, 32 D.F., $P < 0{\cdot}001$).

Sex ratio of T. muris

The mean female: male ratio of *T. muris* in mice was found to be 0·95: 1·00. No significant bias was detected in the sex ratio ($t = 0{\cdot}14$, 33 D.F., $P > 0{\cdot}05$). Regression analysis of the data also indicates that the sex ratio of *T. muris* is independent of worm burden ($t = -0{\cdot}055$, 32 D.F., $P > 0{\cdot}05$) (Fig. 3). Sex ratio is therefore unlikely to have any significant influence on the relationship between worm burden and fecundity.

Helminth fecundity relationships

(i) *Day to day variability in faecal egg counts (days 1–5).* Variability in replicate daily measurements is a striking feature of faecal egg counts (Hall, 1981; Croll *et al.* 1982; Sinniah, 1982; Anderson & Schad, 1985; Keymer & Hiorns, 1986; Bundy, Cooper, Thompson, Anderson & Didier, 1987). It was decided, therefore, in the present study, to examine this feature of faecal egg counts, under the controlled

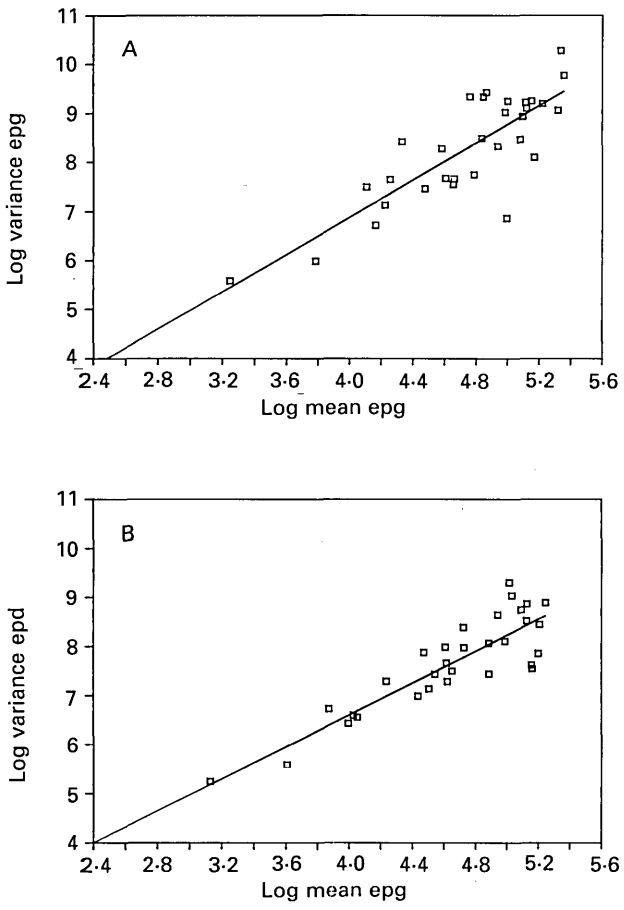

Fig. 4. Day to day variability in *Trichuris muris* egg output from individual mice. The relationship between the logarithms of the variance (V) and the mean (M) epg (A) and epd (B) of 5 repeated estimates from each of 37 mice. (□) Observed values; (—) best-fit linear model of the form $\log_{10}$ (V) = $a + b$ $\log_{10}$ (M) where $a = -0{\cdot}72$ and $b = 1{\cdot}90$ ($r^2 = 0{\cdot}69$), and $a = 0{\cdot}09$ and $b = 1{\cdot}63$ ($r^2 = 0{\cdot}77$), for epg and epd respectively. Values of b close to 2·0 reflect a high degree of variability in the raw counts, while if the day to day counts were randomly distributed (variance approximately equal to the mean), the slope of the linear model would be approximately unity in value (Elliot, 1977).

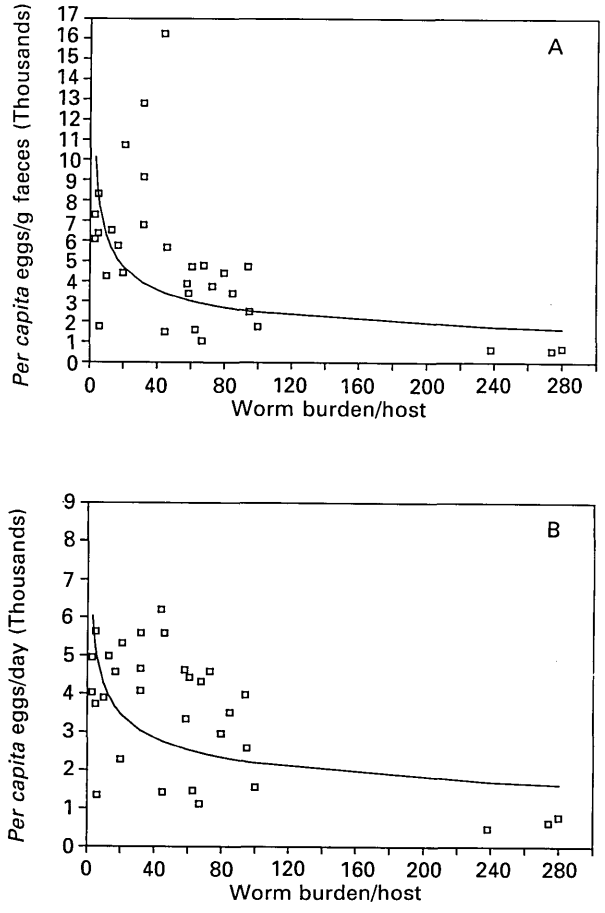

Fig. 5. Egg output/female worm as a function of total worm burden. (A) Relationship based on *per capita* epg; (B) relationship based on *per capita* epd counts. (□) Observed values (mean of 5 replicate measurements for each of 31 patent mice); (——) best-fit power function (Eqn (2) in the text) with parameter values $a = 15{,}812{\cdot}5$, $b = -0{\cdot}40$; and $a = 8{,}317{\cdot}64$, $b = -0{\cdot}29$, for epg/female worm and epd/female worm respectively.

experimental conditions of the laboratory. Fig. 4 shows that the relationship between the variance and the mean on a $\log_{10}$ scale for both epg and epd is linear, with slope b of the linear models being 1·90 and 1·63 respectively. The results indicate that while both the epg and epd counts exhibit high day to day variability, epd counts ($r^2 = 0{\cdot}77$) are marginally less variable than epg ($r^2 = 0{\cdot}69$).

(ii) *Relationships between faecal egg output, host faecal output and worm burden.* The relationship between the mean eggs/g faeces contributed by each female *T. muris* (*per capita* epg) and total worm density is shown in Fig. 5A. The power model of the general form:

$$y = ax^b \qquad (2)$$

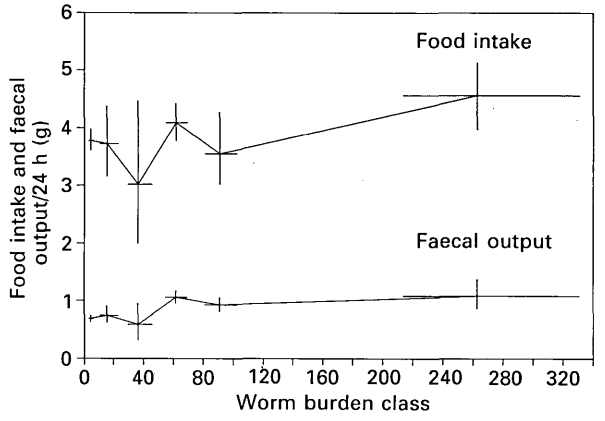

Fig. 6. The relationship between average host food intake, faecal output/24 h and worm burden. The intersections of the vertical and horizontal bars are observed means, the vertical bars denote the asymmetric 95 confidence limits, the horizontal 95 limits arise as a result of grouping individual observations into each of 6 worm classes. The sample sizes for each worm class from left to right were 5, 5, 5, 8, 5 and 3 respectively.

Table 3. Variability in host faeces and egg counts between mice and between days

g faeces/24 h	Between mice	$F = 8{\cdot}85$ 29,119, D.F. $P < 0{\cdot}001$
	Between days	$F = 7{\cdot}90$ 4144, D.F. $P < 0{\cdot}001$
epg	Between mice	$F = 65{\cdot}12$ 29,119, D.F. $P < 0{\cdot}001$
	Between days	$F = 0{\cdot}75$ 4144, D.F. $P > 0{\cdot}05$ NS.
epd	Between mice	$F = 134{\cdot}66$ 29,119, D.F. $P < 0{\cdot}001$
	Between days	$F = 0{\cdot}01$ 4144, D.F. $P > 0{\cdot}05$ N.S.

gave the best empirical fit to the observed data. *Per capita* epg is inversely related to worm burden ($t = -3{\cdot}64$, 29 D.F., $P < 0{\cdot}002$). The parameter z (Croll *et al.* 1982; Anderson & May, 1982; Bundy, Thompson, Cooper, Golden & Anderson, 1985), which varies inversely with the severity of the density-dependent constraints on parasite fecundity ($z = \exp[-b]$, where b is the exponent of the relationship between parasite fecundity and worm burden), for the present population of *T. muris* is found to have a value of 0·992. It is significant that this value of z is almost identical to that observed for *T. trichiura* and for other gastrointestinal helminths of humans (Bundy *et al.* 1985), suggesting that worm fecundity may indeed be a major regulatory force stabilizing the growth of parasite populations (Anderson, 1982; Anderson & May, 1985).

The relationship between mean host faecal output/24 h and worm density is displayed in Fig. 6. The results indicate that mean faecal output/24 h/host exhibited a slight, but statistically significant increase with worm density ($F = 8{\cdot}11$, 1·29 D.F., $P < 0{\cdot}01$). In order to evaluate a possible mechanism behind changes in faecal output in infected hosts, mouse food consumption/24 h was also monitored over each of the 5 days on which faecal collections were made. Host food intake/24 h, however, did not change significantly with worm density ($F = 3{\cdot}53$, 1·29 D.F., $P > 0{\cdot}05$).

The results presented in Figs 5 A and 6 suggest the possibility that the density dependence apparent in the *per capita* epg values could simply depict a diluting artefact of increasing host faecal output at higher intensities of worm infection. This possibility may be tested by considering the relationship between *per capita* daily egg counts (epd) and worm density. If *T. muris* fecundity was independent of worm burden, the *per capita* epd values which, by definition, are independent of faecal output should counteract the diluting effect of increased host faecal egestion at rising intensities of infection. The relationship between mean *per capita* epd and worm density is shown in Fig. 5B. Once again, the egg counts are found to be an inverse function of worm burden; the power model again provides the best empirical fit to the density dependent relationship ($t = -3{\cdot}22$, 29 D.F., $P < 0{\cdot}005$). Significance testing of the slope, b ($= -0{\cdot}29$), with that obtained for

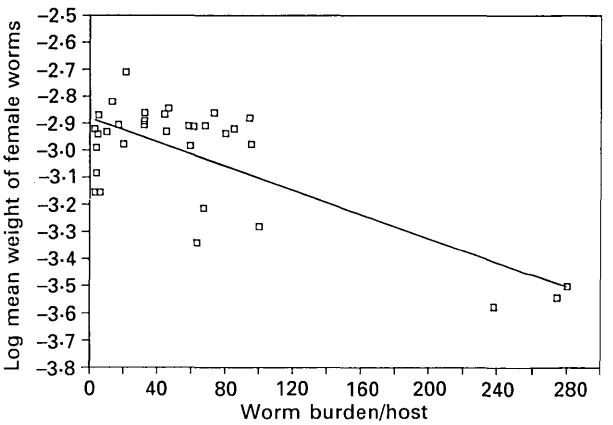

Fig. 7. The semi-logarithmic relationship between mean female worm weight and total burden. (□) Observed values; (——) best-fit linear regression of the $\log_{10}$ worm weight on worm burden with parameter values $a = -2{\cdot}88$ and $b = -0{\cdot}002$.

per capita epg ($b = -0{\cdot}40$) also showed no difference ($t = 0{\cdot}323$, 58 D.F., $P > 0{\cdot}05$), implying that host faecal output had little effect on faecal egg counts in *T. muris* infection.

This conclusion is further supported by the results of an analysis of covariance to assess the variability in egg count and host faecal output from day to day, and from mouse to mouse. The results presented in Table 3 clearly demonstrate the independence of faecal egg counts from host faecal output. While the mice exhibited significant variation in faecal output from day to day, the egg counts, although describing daily fluctuations (Fig. 4), do not vary concomitantly with host faecal output. However, as would be expected, on the basis of the relationships shown in Figs 5 and 6, both parasite egg counts and host faecal output varied significantly between individual mice. These results suggest that the faecal egg counts obtained in the present study are a true reflection of *T. muris* egg production, which varied significantly between mice as a density-dependent function of their worm burdens.

(iii) *Worm size and density-dependent fecundity.* Fig. 7 shows the results of the regression of mean female worm weight upon total worm burden/host. The results indicate a highly significant decrease in female *T. muris* weight with increasing worm density ($t = -6{\cdot}22$, 32 D.F., $P < 0{\cdot}001$).

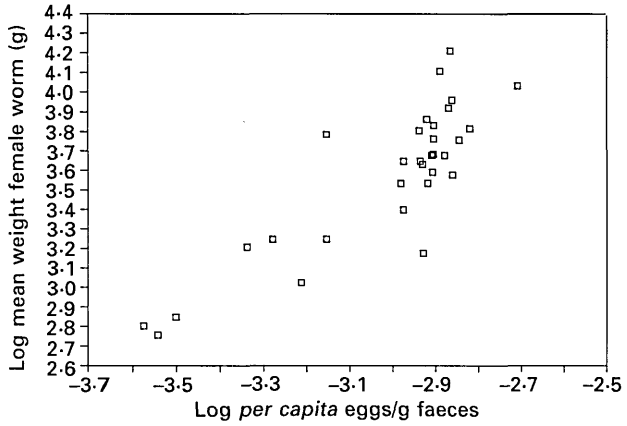

Fig. 8. The logarithmic relationship between mean female *Trichuris muris* weight and *per capita* fecundity. (□) Mean of 5 replicate epg estimates.

To evaluate the significance of the density dependence in adult size on worm fecundity, the mean female worm weights for each burden were plotted against the respective *per capita* epg and assessed for relationship. As can be seen from Fig. 8, the results portray a highly significant positive correlation ($r = 0{\cdot}86$, 29 D.F., $P < 0{\cdot}001$) between mean female worm weight and their mean egg production, indicating that density dependence in helminth fecundity may well arise as a consequence of population effects on individual worm growth or size.

DISCUSSION

The non-linearity detected in the relationship between worm establishment and infection dose in this study (Fig. 1) may be suggestive of a dose-dependent constraint on *T. muris* establishment in the murine host. The suggestion of a possible upper limit to worm establishment in the present model may indicate the probable existence of a physical carrying capacity in the mouse for *T. muris*. Wakelin (1975) found 500 worms to be the maximum burden supportable by non-responder Schofield mice. In the present study, this number was detected to be around 300 for the smaller cortisone-treated CBA/Ca mice. The existence of a finite capacity for *T. muris* in these mice may be related to the large size of the worm (around 8·8–10·1 mm and 14·2–15·8 mm in length for young males and females respectively (Fahmy, 1954)), relative to the dimensions of the mouse large intestine. A similar physical limitation on gut carrying capacity has been suggested for the large human intestinal worm, *Ascaris lumbricoides* (Beaver, 1980). This may indicate a role for parasite size in the mechanisms involved in population regulation.

The typical location for *T. muris* in the mouse host is considered to be the caecum (Panesar & Croll, 1980). However, as host burdens increase, an increasing proprotion of worms are displaced to the colon such that at very high worm densities, there are more worms established in the colon than in the caecum (Fig. 2). It has been suggested that newly-hatched larvae must enter caecal gland openings rapidly or risk being swept with the luminal contents into the colon (Wakelin, 1967; Panesar, 1981). This, and competition between larvae for available caecal gland openings may account for the increasing displacement of establishing larvae to the colon at increasing infection intensities (Fig. 2). Displacement of worms, from the caecum to the colon and even to the rectum has also been described in heavy *T. trichiura* infections of humans (Bundy & Cooper, 1988). This displacement of worms from presumably optimal locations may have important consequences for the population dynamics of trichiuroid infections. It is possible that the physiology of the worm may be affected, with adverse implications for survival, growth and hence fecundity (Krupp, 1962).

In common with many studies of geohelminth infection in humans (Croll *et al.* 1982; Martin, Keymer, Isherwood & Wainwright, 1983; Anderson & Schad, 1985; Bundy *et al.* 1985; Elkins, Hawell-Elkins & Anderson, 1986), no significant bias was found in the sex ratio of *T. muris* in the present study. Furthermore, the sex ratios of both *T. trichiura* (Bundy *et al*, 1985) and *T. muris* are not density related (Fig. 3). These findings support the assumptions of equal sex distributions made for models of mating probabilities of dioecious helminths (Macdonald, 1965; May, 1977; Anderson & May, 1978).

The day to day variability recorded for mean egg counts from individual mice (Fig. 4) is unsurprising. Similar variability has been reported both in human (Croll *et al.* 1982; Anderson & Schad, 1985; Bundy *et al.* 1987) and experimental (Keymer & Hiorns, 1986) intestinal helminth infections. The present data collected under the controlled experimental conditions of the laboratory support the conclusion that daily variability in helminth egg counts is an inherent biological feature of the host–parasite interaction, and not an artefact created through estimation procedures (Keymer & Hiorns, 1986).

In addition to density dependence in worm establishment (Fig. 1), the present results also indicate the existence of density dependence in worm fecundity (Fig. 5). The analyses made in this study (Table 3) demonstrate that variations in faecal egg count between hosts are not artefacts of differences in host faecal output, but are a function of the worm burden.

The above results are in direct contrast to the observations on *H. polygyrus* infection in outbred MF1 mice (Keymer & Hiorns, 1986), where host faecal production exhibited an inverse relationship with worm density, but showed a positive association with parasite fecundity. These findings led the

authors to conclude that density dependence in intestinal helminth fecundity may be a direct outcome of differences in faecal output between infected mice, and thus that helminth fecundity may be functionally related to host faecal production (Keymer & Hiorns, 1986). The present data (Figs 5, 6 and Table 3), however, clearly do not support this conclusion. Instead, they indicate that faecal egg counts are a true reflection of worm egg production, and that density dependence in helminth fecundity is independent of host faecal output; a conclusion which accords with the numerous observations of helminth density dependence in human and veterinary infections (Michel, 1969; Boray, 1969; Anderson & Schad, 1985; Medley & Anderson, 1985; Smith, Grenfell & Anderson, 1987).

The present study also provides some insight into the mechanisms which generate density dependence in worm fecundity. The density dependence of *T. muris* female weight (Fig. 7), and the positive association between worm weight and *per capita* egg production (Fig. 8), suggest that the density-dependent decline in individual worm fecundity may be due to the stunted growth of individual worms when occurring at high population densities. Density dependence in parasite size is generally speculated to arise either as a result of intraspecific competition for finite resources, or through the effects of host immune responses which increase disproportionately in efficiency as parasite burden increases (Anderson & May, 1978; Keymer, 1982; Anderson & May, 1985). While host immune responses, both humoral and cellular, have been implicated in depressed helminth growth and fecundity (Wakelin & Wilson, 1980; Lloyd & Soulsby, 1987), they are probably irrelevant to the present system, in which the mice were rendered immunotolerant to infection by corticosteroid treatment (Lee & Wakelin, 1983). Intraspecific competition for space or food resources, enhanced at high density when the worms are displaced into sub-optimal niches (Fig. 2) is a more probable candidate mechanism. This conclusion indicates the value of laboratory immunotolerance models in the exploration and understanding of the population processes that govern host–parasite interactions in nematode infections.

In conclusion, this study of *T. muris* provides evidence of density dependence in both worm establishment and fecundity; the latter being related to density dependence in individual worm growth and development. These observations are at variance with those on *H. polygyrus* (Keymer & Hiorns, 1986), but are entirely compatible with similar, though necessarily less controlled, studies of human and veterinary helminth infections. The results also accord with theoretical analyses which indicate that the remarkable temporal stability of mammalian helminth populations is a function of tight density-dependent regulation (Anderson, 1982; Croll *et al.* 1982; Keymer, 1982; Anderson & May, 1985; Anderson & Medley, 1985). The practical implication of these conclusions is that helminth populations are intrinsically robust to perturbations (Anderson & May, 1982; Anderson & Medley, 1985), which suggests a gloomy prognosis for attempts to reduce their population size by control interventions.

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