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The development of a mouse model to explore resistance and susceptibility to early *Ascaris suum* infection



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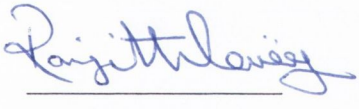
A thesis submitted in fulfilment of the degree of Doctor of Philosophy to the University of Dublin, Trinity College.

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Paul Ranjith Lewis.

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SUMMARY

Ascaris suum and *Ascaris lumbricoides* are widespread geohelminths of pigs and humans, respectively, resulting in severe morbidity and possible mortality. The contribution of migrating *A. suum* larvae to liver damage has been observed in pigs and is problematic, resulting in liver condemnation at slaughter. The larval stages of both species are also associated with allergic and respiratory symptoms and these are an understudied source of morbidity and mortality in people. Bronchovascular damage resulting from migrating larvae may produce secondary damage caused by opportunistic microorganisms. Furthermore, it is believed that susceptibility to the larval population may be a determinant factor in the development of a patent adult population. *A. suum* and *A. lumbricoides* exhibit an overdispersed frequency distribution in their host populations in both the adult and larval stages and individuals appear to be predisposed to either high or low intensities of infection. The impact of host factors on this observed distribution is still poorly understood and difficult to investigate in natural host populations particularly in relation to the larval stages. The use of a mouse model has been supported by the observations that the larval migratory pattern mimics the pattern observed in the pig.

Extrinsic factors were initially explored that might affect the quantitative recovery of larvae during migration. This was developed in order to provide a standard model system facilitating accurate assessment of host genetic variation on this phase of the infection in the mouse model. Larvae accumulated in the livers of both C57BL/6j and BALB/c mice up to and including days 4-5 post-infection (p.i.) and then declined in both strains until day 9. Loss of larvae from the livers corresponded to the arrival and accumulation of larvae in the lungs on days 6-8 in both strains but larval recovery was considerably higher in C57BL/6j mice. It was concluded that day 7 lung recoveries gave the best indication of relative resistance/susceptibility to this parasite. The susceptibility in terms of pulmonary larval burden were then compared in a panel of inbred mouse strains; A/J, BALB/c, CBA/Ca, C57BL/6j, C3H/HeN, DBA/2, NIH, SJL, and SWR mice. C57BL/6j mice were identified as the most susceptible strain and CBA/Ca mice as having the most contrasting phenotype, but with a similar pattern of migration. A strong positive correlation was observed between the size of the initial inoculum and the percentage worm recovery from the lungs in both CBA/Ca and C57BL/6j mice, but the difference between these strains was highly consistent, 66.6-80%, regardless of the initial dose. An assessment of worm length showed a correlation between dose administered and larval lengths, with lower doses resulting in

an increase in the average larval length, especially on day 7p.i. but this was not influenced by strain. These results demonstrated that, using protocols devised in the present study for infection and recovery, between-experiment variation in *A. suum* worm burdens is minimal, and that C57BL/6j mice are highly susceptible to infection compared to other strains examined irrespective of the initial inoculum size.

In the latter part of the investigation possible intrinsic host differences between the resistant (CBA/Ca) and susceptible (C57BL/6j) phenotypes, in relation to pulmonary larval burdens, were examined. The early migration pattern of larvae in C57BL/6j and CBA/Ca mice was examined from 6 hours to day 8 p.i., inclusively. There were more larvae migrating through the large intestine wall of C57BL/6j mice at 6 hours p.i.. This initial difference may reflect a physiological difference in the speed at which larvae and eggs were passed through the intestinal tract between the strains. This observation was confirmed by the comparable larval numbers in the liver in both strains on each post-mortem day. However the larval burden observed in the lungs were significantly reduced in CBA/Ca mice in contrast to C57BL/6j mice between the phase of migration from the liver and into the lungs suggesting some hepatic/post-hepatic factor that varies between the two strains. This demonstrated that the period of migration from the liver to the lungs played a critical role in determining susceptibility/resistance to pulmonary ascariasis.

An examination of the pulmonary inflammatory response at days 8-10 p.i. suggested that this response was not prominently involved in the protection of mice to *A. suum* infection in the latter days of infection in the lungs and confirmed that the eradication of the parasite from host tissues occurs at an earlier stage. C57BL/6j mice produced a Bronchoalveolar lavage response almost twice as intense as that of CBA/Ca mice with stronger neutrophil, lymphocyte and eosinophil but not macrophage components. Therefore mice harbouring more larvae responded more intensely, but this appeared to be an immune reaction to damage caused by migrating larvae rather than a protective response. This was further substantiated by the results of the histological examination which showed a two-fold difference in the inflammatory response of C57BL/6j mice and this did not correspond to an effective entrapment of larvae but a response to a significantly higher larval burden in the lungs of these mice. Susceptibility to the larval stages of *A. suum* was thus determined during the period of residence in the liver or at a post-hepatic stage prior to accumulation in the lungs. This model can now be used with confidence in order to identify the underlying mechanisms of the observed susceptibility/resistance to larval ascariasis.

PUBLICATIONS

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Lewis, P.R., Behnke, J.M., and Holland, C.V. (2004) Optimisation of the *Ascaris suum* mouse model to facilitate subsequent investigation of the mechanisms influencing susceptibility to infection. *Proceedings of the European Multicolloquium of Parasitology (EMOP IX)*, p346.

"It often happens that the universal belief of one age- a belief from which no one was free or could be free without an extraordinary effort of genius or courage- becomes to a subsequent age so palpable an absurdity that the only difficulty is to imagine how such an idea could ever have appeared credible"

-John Stuart Mill

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CHAPTER 1

INTRODUCTION
&
LITERATURE REVIEW

1.1 INTRODUCTION

Ascariasis is described as the infection with the large roundworm of the genus *Ascaris* (Nematoda, Ascaroidea). *Ascaris lumbricoides* is the infectious agent of man and is one of the most prevalent human infections (Crompton *et al.*, 1985) but its sibling species, *Ascaris suum*, is also a widespread geohelminth infection of pigs (Soulsby, 1982). Most authorities agree upon the figure of 1.4 billion people as the global prevalence of *A. lumbricoides* (Chan *et al.*, 1994) with most incidences observed in developing countries (Thein Hlaing, 1993). Morbidity with serious health consequences is observed in 122 million cases (Chan *et al.*, 1994). The clinical manifestations of ascariasis, which have been studied in detail in pigs using *A. suum*, range from acute, life threatening complications such as intestinal obstructions to more subtle symptoms associated with malnutrition and possibly asthma (Crompton *et al.*, 1985). However, many aspects of these parasites and their relationship with their respective hosts are still poorly understood. Furthermore, there is continued debate as to the taxonomic distinctness of the two species.

A. suum infection is important in pig production. Porcine ascariasis interferes with the health and performance of pigs and produces reduced feed to gain ratios which results in economic losses (reviewed by Roepstorff, 2003) and liver condemnation at slaughter due to migrating larvae (reviewed by Boes, 1999). Hale & Stewart (1987) calculated that increased feed and maintenance costs of pigs due to *A. suum* infections varied from $1.92 per raised fattener (pig) with light infections up to $5.56 for heavy infections. The prevalence of *A. suum* in pigs has increased in some regions while it is uncommon to find parasite-free herds (Nansen & Roepstorff, 1999). This is due to increased drug (anthelmintic) resistance, which results in a decrease in drug efficacy, among the parasite populations particularly in areas where anthelmintics are administered regularly as prophylactics (Behnke *et al.*, 2000). Increased drug resistance has important implications for pig producers (Stewart & Hale, 1988) associated with reduced feed conversion efficiency resulting in economic losses through reduced pig prices and worming costs.

The distribution of worms in human hosts is highly aggregated or overdispersed with the minority of individuals harbouring most of the worms and the majority of people bearing few worms. Individuals appear to be predisposed to either heavy or light infection following anthelmintic treatment (Holland *et al.*, 1989; Guyatt *et al.*, 1990; Thein Hlaing, 1993; Crompton, 1994; Palmer *et al.*, 1995). Predisposition is of practical interest since it

has implications for selective approaches to chemotherapeutic control, the distribution of morbidity within the host populations and the dynamics of transmission (Holland & Boes, 2002). Thus far there has been very little work on the impact of larval stages on morbidity and mortality in human hosts and the predisposition of individuals to the migratory stages.

A novel contribution to *Ascaris* epidemiology is the development of a pig model of predisposition to *A. suum* (Boes *et al.*, 1998a). These authors were able to demonstrate that individual pigs were predisposed to low or high *A. suum* worm burdens subsequent to chemotherapuetic treatment. The difficulty in using pigs to explore the contribution of host genetics to susceptibility and resistance to *A. suum* is time, cost and a lack of suitable inbred porcine strains. The mouse was recently re-evaluated as a potential model for the study of *A. suum*, and the associated disease ascariasis, and this was due to the analogous pattern of larval migration in mice and pigs (Murrell *et al.*, 1997a). However, a patent infection does not occur in mice and as such this limits the model to studies of the larval population. Up to now, there has been little definitive evidence that mouse strains vary in their susceptibility to ascariasis. This can be explained by heterogeneity in experimental protocols of previous research. This thesis examines heterogeneity in susceptibility between inbred mouse strains and explores the possible mechanisms of resistance and susceptibility.

1.2 EXPERIMENTAL AIMS

The aims of this project are:

1. to develop an experimental protocol that will remove extrinsic factors as a source of variation in larval burdens of *A. suum* in a mouse model.
2. to provide information on the pattern and kinetics of migration of *A. suum* larvae through the liver and lungs of two reference strains of inbred mice, which were previously identified as resistant (BALB/c) and susceptible (C57BL/6j) with relation to their pulmonary larval burdens, on days 3-9 post-inoculation (p.i.).
3. to select two appropriate strains of mice showing divergent resistant and susceptible phenotypes to *A. suum* infection in the pulmonary tissue on days 6-9 p.i.. This involved the investigation of a panel of inbred mouse strains; BALB/c, C57BL/6j, SWR, SJL, NIH, C3H/HeN, CBA/Ca, A/J, and DBA/2, incorporating those strains highlighted for the mouse genome project, this was used to determine which two strains were most divergent. These two strains were then used in all subsequent experiments.
4. to investigate the effect of initial inoculum size of *A. suum* eggs on relative susceptibility to pulmonary ascariasis in two inbred mouse strains, that are resistant (CBA/Ca) and susceptible (C57BL/6j) to this parasite, on days 6-8 p.i.
5. to examine the effect of mouse strain (using the resistant (CBA/Ca) and susceptible (C57BL/6j) mouse phenotypes), day and dose administered on larval growth in the lungs on days 6-8 p.i..
6. to determine early *A. suum* larval migration in two mouse strains, CBA/Ca and C57BL/6j, from 6 hours p.i. to day 8 p.i..
7. to investigate the influence of the inflammatory immune response on resistance to *A. suum* in the pulmonary tissues of resistant (CBA/Ca) and susceptible (C57BL/6j) mouse strains on days 8-10 p.i.. This will be accomplished using hydrocortisone acetate to immobilise the host's immune response and also by exploring inflammatory and other changes in the lung alveolar spaces through bronchoalveolar lavage (BAL).
8. to examine the histopathological changes in the lungs of CBA/Ca (resistant) and C57BL/6j (susceptible) mice inoculated with *A. suum* on days 8-10 p.i..

1.3 CROSS-INFECTIVITY AND THE QUESTION OF SPECIATION BETWEEN *A. SUUM* AND *A. LUMBRICOIDES*

There has been some uncertainty in the distinction of *A. suum* and *A. lumbricoides* as two separate species. The ambiguity between the two species of *Ascaris* arises commonly in areas that are endemic and where pigs and humans live in close proximity to each other or where excreta of both humans and pigs is used as fertiliser (Peng *et al.*, 1996). A species is defined as "an interbreeding group of biological organisms that are reproductively isolated from all other organisms". A species can be made up of groups in which members do not actually exchange genes with members of other groups (though in principle they could do so). However, if some gene flow occurs along a continuum, the formation of another species is unlikely to occur. Where barriers to gene flow arise (e.g. physical barriers) this reproductive isolation may lead, by local selection, to the formation of morphologically distinct forms, i.e. races or subspecies. These could interbreed with other races of the same species if they were introduced to one another (Allaby, 1999). There is no known morphological distinction between *A. suum* and *A. lumbricoides* that discriminates the two species (Madden & Tromba, 1976). The biochemical differences between their antigens are minute and immunoprecipitation cannot detect major differences (Kennedy *et al.*, 1987; Abede *et al.*, 2002). Instead, there is possible gene exchange between the two species as cross infection does not appear to be as rare as initially perceived (Nejsum *et al.*, 2005b).

Clinical case histories suggest that most cases of ascariasis in non-endemic areas are actually cross-infections from pig *Ascaris*. Molecular data (using Hae III restriction sites on two classes of recombinant DNA or rDNA) and epidemiological data incriminate pigs as the source of infection in the majority of North American cases (Anderson, 1995). Recently this was confirmed in Denmark by a detailed epidemiological and genetic analysis of an international collection of adult worms obtained from pigs and humans (Nejsum *et al.*, 2005b). The genetic analysis and the pattern of contact of Danish patients with pig-manure illustrated that cross-infections from pigs are a common transmission route (Nejsum *et al.*, 2005b). Recent outbreaks of ascariasis in the population of Kyushu Japan, an area that once again began the use of pig manure as fertiliser, is further evidence of constant cross infection and is believed to be caused by larval migrans (Yoshida *et al.*,

2004). There is also the possibility of cross infection in the other direction in endemic areas. Human *Ascaris* was previously shown to be capable of infecting pigs under experimental conditions (Galvin, 1968).

It has been suggested that sympatric populations of *Ascaris* infecting humans and pigs are ecologically/reproductively isolated but that interbreeding can occur. This interbreeding is observed as low levels of gene flow between the species. Anderson *et al.* (1993) observed that nuclear and mitochondrial genomes showed incongruent patterns of divergence suggestive of a secondary introgression (the insertion of the genes of one species into the gene pool of another) following the division of the two host-associated parasite populations. More recently, Anderson & Jaenike (1997) and Anderson (2001) explained the mtDNA profiles as the retention of ancestral polymorphisms within two epidemiologically isolated species but they did not exclude the possibility of gene exchange. Similarly in China the analyses of rDNA and mtDNA from *Ascaris* obtained from pigs and humans in two villages concluded that these were reproductively isolated species (Zhu *et al.*, 1999) but again they did not eliminate the possibility that they were two genetically distinct populations of the same species. Peng *et al.* (2003) carried out a molecular epidemiological investigation of *Ascaris* genotypes in China and recorded five different *Ascaris* genotypes (G1-G5), of which G1 predominately infected humans and G3 was associated with pigs. In contrast, genotypes G2, G4 and G5 represented hybridisation between human and pig affiliated *Ascaris* and have been recovered from both host species (Peng *et al.*, 2003) and these may suggest interbreeding, although the frequencies of the three remaining genotypes was substantially lower than G1 and G3. The common occurrence of cross-infection in non-endemic areas (Anderson, 1995; Nejsum *et al.*, 2005b), and the evidence of a mixed genotype (Peng *et al.*, 2003) may indicate that these are not reproductively isolated species. It is estimated that one individual per generation is enough to prevent significant genetic drift (Wright, 1978).

The overall conclusion particularly from non-endemic areas is that *Ascaris* is zoonotic. From a control perspective, pigs could therefore be considered a reservoir of infection if cross-infectivity was frequent. This would possibly reduce the effectiveness of chemotherapy programmes directed towards humans. Also knowledge of cross-infectivity is required to understand gene exchange and the spread of drug resistance, especially if the two populations are genetically linked. The longevity of viable infective *Ascaris* eggs in the environment, which presents control problems and the use of 'nightsoil' (human faeces

and urine) and pig manure (untreated) as fertiliser for crops means that the possibility of cross-infection in both directions is increased and presents a definite source of zoonotic infection. For the purposes of this review the two species will be identified by their current phylogeny, that of *Ascaris suum* (pigs) and *Ascaris lumbricoides* (humans).

1.4 The Life-cycle of *Ascaris*

Physiology, resistance and development of *Ascaris* eggs

The eggs of *A. lumbricoides* are oval, measuring 50-75 μm by 40-50 μm and they are brown to yellow in colour. The eggs of *A. suum* are similar in size and shape with no discernible differences. The eggs have thick shells of 1.5-2μm (Lysek *et al.*, 1985), the albuminous layer of which bears prominent projections (Soulsby, 1982) and this is a proteinaceous material secreted by the uterus. Beneath these there is a chitinous shell consisting of three layers; the outer vitelline membrane which is derived from the oocyte, the middle chitinous zone and the inner lipid membrane which provides most of the impermeability (Meng *et al.*, 1981; Lysek *et al.*, 1985). This makes them extremely resistant to adverse climatic conditions, except they are vulnerable to desiccation, allowing them to survive in the external environment for up to 7 years (Soulsby, 1982).

In the environment, eggs become infective in 3-5 weeks (Soulsby, 1965; Boes, 1999). The egg development rate is temperature dependent and the lower threshold is approximately at 15°C (at 17°C embryonation takes 50 days) and the maximum rate of development is 12 days for embryonation at 31.1°C and this is also the optimal embryonation temperature (Stevenson, 1979; Crompton *et al.*, 1985). Furthermore, they require the correct environmental conditions of moisture, oxygen, and shade (Crompton, 1994). Thus there is seasonality on the development rates of *A. suum* (Stevenson, 1979; Juris *et al.*, 1991; Wagner & Polley, 1999). Stevenson (1979) observed no egg development in locations around Europe during the winter months and into spring; this can be attributed to the cooler temperatures from October-May which are below the threshold for development (15°C). The position of the eggs in the soil can also attribute to variation in egg development (Juris *et al.*, 1991). This seasonal variation was correlated with the rise in liver condemnation due to white spots during summer and autumn in the UK (Stevenson, 1979).

Adult morphology

Ascaris is the largest of the common human nematode parasites. Morphologically *A. suum* and *A. lumbricoides* are similar (Sprent, 1962; Madden & Tromba, 1976; Crompton, 2001). The males measure 150-250 mm long and 2-5 mm wide and females measure 200-450 mm long and 3-6 mm wide (Sprent, 1962; Galvin, 1968; Seo & Chai, 1980;). Over a period of twelve months, females were found to grow more rapidly than males (Seo & Chai, 1980). The dorsal lip bears two double papillae and each ventrolateral lip, one double and one subventral and a small lateral papilla. Each lip bears a row of minute denticles on the inner surface. There has been an association between the wear of the denticle and age, and it was concluded that the denticles are functional and become worn through use (Madden & Tromba, 1976). The spicules of the male are approximately 2 mm long and wide. There are a large number of precloacal papillae. The vulva opens near the end of the first third of the body (Soulsby, 1982).

Seasonal variation and transmission of *Ascaris* eggs

Seasonal variation appears to be a feature in the transmission of *Ascaris* eggs with higher rates of transmission during the summer and early autumn and this is reduced during the winter and early spring period but this is also dependent on local climatic conditions (Loeffler, 1956; Gelpi & Mustafa, 1967; Spillmann, 1975; Connan, 1977; Stevenson, 1979; Cabrera, 1984; Ali-Shtayeh *et al.*, 1989; Peng *et al.*, 1996; Wagner & Polley, 1999; Roepstorff *et al.*, 2001; Gunawardena *et al.*, 2004). Establishing the seasonal variation in infection in which transmission occurs is an important aspect of control strategies. Warm and moist environments facilitate the survival of *Ascaris* eggs as moisture maintains the viability and infectivity of the eggs (Crompton & Pawlowski, 1985).

The seasonal transmission and persistence of *Ascaris* eggs in the environment is correlated with the observance of Loeffler's syndrome (a respiratory illness associated with pulmonary ascariasis) (Loeffler, 1956; Gelpi & Mustafa, 1967; Spillmann, 1975). In Saudi Arabia, a high rate of pneumonitis was found to coincide with the short rainy period, and this, due to the dry climate, was the only time of the year that *A. lumbricoides* eggs could be transmitted intensively (Gelpi & Mustafa, 1967). Studies in Sri Lanka showed lower rates of transmission during the hot drier months and found that eggs exposed to sunlight became desiccated rapidly and died but this was partly dependent on the soil type and humidity (Gunawardena *et al.*, 2004). Similar seasonal variation in transmission has been observed in pigs on the pasture (Roepstorff *et al.*, 2001).

Infection of *A. lumbricoides* is transmitted to humans readily through the faeco-oral route. This is the same route for transmission of *A. suum* (Crompton *et al.*, 1985; Crompton, 1994). Infective eggs of *A. lumbricoides* can occur in the air and household dust; therefore there is risk of exposure by inhaling and swallowing in hyper-endemic areas (Crompton, 1994; O'Lorcain & Holland, 2000). Young children have greater exposure to infective stages through playing on the ground in areas of defecation and by eating contaminated soil [geophagia]. The parasite is found to thrive in places and communities where poverty is entrenched and as such, systems of sanitation and sewage disposal are unavailable (Crompton *et al.*, 1989). Consequently, there is high prevalence of this parasite in developing countries (Crompton *et al.*, 1989). Widespread contamination of the household environment occurs in these situations. In endemic areas eggs are found attached to utensils, furniture, money, fruit, vegetables, door handles and fingers and this was believed to be a consequence of the "sticky" nature of eggs enabling them to adhere to objects. However, Roepstorff (2003) suggests that this may be caused by contamination through the spreading of faeces or soil and that the apparent adhesive quality of eggs may be an artefact of the methods of obtaining eggs from the uterus of worms (Eriksen, 1981; Oksanen *et al.*, 1990; Eriksen *et al.*, 1992) before the uterine layer is fully developed. Similarly the dissemination of eggs in rural Poland was reportedly spread by soil contaminaton, for example via farmer's shoes (Mizgajska, 1997).

The life-cycle of *Ascaris suum*

Female *Ascaris* are known to produce as many as 200000 eggs daily (Soulsby, 1982; Jungersen *et al.*, 1997; O'Lorcain & Holland, 2000). The entire cycle from the ingestion of infective eggs to the production of fertilised eggs by mature female worms takes 2-3 months. The life span of adult worms is 1-2 years, and females can lay eggs for up to one year (Thein Hlaing, 1993; O'Lorcain & Holland, 2000; Crompton, 2001) although they have been recorded as producing eggs for up to 20 months (O'Lorcain & Holland, 2000). Chai *et al.* (1981) observed that egg production was first detected after female worm's length exceeded 126 mm. Female worm length was positively correlated with increased egg production until about 250 mm at which point egg production reached a plateau and reduced after 275 mm (Chai *et al.*, 1981). This group observed great variation in the eggs per gram of faeces (E.P.G.) among worms of the same length, but this may be associated with the number of encounters with males.

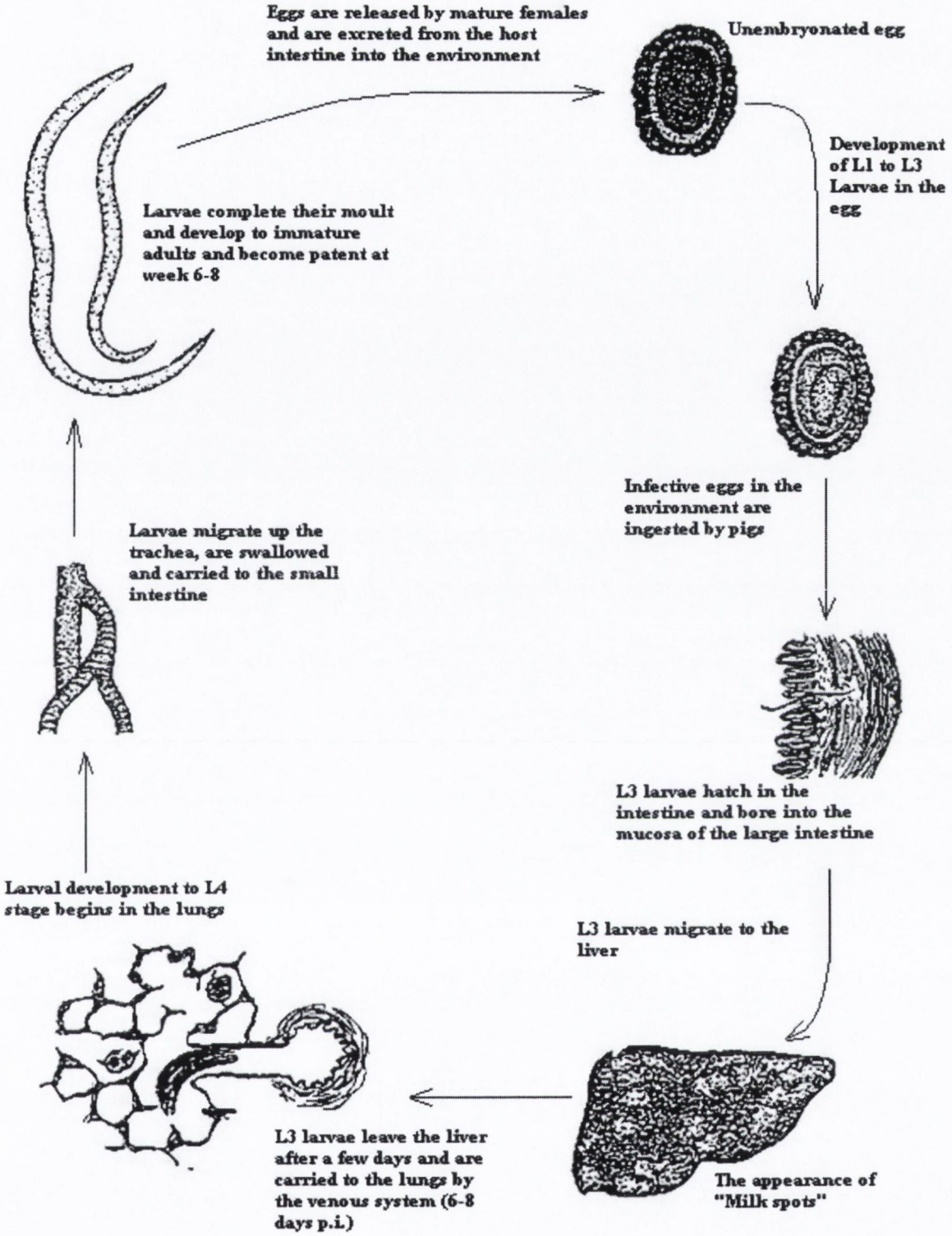


Figure 1.4.1: The migration of *Ascaris suum* through the tissues of pigs from initial infection until patency (original figure).

Studies of the morphology of *A. suum* larvae development revealed that two moults occur in the egg prior to hatching (Maung, 1978; Soulsby, 1982; Geenen *et al.*, 1999; Fagerholm *et al.*, 2000). It appears that the earlier observations of the loss of a cuticle and a moult in the liver (Douvres *et al.*, 1969) are actually the loss of the second stage cuticle which is already present at hatching (Fagerholm *et al.*, 2000). *In vitro* studies of the eggs showed that the first larval stage (L_1) appeared in the egg after 17-22 days of cultivation, the first moult to the second larval stage (L_2) took place from day 22 to day 27, and the second moult to the third stage (L_3) started on day 27 and continued to grow during the 60 day observation period (Geenen *et al.*, 1999) and these were proposed as the infective stage (Maung, 1978; Fagerholm *et al.*, 2000). The L_3 larvae can be up to 500μm in length (Song *et al.*, 1985) and there is a continuous length increase of larvae between the emergence from the egg and the third moult (4^{th} stage) by which time the larvae have returned to the intestine. Total worm length increases from approximately 200 to 1200μm between days 2 and 7 inclusively (Fagerholm *et al.*, 2000). The differentiation of the fourth stage (L_4) larva is initiated during the lung phase but is completed in the small intestine. The cuticles of the L_3 and L_4 stages are similar to the adult stage but both are distinct from the L_2 stage both in terms of the number and molecular weight of the protein bands (Fetterer *et al.*, 1990). Ecdysteroids have been implicated in the moulting process of *A. suum* (Fleming, 1985). Hyraluronic acid has been identified as a component of nematode cuticles and also is involved in cell adhesion, proliferation and differentiation of vertebrate somatic tissues. Hyaluronidase (a hyraluronic acid- specific enzyme) was released into culture fluid during *in vitro* development of L_3 to L_4 stage larvae (Rhoads *et al.*, 2001); thus this enzyme appears to function in the penetration of host tissues, as well as in moulting (Jenkins, 1968). Islam *et al.*, (2005b) indicated a role of inorganic pyrophosphatase of *A. suum* (AsPPase) in larval moulting and development.

Migration is of the hepato-tracheal type (Sprent, 1954) with the larva re-entering the alimentary canal from the lungs via the trachea (Soulsby, 1982). When infective eggs are ingested by a pig they hatch in the digestive tract posterior to the stomach only (**Fig. 1.4.1**); as the optimal pH for larval release is 7-8 (Jenkins, 1968). An *in vitro* examination of egg hatching and migratory activity showed that bile (note this was bovine bile as a precaution against potential antibodies in pig bile) was an important stimulatory factor for both hatching and larval mobility in the various methods of incubation examined. Low bile concentrations (2%, 5% or 10%) stimulated both egg hatching and larval migration (the optimum was 5%), and concentrations >20% impaired egg hatching and larval migration

(Han *et al.*, 2000). Other physical and chemical factors are also involved in the hatching process. One such aspect consists of a host stimulus which acts on a receptor in the infective egg and causes the release of hatching fluid (Soulsby, 1982). One of the first effects after stimulation is an increased permeability of the vitelline membrane which allows the enzymes to reach the shell. *A. suum* hatching fluid contains an esterase, proteinase and chitinase that attack the lipid and chitin in the eggshell (Hinck & Ivey, 1976; Soulsby, 1982). Evidence suggests that the loss of solutes from the egg fluid permits an increase in the water content of the unhatched juveniles and this was responsible for ending their quiescence (Barrett, 1976; Clarke & Perry, 1980).

Newly hatched larvae penetrate the mucosa of the caecum and colon moving through the lamina propria (**Fig. 1.4.1**) (Murrell *et al.*, 1997a). Mucosal epithelium disorganisation is observed at the point of entry at the apices of the papillae and through the basal region between each papilla. The larvae make circuitous burrows in the lamina propria and they appear to follow the line of least resistance avoiding transversing the dense bands of radial connective tissue of the mucosa (Jenkins, 1968). The isolation of larvae in the venules and lymphatic vessels of the caecum suggests a role for these systems in the transfer of worms to the liver and lungs. The failure to find any larvae penetrating the mucosa externa of the intestine provides some evidence that larvae do not pass directly into the body cavity (Jenkins, 1968). From there they migrate via the portal blood to reach the liver (Soulsby, 1965; Jungersen *et al.*, 1999b). One major difference between the lifecycle of the two species of *Ascaris* is the belief that the *A. lumbricoides* lifecycle does not contain obligatory moulting and development in the host's liver and so the potential for migrating *A. lumbricoides* to inflict liver damage in its human host is largely unknown (Murrell *et al.*, 1997a).

The first larvae to reach the liver of pigs have been observed as early as 3 hours p.i.. This may be representative of the time taken to reach a suitable venule or lacteal which is dependent on chance (Jenkins, 1968) or it may be that larvae actively migrate down the gastrointestinal tract (Murrell *et al.*, 1997a). Larvae have been observed in the lungs of mice at 12 hours p.i. but as early as 6 hours p.i. (Jenkins, 1968; Slotved *et al.*, 1998). The larvae leave the liver via the hepatic veins and reach the lungs after passing through the heart. Here they leave the capillaries, resulting in haemorrhage, and enter the alveoli, after which they migrate up the bronchial tree by the microcilliary movements to the pharynx. The larvae are then swallowed and reach the small intestine. Upon re-entering the small

intestine, the majority of the worms are expelled and the remaining larvae develop into adult worms. The prepatent period of *A. suum* in previously unexposed pigs is 6-10 weeks (Soulsby, 1965). It has been suggested that there are differences in the prepatent period between the two *Ascaris* species. Galvin (1968) found that *A. suum* infection pre-patent period was not longer than 45- 53 days and in contrast there appeared to be a longer pre-patent period of development of *A. lumbricoides* in pigs ranging from 52-62 days. However, these results must be interpreted with caution as there may have been cross contamination between the two species as eggs were acquired from areas that were endemic for both species.

Egg excretion from *Ascaris*-infected pigs can be detected as early as 6 weeks post infection (Roepstorff *et al.*, 1997). Fertilisation of eggs in *Ascaris* is known to take place at the junction between the oviduct and the uterus. The surface of immature oocytes and mature oocytes possess different antigens and it is believed that this conditioning prepares the oocytes for fertilisation (Wu & Foor, 1980). The male wraps his curved posterior around the body of the female near her gonophore. Thus positioned, the copulatory spines are inserted into the vagina and sperm is transferred by contractions of the ejaculatory duct. Fertilisation occurs within the uteri (Brusca & Brusca, 1990). *A. suum* has a reservoir for deposited sperm although no such spermatheca is present (Jungersen *et al.*, 1997), thus constant contact with males is required for continued egg production.

The pattern of migration in experimental hosts

The infective eggs of *A. suum* readily hatch in mice of various strains releasing larvae that migrate via the hepato-tracheal route (Jenkins, 1968; Slotved *et al.*, 1998). Recent studies have supported the caecum/ colon as the main sites of penetration for migrating larvae not only in pigs (Murrell *et al.*, 1997a) but also in mice (Jenkins, 1968; Bindseil, 1970a/b; Slotved *et al.*, 1998) and that there was very little difference in the time taken to migrate along this path irrespective of gut length. For example, the first larvae are observed penetrating the pig intestine 6 hours post-infection (p.i.). The first larvae can be observed penetrating the intestine three hours post-infection in the mouse (Jenkins, 1968; Slotved *et al.*, 1998). Larval migration of *A. suum* in mice mimics that of the pig (Murrell *et al.*, 1997a; Slotved *et al.*, 1998) (**Fig. 1.8.1**) although a patent infection does not occur in mice. In pigs the majority of the capable larvae transverse the large intestine by 18 hours p.i. (Murrell *et al.*, 1997a). Although the transit time through the intestine is relatively fast, the maximum liver burden does not occur until 4 days p.i. (Douvres *et al.*, 1969), suggesting

that larvae do not migrate directly from the intestine to the liver. In mice the maximum number of larvae is not recovered until 4 days p.i., a similar transit time to that observed in pigs (Prokopič & Figallová, 1982; Song *et al.*, 1985).

The role of migration

Why do larvae undergo extensive migration which begins and ends in the same location and thus risk being destroyed in inappropriate host tissues (Stephenson & Holland, 1987) or risk a higher level of detection by the host's immune system (Read & Skorping, 1995)? Migration is hazardous as the juveniles become trapped or lost in the host's tissues. Additional to this is the cost of survival in an array of different habitats which requires biochemical and morphological adaptations to these environments (Behnke, 1990). The existing explanation for larval migration is that it is an evolutionary legacy. This may be the loss of an intermediate host or skin penetration event in the evolution of these organisms (Sprent, 1962; Smyth, 1994).

Natural selection should therefore eliminate migration if it was an unnecessary relic of evolution. There must be associated fitness benefits that are selectively favourable associated with tissue migration (Read & Skorping, 1995; Mulcahy *et al.*, 2005). Among the possible associated benefits of tissue migration appear to be more rapid growth and thus greater size and/or rapid maturation. Read & Skorping (1995) demonstrated that nematodes that developed in the tissues of their hosts were bigger and grew faster than their closest relatives developing only in the gastrointestinal tract. This association between larval habitat and size was independent of any effect of adult habitat, worm lifecycle, host size, generation time or diet. It was suggested that this provided a selective advantage to migration, as body size is closely linked with fecundity in nematodes. Other benefits could be the avoidance of severe chemical, immunological and physical conditions in the intestine. The host responses generated by helminth infection seem best suited to dealing with threats at the mucosal surface. A logical corollary is that helminths might have developed elaborate tissue-migratory pathways as immunoevasive strategies (Mulcahy *et al.*, 2005). Those parasites that reside in the gastrointestinal (GI) tract may have to expend more resources into combating these harsher conditions and resulting in reduced growth in contrast to their tissue migrating cousins. L_3 *A. suum* larvae that are inoculated into the lungs of mice develop normally bypassing the liver tissue, but development occurs at a slower rate (Jungersen *et al.*, 1999b) and these worms do not grow to the same extent as their counterparts that are allowed undergo normal migration.

1.5 EPIDEMIOLOGY OF *ASCARIS*

The epidemiology of ascariasis is primarily concerned with *A. lumbricoides* because of its public health significance and involves the study of prevalence, intensity and the distribution of infection. *A. lumbricoides* is illustrative of a typical aggregated/ overdispersed distribution. Anderson & May (1991) identified four consequences of aggregation in *Ascaris* that are divided into those affecting the host and those impacting on the parasite community. In the host, aggregation influences morbidity and mortality, especially in children who tend to be more predisposed to heavy worm burdens. It is also relevant to the design of control strategies. In the parasite community, it influences mate finding and it increases regulation of the parasite population through density dependent effects. Established worms may inhibit the development of newly acquired *Ascaris* larvae, thus regulating their own abundance in a density-dependent mechanism (Elkins & Haswell-Elkins, 1989) or vice-versa. Factors that influence intensity and prevalence include socio-economic factors, predisposition and agricultural practices.

Prevalence

The estimated global prevalence of *A. lumbricoides* is 1.4 billion people (Chan *et al.*, 1994; Chan, 1997). An estimated prevalence of 73% of *A. lumbricoides* infection is present in Asia, while 12% is in Africa and 8% in Latin America (Peters, 1978). The increasing world population density has contributed to the increasing prevalence of intestinal parasites and the problem is not abating (Chan, 1997). The typical age-prevalence curve of ascariasis from birth to preschool age reaches a peak of 80-90% among school children in highly endemic areas (Thein Hlaing, 1993). A typical pattern of infection is first contact and acquirement of the parasite in children in their second year of life because this is the first time period when infants begin to explore away from the mother and hence are crawling on the ground (Crompton, 1994). Prevalence reaches a plateau after the ages of 5-10 and remains reasonably stable throughout life (Crompton *et al.*, 1989).

There is a direct correlation between prevalence and location, with prevalence being more pronounced in rural rather than urban communities (Crompton & Tulley, 1987; Crompton *et al.*, 1989; Crompton, 1994). Recently this situation has shifted in some areas with urbanisation and the uncontrolled migration of people from rural villages to urban areas (Crompton *et al.*, 1989; Crompton, 1994) and the development of shantytowns with poor services such as a lack of sanitation. There are a number of other possible contributing

factors to prevalence. Studies have linked prevalence of *A. lumbricoides* to gender. It appears that females tend to be infected more than males, independent of age (Crompton, 1994; Peng *et al.*, 1996; Saathoff *et al.*, 2004). This may be a social issue related to contact with the children. As highlighted earlier, climatic factors have a role in prevalence rates due to their control of embryonation and survival of eggs (Crompton, 1994; Wagner & Polley, 1999). In Africa, the prevalence is generally low in countries with arid climates and high in those with consistent wet weather and warmth (Crompton & Tulley, 1987). The distribution of *A. lumbricoides* will thus be reflected also in the dependence of human communities for rainfall and water supplies (Crompton, 1994). Further data indicated that altitude also influences prevalence with people living at higher altitudes having a higher prevalence (Meakins *et al.*, 1981).

Overdispersion and predisposition

The frequency distribution of *Ascaris* in humans tends to be highly aggregated in which a few people are host to the majority of worms (Crompton & Tulley, 1987; Guyatt *et al.*, 1990; Thein Hlaing, 1993; O'Lorcain & Holland, 2000). Studies in human populations have provided evidence that the intensity of reinfection with *Ascaris* is not random, individuals are predisposed to a level of infection, as well as being an age-related effect, this appears to be under genetic control (Holland *et al.*, 1989; Guyatt *et al.*, 1990; Wong *et al.*, 1991; Hallam & Anwar, 1992; Chan *et al.*, 1993; Palmer *et al.*, 1995; Peng *et al.*, 1996; Williams-Blangero *et al.*, 1999; Williams-Blangero *et al.*, 2002) and this is also relevant in experimental and natural infections in pigs (Boes *et al.*, 1998a). Other factors which can also generate aggregation include heterogeneity in host behaviour, influencing the uptake of infective stages, heterogeneity in immunity due to previous exposure, or socio-economic factors (Crompton, 1994; Roepstorff *et al.*, 1997; Nishiura *et al.*, 2002). Data on the intensity of infection is important because of its correlation with the regulation of the parasite population (density dependent factors). It is also crucial in public health terms since morbidity and mortality are associated with parasite intensity (Crompton & Tulley, 1987; Pawlowski & Davis, 1989; Crompton, 1994) and implementation and monitoring of control programmes (Crompton *et al.*, 1989).

During childhood, the intensity profile is similar to that observed in the relationship between age and prevalence, but intensity usually declines markedly with age. The mean worm burden peaks in children aged 5-14 years old and is significantly lower among adults (Crompton, 1994; Palmer *et al.*, 1995; Peng *et al.*, 1996; Nishiura *et al.*, 2002). This

reveals an interesting fact that older people, although infected, tend to be predisposed to lower worm burdens in contrast with children. Immunological and socio-environmental hypothesis are proposed as explanations for this observation (Crompton, 1994; Nishiura *et al.*, 2002). Exposure to repeated infections with *A. lumbricoides* during early life may induce some level of protective immunity (McSharry *et al.*, 1999). Pigs (Eriksen, 1981) and mice (Bindseil, 1969a) are known to develop an immune response to *Ascaris* antigens with continued exposure. The socio-environmental explanation is based on personal hygiene and behaviour of adults compared to children. It is clearly evident that children tend to be more susceptible to infection through greater contact and also by the lack of temporal exposure, thus they have not developed an adaptive immune response, to infective stages than adults particularly where sanitation is already poor. However, when age and sex are accounted for, some individuals are persistently susceptible to *Ascaris* infection (Jackson *et al.*, 2004) and this implies an underlying genetic effect. The adaptive (acquired) immune response of a host is fundamentally constrained by the genetic constitution of an individual (Kennedy, 1989; Behnke, 1990). It is the individual host genetic repertoire which is polymorphic in members of a population or host species and results in variation in how the immune system responds to infection.

Individual predisposition can be determined by measuring the intensity of infection following anthelmintic treatment and then assessing the intensity post-infection after an extended period in a follow-up treatment and comparing the two results. Studies in India, Myanmar, Mexico, Malaysia, Nigeria, and Thailand confirm that individuals are predisposed to high/low worm burdens, showing that pre-treatment intensities positively correlate with post-treatment intensities (Crompton, 2001). Previously, Holland *et al.* (1992) continuing from their earlier studies (Holland *et al.*, 1989) discussed a possible genetic basis for predispositon. On the basis of detection of eggs around the subjects homes, all subjects were determined to have likely been exposed repeatedly to re-infection, but some individuals remained uninfected, thus inherent (presumably genetic) host features were proposed to be involved. They found that the distribution of class I human leucocyte antigens among 3 groups of children correlated with predisposition to ascaraisis. None of the children who were predisposed to remain uninfected were found to possess the A30/31 antigens in contrast to those children who remained infected. However, convincing evidence requires pedigree studies, such as those carried out by Williams-Blangero *et al.* (1999; 2002), that are capable of analysing the effect of relatedness and shared environment across many households thus providing greater statistical evidence. Results to

date suggest that a proportion, up to 44% of the variance in worm burden is explicable by additive genetic effects, compared to 3-14% of variance explicable by measured environmental effects (Quinnell, 2003). For example, Williams-Blangero *et al.* (2002) identified quantitative trait loci (QTLs) associated with predisposition to *Ascaris* in human populations. A preliminary study demonstrated a strong genetic component accounting for between 30% and 50% of the variation in *A. lumbricoides* worm burden among 1200 individuals from a single pedigree in the Jirel population of East Nepal. While shared environmental effects such as a common household accounted for between 3 and 13% of the total phenotypic variation (Williams-Blangero *et al.*, 1999). This work resulted in the localisation of two genes (one on chromosome 1 and another on chromosome 13) with observed effects on susceptibility to adult worms. These genes are believed to regulate the immune response to *Ascaris* infection (Williams-Blangero *et al.*, 2002). Resistant genotypes in relation to susceptibility to parasites are usually considered dominant as animals have been selectively bred for resistance to helminths (Mitchell *et al.*, 1976; Behnke *et al.*, 2003) through innate or acquired immunity. However, in some individuals there may be the additive effect of genetic influence on exposure to infection, through, for example, behavioural genes affecting hygiene or geophagy (Quinnell, 2003).

Recent studies have implicated heterogeneity in the Th2 immune response to intestinal helminths as a possible cause of predisposition to adult worm infection. In pigs, an elevated Th2-like pattern is expressed during the expulsion of L_4 larvae from the small intestine (Dawson *et al.*, 2005). McSharry *et al.* (1999) identified some children in an area of hyper-endemicity of *Ascaris* in Nigeria who appeared to be naturally immune and this was associated with IgE antibody to ABA-1 allergen as well as inflammatory indicators. The naturally immune group had significantly more IgE antibody to the allergen than susceptible individuals. Immunity was correlated with higher levels of serrum ferritin, C-reactive protein and eosinophil cationic protein, which indicated an on-going inflammatory process. Susceptible children showed little serological evidence of inflammation regardless of their high worm burden. Recent evidence has shown that the upregulation of STAT6 (signal transducers and activators of transcription) which, is involved in Th-2 immune signalling, predicts increased resistance to adult worm infections (Peisong *et al.*, 2004). However, the chromosomal location of STAT6 (chromosome 12) (Peisong *et al.*, 2004) does not match chromosomal linkages observed between *Ascaris* burden and chromosome 1 and 13 in the Jirel population of Nepal (Williams-Blangero *et al.*, 2002) indicating that other genes may be involved in protection of innately resistant individuals. This Th2

associated resistance has been further substantiated by Jackson *et al.* (2004), who linked the host cytokine response to susceptibility with *A. lumbricoides* reinfection in a Cameroonian population. When age and sex were accounted for, Th2 cytokine responses immediately before deworming had a significant negative effect on the probability of reinfection. Thus individuals that were persistently susceptible to patent adult infections were characterised by a weak Th2 response. A measure of overall susceptibility was significantly related to Th2 cytokine response in 4-13 year-old hosts. These results validate the relevance of the murine models that have indicated a causal link between Th2 responses and resistance to GI nematodes (Grencis, 2001).

Features of *Ascaris* infection in pigs in a Chinese province, such as prevalence, intensity and overdispersion were analogous to those of the human population except for a lower mean intensity of infection (Peng *et al.*, 1996). The Danish Centre for Experimental Parasitology (DCEP) has developed the pig model for the study of predisposition to *A. suum* (Boes *et al.*, 1998a). This group described the distribution of *A. suum* in experimentally and naturally infected pigs and offered a comparison with *A. lumbricoides* in humans. The prevalence and intensity as well as the distribution observed for *A. suum* infection in this pig model was comparable to results obtained for *A. lumbricoides* in endemic areas and evidence suggested that predisposition influenced this observation in pigs. Initial worm burdens and those on reinfection were significantly correlated suggesting that individual pigs were predisposed to heavy or light infections (Boes *et al.*, 1998a). This indicates the importance of this pig model in the study of population dynamics and predisposition. When the conditions of natural exposure are similar, differences in immunocompetence may be attributable in large part to differences in alleles of some key genes in the gene cascade that drives the immune response (Behnke *et al.*, 2003).

However, the predispostion of individuals has not been examined in relation to larval ascariasis and the effects of heavy larval burdens on the host. Larval migration is associated with serious morbidity and mortality and this is especially identifiable in the inflammatory response of the lungs which can lead to pulmonary distress and secondary infections associated with opportunistic pathogens. Individuals that are susceptible to the larval stages could be at risk from increased disease caused by migration. Furthermore the immune response to larval stages may determine the success of patent adult infections in the intestine (Jungersen *et al.*, 1999a). Therefore, the host response to the presence of

migrating larvae may determine the possibility of establishment of patent adult worms in the intestine following migration.

Control strategies

Current helminth control programmes are based on reducing infection and transmission potential in order to reduce morbidity and avoid mortality but not on eradicating the disease. There is evidence that households with more education enjoy better health with reduced *Ascaris* prevalence (Tshikuka *et al.*, 1995; Crompton, 2001; Savioli *et al.*, 2002). Anthelmintic treatments are available for the alleviation of intestinal worm burdens but they cannot target the larval population. Thus prevalence can remain constant in most areas due to constant exposure to infective eggs and migrating larvae (Boisvenue *et al.*, 1968). Epidemiological evidence suggests that anthelmintic treatment should be administered in one of three ways in community control programmes. These are universal (chemotherapy offered to all individuals in endemic area regardless of infection status, age etc.), targeted (treatment of a defined group e.g. school children) or selective treatment (based on intensity of infection or past infections). Studies in endemic populations comparing the efficacy of treatment types suggested that the various methods reduced the intensity of adult worms in the population (Asaolu *et al.*, 1991; Hallam & Anwar, 1992; Beltramino *et al.*, 2003). Anthelmintic treatment of pigs is used for the reduction of *A. suum* infections in conjunction with management policies and has been quite effective (Boes, 1999). However, the risk of anthelmintic resistance can be associated with the methods of treatment.

There are several risk factors associated with the development of anthelmintic resistance. These include; the frequency and method of the dosing regime, large parasite burdens, small external reservoirs, and the short life (generation) time of parastic organisms. The fundamental strategy is to apply chemotherapy such that the emergence of drug resistance is delayed or circumvented while still providing the necessary requirements to the population, namely the reduction in the prevalence of the parasite and the alleviation of disease. The use of universal chemotherapeutic treatment has primarily been correlated with the development of resistance. The design of chemotherapeutic strategies is perhaps more effective if it is targeted at individuals that are predisposed to heavy worm burdens. For example the targeting of schoolchildren will ensure that nematodes remain in the community and that the genes of the survivors will dilute those of the nematodes experiencing selection pressures (Savioli *et al.*, 1997). The identification of genetic

relatedness as a primary determinant could justify the costs involved in selective and individually targeted treatment regimes (Holland *et al.*, 1989).

Anderson *et al.* (1995) suggested that overdispersion leads to homozygosity in the worm genotype and possible anthelmintic resistance. There is a propensity for individuals to infect the same host due to clustering of infective stages which may lead to interbreeding and as a consequence result in over-representation of homozygotes in the population. Homozygosity results in the phenotypic expression of recessive traits and thus exposes them to selection. Many genes conferring resistance to pesticides are recessive (Plapp, 1986 cited by Anderson *et al.*, 1995) and if this was the case for anthelmintic resistance then such genes could become established in subdivided populations of parasite. Recently, Nejsum *et al.* (2005a) following the population structure of *A. suum* in pigs in Denmark by whole genome DNA fingerprinting found that there was homogenisation of the worm gene pool due to the movement of pigs. They concluded that the evolution of resistance to anthelmintic drugs in breeding herds could spread rapidly through the population.

1.6 THE PATHOLOGY OF *ASCARIS*

The clinical manifestations of ascariasis, caused by the intestinal roundworms *A. lumbricoides* in man and *A. suum* in pigs, range from acute, life threatening complications such as intestinal obstruction to more subtle manifestations associated with the persistence of impaired nutritional status especially during childhood (Stephenson, 1980; Crompton, 1985; Nesheim, 1985; Thein Hlaing, 1993; Crompton, 1994). Acute ascariasis is observed as allergic and respiratory symptoms that are associated with migratory larvae (Beaver & Danaraj, 1958; Spillmann, 1975; Crompton *et al.*, 1989; McSharry *et al.*, 1999; Cooper *et al.*, 2000) or as obstructions and surgical complications caused by adult worms (O'Lorcain & Holland, 2000; Valentine *et al.*, 2001; Esser-Köchling & Hirsch, 2005; Sangkhathat *et al.*, 2003). In contrast, chronic ascariasis primarily involves adult worms and is accepted to contribute to the aetiology and persistence of impaired nutritional status especially during childhood (Connolly & Kvalsvig, 1993; Kvalsvig, 2002). In relation to chronic ascariasis other variables such as host age, nutritional status and socio-economic factors must be considered when determining the severity of morbidity (Holland *et al.*, 1989) as ascariasis is often associated with poverty and is almost always an aspect of the environment where childhood malnutrition is prevalent (Stephenson, 1980). The estimated mortality rate of *A. lumbricoides* infection is 100,000 people annually (Pawlowski & Davis, 1989) although

these estimates are from areas with other endemic diseases and as such there may be other causative agents. Ascariasis is generally classed as asymptomatic, oligosymptomatic (host can balance pathological changes), symptomatic, or fatal.

Pathological impact of *Ascaris* in the liver

Murrell *et al.* (1997a) believed that there was no unequivocal evidence to suggest migration and development of *A. lumbricoides* in man was different in any way to that of its sibling species in the pig. Given the genetic similarities between the two species and the evidence that cross-infection can and does occur between the human and pig *Ascaris* (Anderson, 1995; Nejsum *et al.*, 2005b), the potential for liver lesions, which has never considered to be a pathological consequence of human *Ascaris*, should be considered a potentially important risk, until more information regarding *A. lumbricoides* migratory behaviour is known. Cases of lesions on the livers of humans are believed to be the result of cross-infection with pig *A. suum* rather than the result of *A. lumbricoides* infection (Kakihara *et al.*, 2004). Thus far there has been no definitive report of human *Ascaris* migrating to the liver.

The contribution of migrating larvae to liver damage is well documented in pigs and is problematic. A marked histopathological response occurs in the liver of pigs and is an important consequence of porcine ascariasis and represents an immune reaction by the host (Ronéus, 1966; Eriksen *et al.*, 1980; Eriksen, 1981; Frontera *et al.*, 2003). "Whitespots (WS)", "milkspots" or chronic focal interstitial hepatitis are the terms used to describe the liver lesions in pigs that have been exposed to infective *A. suum* eggs. These lesions occur on the surface and deeper in the hepatic tissue. The formation of WS is due to mechanical injury and the inflammatory process. The longer the exposure to infective eggs, the greater the number of WS that will develop, but the number declines with continuing exposure indicating the induction of a dose dependent protective immune response in the host (Eriksen, 1981).

Ronéus (1966) described liver lesions and suggested that granulation-tissue type WS (GT-WS) are formed around larvae and these are subcategorised as small GT-WS and large GT-WS, while the small lymphnodular type WS (LN-WS), without a compact centre, are generated along the migratory routes of the larvae. This inflammation is characterised by an intensive infiltration with eosinophils and mononuclear cells (Frontera *et al.*, 2003). Early lesions are composed of necrotic haemorrhagic foci, surrounded by infiltrates of

eosinophils, neutrophils and small numbers of macrophages. These foci progress to granulomatous lesions composed of a necrotic centre, sometimes with larval debris, surrounded by eosinophils, macrophages and lymphocytes, arranged either in a diffuse pattern or in aggregates of lymphoid follicles. Older lesions are composed of portal fibrosis with diffuse infiltration of lymphocytes and plasma cells (Pérez *et al.*, 2001). Small GT-WS dominate the early phase of infection, while the much lower numbers of large GT-WS and LN-WS tend to make up a higher proportion of the total number of WS in the late phase (Ronéus, 1966, Jungersen *et al.*, 1999a). Liver damage has also been demonstrated in abnormal hosts (Eriksen, 1981). It is these liver lesions which result in liver condemnation at slaughter. There have been few reports of hepatic lesions caused by *A. suum* in human livers (Kakihara *et al.*, 2004).

Pathology of *Ascaris* in the pulmonary tissues

Considerable pathology is associated with lung migration of *A. lumbricoides* in human infections as well as *A. suum* in pigs (Matsuyama *et al.*, 1998). The pathology provoked by the larvae of *Ascaris* in the lungs of humans and pigs is an important but understudied source of morbidity, a condition that can cause potentially lethal hypersensitivity responses in individuals (Vogel & Minning, 1942; Beaver & Danaraj, 1958; Spillmann, 1975; McSharry *et al.*, 1999; Cooper *et al.*, 2000). Cooper *et al.* (2000) suggested that pulmonary migration of *Ascaris* larvae could create a highly polarised Th2 immune environment in the lung mucosa, which might enhance Th2 cytokine production to non-parasite antigens such as aeroallergens or microbial pathogens associated with these lesions thus increasing the host reaction to common allergens and so resulting in a hypersensitive reaction. A recent histopathological study of lung involvement to *Toxocara canis* infection in mice observed that infection with *T. canis* in two strains of mice, BALB/c and C57BL/6, resulted in both strains of mice showing marked histological changes and increased levels of total serum IgE (Pinelli *et al.*, 2001). An IgE-mediated hypersensitivity reaction to *Ascaris* occurs, as illustrated by Vogel & Minning (1942), even if only 6 to 45 larvae are in the lungs.

Pulmonary ascariasis results in symptoms which include respiratory distress, pneumonitis, substernal pain, fever, rash and eosinophilia that were linked with hypersensitivity to antigens. The parasite has been associated with the occurrence of asthma (however some authors doubt this association). Antigens can also result in symptoms resembling anaphylactic shock. In 1922, Koino experienced an increased respiratory rate and a cough

after ingesting *Ascaris* eggs. These symptoms were present for 8 days after which they quickly disappeared, leading to the conclusion that there was transient pulmonary involvement in larval migration even at low dosages of administration. In 1932, Loeffler described a respiratory illness characterised by transient and rapidly changing infiltrates (which where termed pulmonary transitory shadows) and marked peripheral eosinophilia. Loeffler's syndrome, as it was termed later, was subsequently shown to be frequently caused by the migrating larvae of *A. lumbricoides* in human populations (Vogel & Minning, 1942; Loeffler, 1956). These symptoms of respiratory distress were also distinguished in pigs infected with larval *A. suum* (Slotved, 1997). Massive numbers of migrating larvae in the lungs of pigs were associated with symptoms such as severe dyspnoea, caused by oedema and emphysema of the lung tissue (Taffs, 1968; Eriksen, 1981). This was accompanied by prominent abdominal breathing, coughing and elevated body temperature beginning on day 7 p.i. and peaking on days 8/9 p.i. (Roepstorff, 2003). In hyper-endemic areas this can often go unnoticed due to the constant exposure of individuals to low numbers of infective eggs, which results in very few incidences of acute pulmonary manifestations (Keller *et al.*, 1932; Spillmann, 1975). Although individuals repeatedly infected with *Ascaris* in endemic areas may appear asymptomatic, bronchovascular damage caused by the parasite may result in secondary damage caused by opportunistic microorganisms which invade the pulmonary tissues and proliferate due to the damage caused by larvae escaping from the blood vessels into the alveolar lumen (Keller *et al.*, 1932; Liljegren *et al.*, 2003). Recently a case study reported on the injuries induced by *Ascaris* larvae migrating through the lungs of hosts which had an additional fatal effect on an individual with severe smoke-induced lung injury and it was believed that the presence of *Ascaris* caused unrecoverable respiratory failure (Heggers *et al.*, 1995).

Visceral larval migrans (VLM) caused by *A. suum*

Maruyama *et al.* (1997) suggested that *A. suum* has the potential to cause severe VLM. There have been a number of reports of pathology caused by suspected VLM caused by *Ascaris* particularly in Japan. Maruyama *et al.* (1997) developed an immunological assay to determine if antigens in the sera from VLM patients were due to *A. suum*. They used a number of antigens as the employment of a single antigen can result in misdiagnosis because of cross reactions with other ascarid/nematode antigens (Kennedy *et al.*, 1987; Kennedy, 1989). In conjunction with this analysis, etiological examination and the presence of stage-specific larval antigens (Kennedy & Qureshi, 1986) it was concluded that *A. suum* was the cause of VLM in Japanese patients. One case reported an individual

with encephalopathy associated with VLM caused by *A. suum* in Japan (Inatomi *et al.*, 1999). Another patient in Tokyo developed myelopathy (disease associated with the spinal cord) caused by VLM of *Ascaris* also. This diagnosis was made following the positive result for *Ascaris*-specific antibody in the patient's cerebrospinal fluid (CSF) and serum. Furthermore the resolution of spinal cord lesions following albendazole treatment supported this hypothesis (Osoegawa *et al.*, 2001). There have been further cases of myelopathy caused by *Ascaris* from Japan (Yoshida *et al.*, 2004). However there are very few reports of this kind in the medical literature outside Japan. Improved diagnostic tests have been provided to ensure these are genuine cases of VLM caused by *Ascaris*, since cross-reactions do occur for example between *A. suum* and *Toxocara canis*. Fan & Su (2004) provided a more specific and sensitive diagnostic method that showed that *A. suum* larval ES (Excretory-Secretory) antigen (AsES) was superior to two other crude somatic antigens for the diagnosis of *A. suum* induced-VLM in outbred ICR mice, because no cross-reactions in serum from *T. canis*- and *Angiostrongylus cantonensis*-infected mice were found. These are in agreement with the results of Cuéllar *et al.* (1995) who indicated that the sera of inbred BALB/c mice orally infected with either a single or multiple doses of *T. canis* eggs showed no cross reactions to AsES antigen but that there were intensive cross reactions to adult somatic antigen (AsAA). *Ascaris* larval somatic antigen was inappropriate as a diagnostic tool as serum IgG from *T. canis* and *A. cantonensis* was cross-reactive with this latter antigen (Fan & Su, 2004).

Complications associated with adult *Ascaris* worms

The intestinal phase of *A. lumbricoides* is one of the most common causes of abdominal surgical emergencies in children in developing countries. In endemic areas such as India up to 25% of hospital admissions for intestinal obstruction could be accounted for by ascariasis (Mukhopadhyay *et al.*, 2001). The greater the worm burden the greater the possibility of risk from life threatening obstruction. Coupled with the presence of these worms in the GI tract is the observation that *Ascaris* is associated with mucosal abnormalities such as crypt elongation, villous deformation and cellular infiltration of lamina propria resulting in nutritional imbalance in pigs and humans (Tripathy *et al.*, 1972; Stephenson, 1980; Nesheim, 1985) and this is associated with gastrointestinal symptoms such as diarrhoea, nausea, abdominal pain, vomiting and anorexia. There have been reported cases of a rare complication of massive gastrointestinal bleeding associated with *A. lumbricoides* (Sangkhathat *et al.*, 2003). This is likely to be the result of perforation of the small intestine by adult worms which migrate into the peritoneal cavity causing

peritonitis. This develops due to pressure on the wall of the intestine in heavily infected individuals leading to ulceration, necrosis, and perforation (Chawla *et al.*, 2003).

Biliary obstruction is an important complication of *A. lumbricoides* infection and rates only second to intestinal obstruction in presentation of complications related to ascariasis (Pawlowski & Davis, 1989; Misra & Dwivedi, 2000; O'Lorcain & Holland, 2000; Crompton, 2001; Valentine *et al.*, 2001; Esser-Köchling & Hirsch, 2005). The obstruction of the biliary tree causes cholecystitis, jaundice, pancreatitis, cholangitis, and liver abscess (Pinilla *et al.*, 2001). The occurrence of hepatic abscesses and pancreatitis are often induced by adult worms since the common bile duct provides a route from the small intestine to the biliary system, liver and pancreas. It is believed that they invade the parenchyma, leading to local inflammation, necrosis, and abscess formation (Akata *et al.*, 1999; Javid *et al.*, 1999). Case studies reported interstitial nephritis and in one instance this was associated with acute renal failure with the presence of *A. lumbricoides* infection (Meister *et al.*, 1995). Morbidity and mortality associated with chronic ascariasis is related to the intensity of infection (Pawlowski & Davis, 1989; O'Lorcain & Holland, 2000; Crompton, 2001). Biliary ascariasis appears to be more common in females and one possible explanation for this has been linked to the hormone progesterone which leads to smooth muscle relaxation of the sphincter of oddi, allowing easier access for the worms into the bile duct (Javid *et al.*, 1999; Sunil *et al.*, 2004).

Chronic ascariasis

Chronic ascariasis generally manifests itself as nutritional impairments especially in children and the parasite is usually endemic in areas where malnutrition is entrenched (Crompton, 1985). Other factors and not only the presence of *Ascaris* must be considered when accessing malnutrition such as climatic, agricultural and socio-economic factors. The infection is most likely to affect bodily growth, fat absorption, vitamin A absorption, iodine absorption, lactose digestion and protein absorption (Stephenson, 1980; Stephenson & Holland, 1987; Thein Hlaing, 1993; Crompton, 1994; Crompton, 2001; Quihui-Cota *et al.*, 2004). Ascariasis also reduces appetite and effects digestion due to the intestinal pathology that occurs in malnourished children such as intestinal atrophy and cellular infiltration of the lamina propria (Tripathy *et al.*, 1972). It is clear from deworming experiments on infected malnourished hosts that improved growth rates are observed after deworming (Stephenson, 1980, Thein Hlaing, 1993; Hadidjaja *et al.*, 1998; Sarkar *et al.*, 2002). There is also a proposed association between *Ascaris* and reduced cognitive

performance but this is yet to be firmly established especially when confounding variables such as age, sex, socio-economic status, baseline nutritional status, food intake, polyparasitism and place of residence, can impinge on the results of possible association (Connolly & Kvalsvig, 1993; Kvalsvig, 2002).

Interactions between *Ascaris* and other infections in the host

Recent evidence suggests that there is an important interaction between pathogens and the development of the host immune response. The hygiene hypothesis proposes that people in developed countries particularly in post-natal life are exposed to less viruses, bacteria, ectoparasites and endoparasites, particularly helminths due to the ultra-hygienic environments they inhabit (Cooper *et al.*, 2003; Palmas *et al.*, 2003) and this may be responsible for the imbalance in immune control mechanisms and contribute to predisposition to other diseases. It is believed that increased cases of asthma in the western world were related to decreases in early life infections that have led to a reduction of Th1 responses and an increase in Th2 responses in relation to common allergens (Cooper *et al.*, 2001; Perzanowski *et al.*, 2002). Conversely it is proposed that Th2-dominated responses initiated by helminth infections suppress the cellular response and can make the host more susceptible to other diseases such as Human Immunodeficiency Virus (HIV) and Tuberculosis (TB) (Fincham *et al.*, 2003). The chronic activation of Th2 lymphocytes and their cytokines as well as other immune responses to adult helminths probably provide an optimal environment for the progression of HIV and its ultimate success (Kaminsky *et al.*, 2004). The impairment of cellular immunity by helminth infections with eventual anergy or exhaustion impairs the efficacy of the host to combat HIV.

Conversely, *Ascaris* infection may be tolerated in some areas as it has been associated with the prevention of deleterious pathological responses associated with co-infections. Concurrent infection with *A. lumbricoides* appears to alleviate the pathology of cerebral malaria infections (Nacher *et al.*, 2000). Exposure to *Ascaris* appears to shift the immune response towards Th2 and inhibit Th1 lymphocytes and their ability to induce cyto-adherence (Nacher *et al.*, 2000). It is believed that the genetic predisposition to *Ascaris* infection may confer a benefit against the more harmful cerebral malaria infection. This would explain the persistence of *A. suum* predisposition in malaria endemic areas.

Pulmonary migration can lead to significant increases in microbial infections. This has been demonstrated by the association between *A. suum* and *Pasteurella multocida* in the

lungs of mice. Following aerosol exposure to this pathogen, mice with pulmonary ascariasis developed more severe pneumonia and septicaemia than did parasite-free mice (Tjørnehøj *et al.*, 1992). This may be due to the transient damage to the alveolar epithelium or poor mucosal clearance of microbes. It could also be a direct result of immunosuppression by the parasite. In weanling pigs, the presence of *A. suum* larvae in the lungs was associated with impaired pulmonary clearance of *Escherichia coli* (strain B) but the deposition of inhaled bacteria did not differ between controls and *A. suum* infected pigs (Curtis *et al.*, 1987). Another possible route of bacterial infection is the transference of microbes during larval migration. Lee & Liu (1976) isolated *E. coli* from the eggs of *A. suum* and suggested that these bacterial isolates were able to thrive in the egg thus they suggested that *A. suum* eggs and thus the newly hatched larvae can transmit bacterial disease. Similarly, Adedeji *et al.* (1989) showed that the intestinal microflora was carried to the lungs by the migrating *A. suum* larvae in pigs via the normal migratory pathway. *E. coli* infection can result in septicaemia, enteritis, diarrhoea, and oedema.

1.7 Immunological responses to parasitic infection

In host-parasite relationships where natural resistance is low, the host can regulate the degree of infection to which it is subject only through the activities of its immune system; that is by developing an acquired resistance to infection (Wakelin, 1996). Heterogeneity in immunological responses contributes to susceptibility or resistance to re-infection and this influences predisposition. The adaptive immune response is fundamentally constrained by the genetic constitution of an individual and the need to avoid a reaction to self or beneficial antigens (such as commensals of food). This means that every individual probably has their own unique immunological repertoire (Kennedy, 1989).

There are two major branches of the adaptive immune responses: humoral immunity and cell-mediated immunity. Humoral immunity involves the production of antibody molecules in response to an antigen and is mediated by B-lymphocytes. Cell-mediated immunity involves the production of cytotoxic T-lymphocytes, activated macrophages, activated NK cells, and cytokines in response to an antigen and is mediated by T-lymphocytes (Wakelin, 1996). Th1-lymphocytes recognize antigens presented by macrophages and function primarily to activate and heighten cell-mediated immunity by producing cytokines such as interleukin-2 (Il-2), interferon-gamma (IFN-γ), lymphotoxin, and tumor necrosis factor-beta (TNF-β) (Sell, 1987). Collectively these cytokines enable T8-lymphocytes to

proliferate and differentiate into cytotoxic T-lymphocytes capable of destroying infected host cells and mutant cells, activate macrophages enabling them to destroy intracellular pathogens, stimulate the production of opsonizing and complement-activating antibodies for enhanced attachment during phagocytosis, activate neutrophils , stimulate increased production of monocytes in the bone marrow, and function as chemoattractants for phagocytes (Sell, 1987). Th2-lymphocytes recognize antigens presented by B-lymphocytes. They produce cytokines such as interleukins 2, 4, 5, 10, and 13 that promote antibody production. Collectively these cytokines enable activated B-lymphocytes to proliferate, stimulate activated B-lymphocytes to synthesize and secrete antibodies, promote the differentiation of B-lymphocytes into antibody-secreting plasma cells, and enable antibody producing cells to switch the class of antibodies being produced. Another major function of the cytokines produced by Th2 cells is to enable B-lymphocytes to activate eosinophils and produce increased amounts of IgE against adult helminths and IgE acts as an opsonizing antibody to stick phagocytic eosinophils to helminths for extracellular killing of the helminths (Wakelin, 1996). The balance between these two subsets plays a role in how well the body reacts to infection. Th1 cells help remove microorganisms. When Th1 cells produce IFN-γ, this prompts macrophages to produce TNF and toxic forms of oxygen which destroy the microorganisms. However, Th2 cells produce IL-4 and IL-10, these cytokines block the microbe killing that is activated by IFN-γ.

The immune response to larval *Ascaris*

Eriksen (1981) indicated that the elimination of *A. suum* larvae from the pulmonary tissues of mice during primary and secondary infection was influenced by the thymus-dependent immune response. Protective immune responses may operate at the different stages of the life cycle. Parasite killing in the intestinal lamina propria, in the liver, or in the lungs, may occur via mast cell mediated mechanisms with the recruitment of cytotoxic effector cells such as activated eosinophils, neutrophils and macrophages. The migrating larvae may become mechanically trapped in the tissues or be immobilised by a combination of antibody and cellular mechanisms with larval destruction occurring via anti-body dependent cellular cytotoxicity and other cellular pathways such as the release of nitrous oxide and reactive oxide products by activated macrophages (Cooper, 2002). The Th2-response appears to be more dominant in the expulsion of GI stages but it appears that the Th1 type response may be paramount in the reaction to migratory phases (Eriksen *et al.*, 2004) but that these responses are antagonistic and the Th2 responses may effectively

suppress the Th1-response to migrating larvae. In most cases, the protective capacity of immune responses against mucosal dwelling helminths is greater than that against stages dwelling in tissue spaces. For example, a protective memory immune response to *A. suum* challenge inoculation was induced in pigs using adult worms and had little or no effect on the migration and expulsion of larvae. This may indicate that antigens of adult worms do not penetrate intestinal lining and thus they do not stimulate the immune system. The reduced recovery of transferred adult worms after challenge with larval stages indicated that the strong immune responses to larval migration could have a detrimental effect on the resident adult worm population (Jungersen *et al.*, 1999a). More recently, the recovery of anti-*Ascaris* IgA and IgM antibody secreting cells (ASC) in the lamina propria of the proximal and distal jejunum, indicated the recognition of the presence of the parasite in the lumen of the small intestine by the host and furthermore that this response is dependent on both antigen specific T- and B- cells (Miquel *et al.*, 2005). Newly hatched *A. suum* are known to penetrate the caecum wall en-route to the liver and it has been speculated that increased levels of IgA anti-L3-ES ASCs detected in the lamina propria of the distal jejunum on day 10 p.i. are likely due to the immune response developed against migrating larvae (Miquel *et al.*, 2005) and this impacts on the expulsion of L_4 larvae from the intestine.

The gastrointestinal response to adult worms

McSharry *et al.* (1999) indicated that ongoing acute phase or inflammatory processes were associated with immunity to adult *A. lumbricoides*. Immunity status was determined by the number of worms developing to maturity. Resistant individuals produced significantly more IgE antibody to ABA-1, a recombinant allergen of *Ascaris*, than their susceptible counterparts. Susceptible individuals showed very little serological evidence of inflammation although their parasite burdens were high. They suggested that these two factors, i.e. increased IgE and ongoing acute inflammatory processes, were associated with natural resistance to adult intestinal *A. suum* infection. This has been shown to be a possible resistance mechanism for other helminths such as *Schistosoma haematobium* (Hagan *et al.*, 1991). However, these studies were performed in areas not only hyper-endemic for ascariasis but that have concomitant infections. Conversely, higher concentrations of parasite specific IgG1, IgG4 and IgE were found to occur in children heavily infected with *Ascaris* in a study of Bangladeshi children (Palmer *et al.*, 1995). The implication was that these antibodies simply reflected the intensity of infection and did not play a significant role in protecting against heavy infections. There is no direct evidence to

suggest that IgE is protective as it is just one of the responses driven by the Th2 response and helminth infections are among the most potent stimulators of the Th2-type immune responses (Paterson *et al.*, 2002).

Immunomodulation and parasite evasion of the host immune response

Read & Skorping (1995) have suggested that tissue migration is an expensive strategy in terms of energy expenditure and requires physiological adaptations to numerous sites with increased attrition rates of larvae in the tissues. However, it offers advantages in relation to parasite size and thus fecundity. Parasites that undergo tissue migration were correlated with greater body-size which has been associated with more fecund females and generally faster maturation. The survival advantage could be an immune-evasive mechanism that is specific to avoid the innate immune mechanisms associated with the Th2 immune response that results in inflammation at the mucosal surface and reinfecting larvae may be less vulnerable to localised intestinal defences induced by previous adult infections.

A. suum extracts have been shown to be involved in immunosuppression of the hosts immune response and this has been examined by a number of authors (Crandall *et al.*, 1978; Barta *et al.*, 1986; Macedo & Barbuto, 1988; Soares *et al.*, 1990; Rhee *et al.*, 1991; Soares *et al.*, 1992; Faquim-Mauro & Macedo, 1998; Faquim-Mauro *et al.*, 1999; Paterson *et al.*, 2002; Souza *et al.*, 2002, Oshiro *et al.*, 2005). Ferreira *et al.* (1995) found that the immunisation of DBA/2 mice with *A. suum* extract impaired T-cell functions for cell-mediated as well as humoral immune responses to other antigens through the induction of a predominately Th2-like response. Cooper *et al.* (2000) observed a highly polarised Th2 immune environment caused by the pulmonary migrations of *Ascaris* larvae observed as greater levels of IL-5 in the infection group in response to L_3/L_4 *Ascaris* antigens (lung-stage) and suggested that migrating larvae caused an inappropriate immune response (Cooper *et al.*, 2000) by the preferential stimulation of a Th2 response to their own components (Souza *et al.*, 2002).

The arms race between the host and parasite that results in host immunity and parasite evasive techniques is a constant struggle and the victor is reflected in the intensity of infection. Evasive strategies include both measures to protect the parasite from host effectors (e.g. resistant cuticle, oxygen scavenging enzymes, anti-IgA proteases etc.) and measures to incapacitate the host response itself (immunomodulatory strategies) (Behnke, 1990). The idea that hosts may vary in their susceptibility to parasite evasive strategies

(Kennedy, 1989) is important because a host with a genotype capable of generating a strong immune response to a parasite may nonetheless fail to do so if the host is suscpetible to the parasite's immunomodulatory strategy (Behnke *et al.*, 2000). This concept, that some host genetics may be susceptible to a parasitic infection, also applies in the opposite direction, in that parasites, which by nature are polymorphic (particularly for surface antigens), can be considered to be more or less vulnerable to certain hosts immune responses depending on their genetic makeup. So the parasite will only be successful in those individuals that bear particular alleles or certain genetic characteristics. The *Trichuris muris* mouse model system provides clear evidence that the interplay between host responses and parasite survival strategies lead to specific infection levels. A host genotype that leads to the development of a strong and dominant Th2 response following infection will lead to resistance. A host genotype that does not do this (e.g. high IL-18 production) will lead to the development of a Th1 response. This is associated with susceptibility to infection. In the latter case, or particularly in cases of "intermediate" host genotypes, the influence of the parasite may become paramount and novel immune evasion strategies such as those hypothesised above will play crucial roles in promoting parasite survival (Grencis, 2001). Whether similar mechanisms are involved in other intestinal nematode infections remains to be elucidated.

1.8 The murine model

The development of the mouse as a model for ascariasis

The definition of an animal model is a living organism with an inherited, naturally acquired or induced pathological process that in one or more respects resembles the same phenomenon in man (Wessler, 1976 cited by Boes & Helwigh, 2000). The choice of animal model for parasitic diseases should mimic (a) the human host, (b) the parasite, (c) the human host-parasite system or the way in which the host and parasite interact (Boes & Helwigh, 2000). A number of animal models are available for investigation of aspects of disease for numerous species of parasite but the mouse is a regularly utilised system (Mitchell *et al.*, 1982).

Modern mouse genetics began in 1902 when William Castle began studying inheritance in mice. The first inbred mouse strain, DBA, was started by Clarence Little in 1909 (Festing, 1996). Other inbred strains, were generated over the next decade, including C57BL/6, C3H, CBA and BALB/c (Festing, 1996). The first guidelines for generating inbred strains

were published in 1952, and a strain is "regarded as inbred when it has been mated brother x sister for 20 or more consecutive generations and can be traced to a single ancestral breeding pair in the 20th or a subsequent generation" (Beck *et al.*, 2000). Using single-nucleotide polymorphism, a study at Jackson laboratories involving a number of mouse strains, such as C57BL/6j, provided information to confirm the background of some 300 strains with high confidence (Petkov *et al.*, 2004). At 20 generations, on average at least 98.6% of the loci in each mouse are homozygous. Many strains have been bred for more than 150 generations and are essentially homozygous at all loci (isogenic) because all individuals trace back to a common ancestor in the 20th or a subsequent generation. Thus inbred mouse strains can be used in the knowledge that individuals of the same strain should consistently show the same ability to control parasitic infection.

The murine model has previously been exploited for the study of larval *A. suum* and the acquired immune response to this parasite by a number of authors (Boisvenue *et al.*, 1968; Jenkins, 1968; Bindseil, 1969a/b; Bindseil, 1970a/b; Douvres & Tromba, 1971; Mitchell *et al.*, 1976; Johnstone *et al.*, 1978; Eriksen, 1981; Slotved, 1997; Slotved *et al.*, 1997a; Slotved *et al.*, 1998; Boes, 1999) as have a number of other abnormal hosts including guinea pigs, rabbits and rats (Jenkins, 1968; Douvres & Tromba, 1971). However, the mouse is favoured for a number of reasons. Firstly ruminants and carnivores are considered less suitable models for modelling human disease because of their dissimilarity with humans (Boes & Helwigh, 2000). The pattern of migration of larval *Ascaris* in mice mimics their behaviour in pigs except the timing of larval migration in pigs is extended over slightly longer periods of time (**Fig. 1.8.1**; Eriksen, 1981; Murrell *et al.*, 1997a; Slotved *et al.*, 1998). This supports the use of the mouse as a model for studies on aspects of the biology of *A. suum.* Furthermore, larval recovery from the organs of mice has been shown to be higher in contrast to other abnormal hosts (Douvres & Tromba, 1971). The mouse internal organs by comparison to rabbits, guinea pigs, rats and pigs can be examined with great ease as they are smaller and hence are more efficient for experimentation purposes. Finally, a great deal of knowledge on mouse genetics has been gathered throughout the years as they are a primary source of study in laboratories. Susceptibility to infection can be influenced by a number of factors which include host genetic constitution which influences innate and acquired resistance (Taffs, 1968) and physiological/morphological differences expressed by the host at stages in the migration of the parasite. The availability of inbred strains of mice allows for specific intrinsic host

factors to be examined in determining the mechanisms of host susceptibility/resistance to larval *Ascaris* infection.

The observations of Mitchell *et al.* (1976) concerning genetically resistant and susceptible inbred mouse strains to migrating larvae of *A. suum* have been paramount in the investigation of predisposition of mice to pulmonary larval burdens. They studied larval recovery in the lungs on days 6-9 post-infection (p.i.) and in the liver on days 5-7 p.i. in various strains of inbred mice. They reported higher larval recovery in the lungs compared to the liver. Thus they concluded that susceptibility was reflected by the number of larvae recovered in the lungs on day 7, with the strains of mice showing heavier lung larval burdens said to be more susceptible. This demonstrated a direct link between the genotype of the host and the larval burden experienced in the lung. The strain C57BL/6 was identified as being the most susceptible strain of mouse.

Although the results of earlier research using inbred mouse strains inoculated with infective doses of *A. suum* suggested that there was an innate heterogeneity in mouse strains in their pulmonary larval burden (Mitchell *et al.*, 1976), there was uncertainty associated with the results due to a high rate of variability between groups associated with the experimental procedures employed. Subsequently, Johnstone *et al.* (1978) evaluated C57BL/6 mice as a model for the study of resistance to infection. The comparison of larval recovery methods (Baermann and spin methods) in this mouse-model led to the observations that certain parameters influenced the number of *A. suum* larvae recovered. These parameters were dose administered (more larvae were recovered as the dose of ova administered increased), organ of recovery (more larvae were discovered in the liver compared to the lungs, this was the opposite to the observations of Mitchell *et al.* (1976)), and host age (with more larvae retrieved from 8 weeks old mice compared to 3 week old mice which was contradictory to the observations of Bindseil (1969b)). The consequence of the disparity in larval burdens between these groups highlighted the effect of extrinsic factors on larval burdens and thus the role of inherent host factors could not be determined.

Heterogeneity in the response to parasitic infection between mouse strains

Heterogeneity in the immunological response to larval antigens is one possible source of variation between hosts. Kennedy & Qureshi (1986) found that excretory/secretory (ES) antigens of developing L_3/L_4 lung-stage *A. suum* were stage specific and were the target of the antibody response mounted in experimental animals. There was noticeable

responsiveness of three experimental animals to *Ascaris* antigens. A comparison of the humoral response between rabbits, mice (NIH) and rats showed that rabbits recognised all ES components, while mice and rats responded to distinct subsets of these. The mouse was distinguished by non-recognition of a major M_r 14,000 antigen. They suggested that an analogous disparity may exist between strains of mice in their response to the presence of migrating larvae in the lungs that results in fluctuations in the development of an appropriate immune response. In subsequent investigations involving strains of mice of different genetic backgrounds, variation in the humoral response to different components of the ES antigenic material of *Ascaris*, and the antigen recognition patterns, varied considerably from strain to strain. Only H-2^s strains on BALB/c and B10 backgrounds responded with IgG to an ES component of M_r 14,000 and this major histocompatibility complex (MHC) restriction was confined to infection. This has implications for the heterogeneity of responses not only in the mouse model but also offers one possible explanation for the susceptibility of individuals in an infected stock of pigs (Tomlinson *et al.*, 1989; Kennedy *et al.*, 1990).

Recent evidence suggests that, at least in part, genetic heterogeneity explains the susceptible phenotype in relation to worm expulsion from the GI tract. Pemberton *et al.* (2004) described the up-regualtion of a novel epithelially-derived lectin, intelectin-2 that is exclusively expressed in the intestine of certain mouse strains, at the site of infection with *Trichinella spiralis*, when worm expulsion was at its maximum level. The mucus layer that coats and protects the simple epithelia of the gut and lung is composed of mucin glycoproteins and additional components that help to protect the epithelium against invasion by pathogens. For example, the mucus layer plays a significant role in the response to intestinal nematodes in which the worms become physically entrapped in mucus and are expelled (Pemberton *et al.*, 2004). The up-regulation of this protein as early as day 3 p.i. suggests that it may be a component of the early innate response to nematode infection. They compared the expression of lectins in the low-responder C57BL/10 strain which fail to efficiently expel the parasite in contrast to the resistant BALB/c strain. The results indicated that the former failed to up-regulate intelectin-2. Although this is unlikely to be the sole difference between the two strains as there are cytokine profile variations it is likely it is involved in early innate immune response to infection.

Similar heterogeneity in the level of infection has been observed in mice infected with other parasites. Incani *et al.* (2001) investigated parasite and host genetic heterogeneity and

determined that both features influenced the outcome of infection by *Schistosoma mansoni*. They investigated host variation by examining infection in C57BL/6j and BALB/c mice, and parasite heterogeneity by infecting mice with different laboratory strains of *S. mansoni*. They found that in this model the host genetics determined susceptibility and parasite fecundity. On the other hand, the strain of parasite determined infectivity, the sex ratio and the ability to cross the intestinal wall. In *Trichinella spiralis* infected-mice it was determined that genetic differences between isolates of the parasite exerted significant influences upon the outcome of infection and that this outcome was determined by the genetically-controlled response capacity of the host when examined in inbred mouse strains (Wakelin & Goyal, 1996). C57BL/6 appears to be a highly susceptible group to many parasites including *A. suum*, *Brachylaima cribbi*, *Plasmodium berghei*, *Toxoplasma gondii*, and *T. spiralis* (Johnstone *et al.*, 1978; Mitchell *et al.*, 1976; Butcher *et al.*, 2002a; Butcher *et al.*, 2002b; De Souze & Riley, 2002; Lee & Kasper, 2004; Dehlawi & Goyal, 2003). However, the reason for the highly susceptible nature of this mouse strain to these parasites is yet to be clarified.

Immunological response to migrating larvae in mice

The role of the immune response to migrating stages of *A. suum* is poorly understood and there is a lack of detailed analysis on the responses associated with susceptibility/resistance to these stages. Prominent antibody and cellular responses appear to characterise the human immune responses against *Ascaris* and this is directed primarily against the larval stages of infection (Cooper, 2002). Bindseil (1970a) suggested that the difference between the number of *A. suum* eggs and larvae in the caecal contents in immune mice strongly indicated that protective immunity was already working in the gut lumen itself by influencing the process of hatching and the penetration of the gut mucosa by the larvae. It was believed that these observations gave further support to the view that the early larval migration in immune mice is characterised by a decreased number of larvae starting migration and that the penetration into the gut wall is delayed (Bindseil, 1969b). Alternatively, Taffs (1968) considered the liver to be an important site for destruction of migrating larvae in both primary and secondary infection. This organ, at least in part, plays a role in the defensive mechanism against this parasite. Resistance to *A. suum* in pigs has been suggested to involve the co-operation of the humoral and the cell mediated immune response and the parasite is eliminated both during migration by white spot formation in the liver and during the early pre-patent period expulsion from the gut (Eriksen *et al.*, 1980). Eriksen (1981) found that in mice inoculated with *A. suum* an increased liver

reaction and decreased lung reaction was observed after reinoculation suggesting that larval reduction occurs during the liver migration and these results are contrary to those of Bindseil (1970a). Migration through the liver may stimulate or contribute to the development of an anti-parasite response in the later stages of larval migration as well as trapping migrating larvae and preventing further migration to the pulmonary tissues. The intravenous administration of hatched *A. suum* larvae that by-passed the liver in pigs resulted in an increased chance of survival in the critical phase after the return to the intestine (Jungersen *et al.*, 1999b) providing further evidence for a strong contribution of the immune responses of the liver in larval entrapment.

Investigations of the inflammatory response in the lungs and the correlation with larval burden showed that the cellular reaction to migrating *A. suum* larvae in resistant and non-resistant mice (mice were made resistant by exposure to viable *A. suum* larvae) indicated that the response differed in nature, showing that at least in challenge infections there is some level of immunity observed during the pulmonary stage (Ronéus, 1966; Eriksen, 1981). Ultimately the role of the lungs is poorly understood in relation to their impact on the successful migration of larvae during primary infection. Eosinophils were thought to play an important role in the inflammatory response directed against pulmonary stages as has been observed in other helminth infections. However, it appears that eosinophils are associated with the encapsulation of dead larvae but they do not appear to be destructive to living larvae as these leukocytes were not prominent in the inflammatory reaction in the liver and lungs of primary infected mice (Sprent & Chen, 1948). Andersen *et al.* (1973) observed a corresponding increase in eosinophil response in the lungs but it was suggested that, although reflecting immune reactions, they were not involved in the defence mechanisms against *A. suum* larvae. *A. suum* larval migration also induces a dose-dependent blood eosinophilia, in primary and secondary infections peaking at day 14 and 10 p.i. respectively (Jungersen, 2002) but there was no indication of its role in protection against larval stages.

The current hypothesis is that immunity may be the result of a combined Th1/Th2 response to migrating larvae and that the balance of these responses is crucial. Islam *et al.* (2005a) demonstrated that mice vaccinated with recombinant *A. suum* 24-kilodalton antigen (rAs24), an antigen expressed at all stages of the life-cycle, exhibited increased levels of splenic T cells of both T-helper types, both IFN-γ (a Th1 response marker) and IL-10 (a Th2 response marker) cytokines. Mice vaccinated with rAs24 were protected against

migration of *A. suum* larvae, suggesting that rAs24 was able to stimulate a response capable of killing the larval stages of *A. suum* by immune responses and that protective immunity, at least, induces a mixed Th1/Th2 response against this antigen. IgE responses were not detected in these mice, suggesting that immune responses to rAs24 were not mediated by hypersensitivity reactions. This contrasts with previous studies which associated parasite specific IgG and IgE responses to protective immunity to human ascariasis (McSharry *et al.*, 1999; Cooper *et al.*, 2000). Recent data from *Heligmosomoides polygyrus* infection in a number of mouse strains indicated that expulsion is controlled by both the Th2 and Th1 responses following primary infection and these responses operate differentially to a greater or lesser extent within the strains. While expulsion was most commonly associated with a Th2-type pattern of mediator response, in certain strains Th1 responses predominated and appeared to mediate expulsion. Thus chronic infections could be the result of suppression of both arms of the T helper response in susceptible strains (Ben-Smith *et al.*, 2003). This suppression of the arms of the immune response is possibly initated by the host to prevent immunopathology. A similar suggestion can be theorised for the host response to larval *A. suum*. Bancroft *et al.* (2000) found that both gender and strain play an important role in cytokine-mediated immunity in mice infected with *T. muris*. The reason why gender plays such an important role is probably due to the effects of male and female sex hormones on the Th1/Th2 cytokine balance.

Thus there appears to be a combined role of the two arms of the immune response in controlling both migratory and intestinal stages of helminths. Heterogeneity in the immune response to parasite infections may be a reflection of an immunomodulatory response induced to limit immunopathology elicited by the protective Th2 response or by the migrating larvae themselves (immunoevasion). However, it may be a failure by the host to produce an appropriate response to the presence of migrating parasitic helminths. IL-10 was recently demonstrated to be a key regulator of protective immune responses against *T. spiralis* (Helmby & Grencis, 2003) and *Trichuris muris in vivo* (Schopf *et al.*, 2002). IL-10 knockout mice or normal mice treated with a neutralizing anti-IL-10 receptor antibody are highly susceptible to a primary *T. spiralis* infection and show significantly delayed adult worm expulsion. Depletion of IL-10 resulted in elevated Th1 and Th2 cytokine responses but significantly reduced numbers of mucosal mast cells in the jejunum. Interestingly, an increase in IFN-γ detected in the absence of IL-10 resulted in increased immunity to larval stages of *T. spiralis* (Helmby & Grencis, 2003) thus associating Th1 responses with protection against the larval stages. This is a response previously associated with microbial

pathogens. Hence, IL-10 has a negative effect on immunity to the tissue dwelling larval stages of *T. spiralis* but plays a significant biological role as an *in vivo* regulator of intestinal mast cell responses and was crucially involved in protection against adult stages of intestinal parasites *in vivo*.

An examination of *T. muris* infected mice demonstrated that IL-$10^{-/-}$, IL-$4^{-/-}$ and double IL-$10^{-/-}$/$IL4^{-/-}$ deficient mice failed to expel the parasite. IL-$10^{-/-}$ deficient mice displayed marked morbidity and mortality. In contrast, double IL-$10^{-/-}$/IL-$12^{-/-}$ deficient mice were completely resistant and mounted a highly polarised Th2 cytokine response. This suggested that increased susceptibility of IL-10 deficient mice was dependent on IL-12. This was attributed to a marked increase in type 1 cytokine production (Schopf *et al.*, 2002). In humans, IL-10 is produced by Th1 and Th2 clones and reduces proliferation of both types of cells. It inhibits synthesis of IFN-γ by Th1 and IL-4 and IL-5 (Th2 regulators) by antigen stimulated Th2 clones (Ferreira *et al.*, 1995) and this actively downregulates pathological immune responses (Paterson *et al.*, 2002; Specht *et al.*, 2004; Oshiro *et al.*, 2005). This supports the theory that increased Th2 responses would down-regulate an appropriate Th1/Th2 response to larval stage *A. suum*.

The distribution of *A. suum* in the pig model demonstrated that in experimentally and naturally infected pig populations the worm population is over-dispersed and there is a tendency for a few individuals to consistently harbour the majority of worms even following anthelmintic treatment (Boes *et al.*, 1998b). However, the mechanistic basis for predisposition has yet to be elucidated but it appears to be genetically linked (Williams-Blangero *et al.*, 2002). Since mice are known to vary in their susceptibility to infection with *Ascaris* (Mitchell *et al.*, 1976) they provide a convenient model for investigating the basis of variation in establishment and the successive passage of migrating larvae through the liver and lungs of a host that has variable genotypes. Previous studies have contended with issues related to reliability of the observed larval burdens, resulting in inconsistent observations relating to the susceptibility of mouse strains. Thus the definition of a murine model that can be reproduced with consistent larval recoveries within strain provides a foundation to explicate the mechanisms that determine the relative susceptibility of a host to the larval phase of *A. suum*.

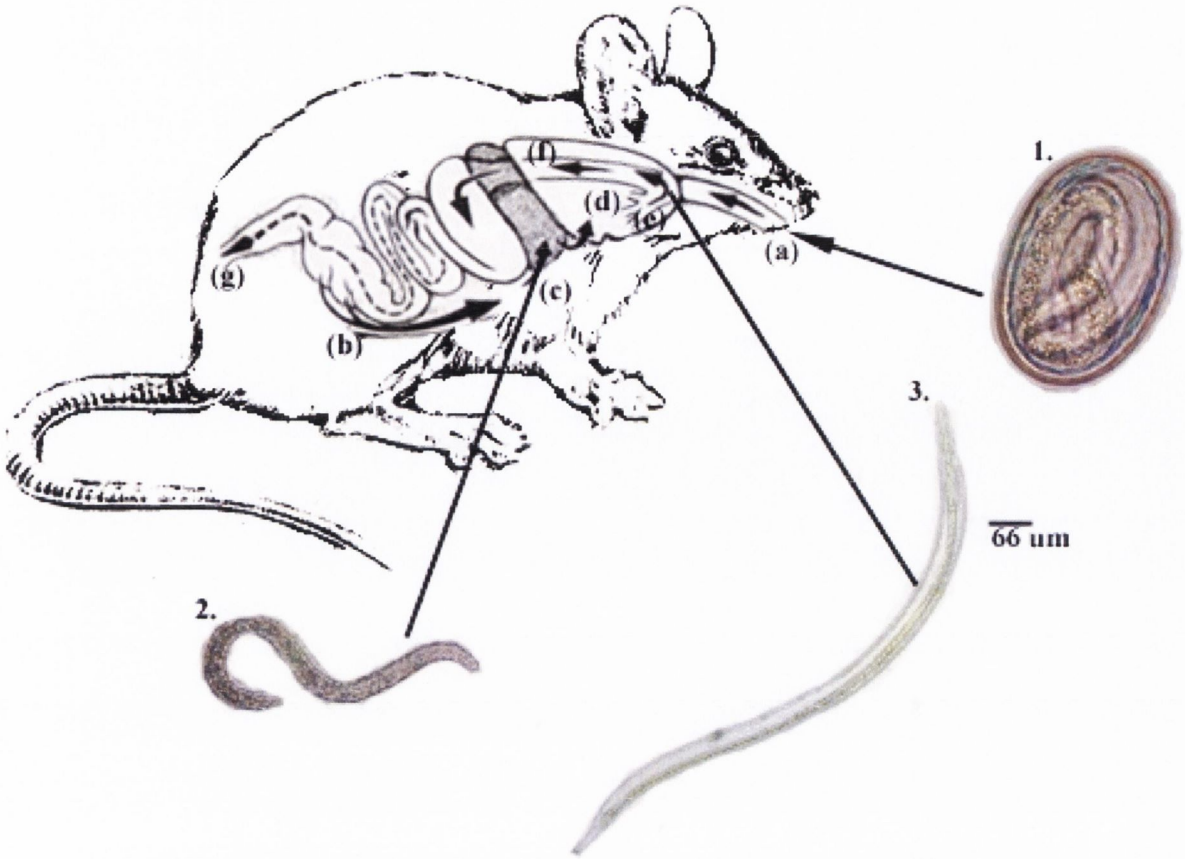


Figure 1.8.1: Embryonated eggs (**1**) were administered to the mice by gastric lavage (a). Infective eggs were swallowed; the larvae hatched (**2**), invaded the intestinal mucosa (b), and were carried via the hepatic veins to the liver (c) (0-5 days), then via the portal system to the lungs (d) (6-8 days). The larvae matured (**3**) further in the lungs (e), penetrated the alveolar walls, then ascended the bronchial tree to the throat (f), and were re-swallowed. Upon reaching the small intestine they are quickly expelled in the faeces (g) [parasites and egg not to scale. Scale bar refers to the matured larva] (original figure).

CHAPTER 2

DEVELOPMENT OF THE EXPERIMENTAL PROTOCOL AND ANALYSIS OF LARVAL *ASCARIS SUUM* MIGRATORY KINETICS IN TWO STRAINS OF MOUSE (C57BL/6j AND BALB/c)

2.1 INTRODUCTION

An increase in the knowledge garnered concerning parasite infections of humans in endemic areas can be gained by studying the parasite (or its close relatives) in an animal model (Boes & Helwigh, 2000). A novel contribution to *Ascaris* epidemiology is the development of a pig model of predisposition to *A. suum* (Boes *et al.*, 1998a). These authors studied pigs subjected to trickle inoculation with high doses of eggs and naturally exposed pigs, both of which exhibited an aggregated distribution of worm burdens. Furthermore, they were able to demonstrate that individual pigs were predisposed to low or high *A. suum* worm burdens after treatment. This model is clearly important and in the future may be used to dissect the relative contributions of exposure and susceptibility to predisposition (Holland & Boes, 2002). As evidence from the Jirel population illustrates the genetic contribution to the phenomenon of predisposition is considerable (Williams-Blangero *et al.*, 2002). The difficulty in using pigs to explore the contribution of host genetics to susceptibility and resistance to *A. suum* is time, cost and a lack of suitable inbred porcine strains.

Only when the lifecycle and dynamics of parasite infection in the animal model are clearly defined can valid conclusions be drawn from the model. The animal model may actually provide new knowledge about the natural animal host-parasite system. An interesting example was the elucidation of the predilection of *A. suum* larvae to penetrate the wall of the caecum/ colon and not the small intestine as previously believed (Murrell *et al.*, 1997a). This preference of larvae for the large intestine was first proposed from investigations in the mouse model (Jenkins, 1968; Bindseil, 1970a). Initially it was suggested that the distribution of larvae within the mouse gut may be influenced by the relative shortness of the alimentary canal. After investigation of 6-month old rats and guinea pigs it was found that distribution was similar in all three animals irrespective of gut length and emptying time (Jenkins, 1968) and more recently, Murrell *et al.* (1997a) showed that the majority of newly hatched larvae penetrate the large intestine wall of pigs also. This was later confirmed in the mouse using more accurate experimental protocols (Slotved *et al.*, 1998). This has led to the re-evaluation of the mouse as a potential model for the study of early *A. suum*, and the associated disease ascariasis, due to the similarities of the early stages of the life cycle in this host compared to the natural host, the pig (Slotved *et al.*, 1998). The establishment of the mouse model in relation to predisposition

to *Ascaris* has been made possible by the observation that mouse strains can be either susceptible or resistant to the migrating stages of this parasite (Mitchell *et al.*, 1976).

The examination of two contrasting phenotypes, in relation to larval burdens, provides a basis for studies of genetic variation, immunology and pathology. Extrinsic factors were explored that might affect the quantitative recovery of larvae from the visceral organs in order to standardise a model system facilitating accurate future assessment of the impact of host genetic variation on the migratory phase of infection. Inoculation doses were standardised to ensure each individual mouse received an identical number of embryonated *A. suum* eggs. This section also explored the kinetics of early *Ascaris* infection in the liver and lungs of inbred mice. The two reference strains of mice employed (C57BL/6j and BALB/c), were chosen based on the older literature (Mitchell *et al.*, 1976; Johnstone *et al.*, 1978) and were believed to represent susceptible and resistant phenotypes respectively.

2.2 MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Male mice were purchased from Harlan, UK at 7 weeks of age and were 8 weeks old upon commencement of the initial inoculations. Seventy inbred mice (35 of the strain C57BL/6j and 35 of the strain BALB/c) were purchased to examine the pattern of migration in the liver and lungs on days 3-9 post-inoculation (p.i.). The mice were housed in the Bioresources unit, Trinity College Dublin for the duration of the experimental procedures. Groups of five mice were kept in standard plastic cages (size 35 x 15 x 13) with sawdust bedding (Lilico- gold chip). Each mouse was assigned to one of seven cages randomly within the strains and then assigned an identification number. Individual mice in the cages were identified by ear markings which were a series of holes made by an ear punch on the day of arrival (**fig. 2.2.1**). These marking were: right ear full moon (1), Left ear full moon (2), right ear half moon (3), left ear half moon (4) and no marking (5) (**fig. 2.2.1**). Conditions in the unit were standard and constant; approximately 20°C (±2°C) with humidity of 50% (±10%) and a daily 12 hour photoperiodicity. Water and pelleted commercial food (Redmills rat and mouse pelleted diet) were provided *ad libitum*. The cages were cleaned and the sawdust changed regularly. The mice were habituated to these conditions for one week prior to infection to reduce stress on the animals and the mice were provided with play items.

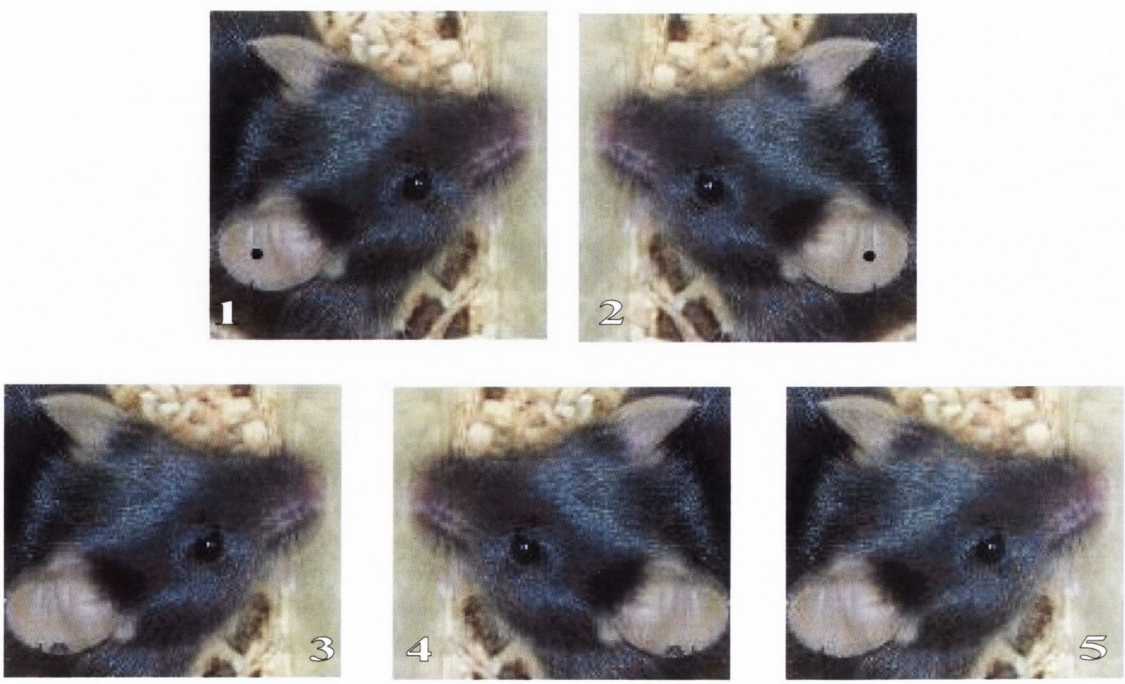


Figure 2.2.1: Ear marking scheme for the identification of individual mice.

PARASITE

Approximately 4,000,000 embryonated ova (batch number: 8/2002) were provided by the Danish Centre for Experimental Parasitology (DCEP), The Royal Agricultural and Veterinary University, Copenhagen, Denmark. The eggs were removed from an isolate of *Ascaris suum* taken from pigs at a Danish slaughterhouse. The worms were rinsed in tap water, then brought to the laboratory and processed on the same day. After a short wash in 96% ethanol, the cranial 2-4 cm of the uteri was removed by dissection. These segments were washed in tap water, cut with a pair of scissors and strained through a brass sieve (80mesh/inch). They were then rewashed and sedimentation was allowed to occur in 2 litres of distilled H_2O for 30 minutes. The supernatant was removed and shaken in 0.5% sodium hypochlorite for 5 minutes in order to decoat the eggs (**fig. 2.2.2**). After sedimentation for 30 min, the eggs were then washed in distilled H_2O until no smell of chlorine was detected (usually 3-4 washings) (Eriksen, 1981).



Figure 2.2.2: Unembryonated egg and decoated embryonated egg.

After the final wash the eggs were suspended in ½ litre of 0.1 N H_2SO_4 and allowed to settle for 30 minutes after which the egg suspensions were transferred to Petri dishes with a ½ cm layer of 0.1 N H_2SO_4. The eggs were incubated at room temperature (20 °C) and aerated 2-3 times weekly by shaking. After 3 months of incubation (in a dark room at room temperature), conditions which have shown to result in fully infective eggs (Oksanen *et al.*, 1990), larvated eggs had developed and the infectivity of the different batches of eggs was tested by mouse inoculation. Eggs are most infective and stable after a year in sulphuric acid (Eriksen, 1981).

The eggs were shipped to Dublin undiluted. Upon arrival in Dublin, these eggs required dilution to a concentration below 25 eggs μl^{-1} as this was shown to be the maximum egg density which does not inhibit the infectivity or viability of the eggs (Eriksen, 1990). The

4,000,000 embryonated ova were diluted in 400 ml of sulphuric acid (0.1 N H_2SO_4) which gave a concentration of 10 eggs μl^{-1} (Eriksen, personal correspondence). This stock solution was used for all subsequent experimental procedures.

PREPARATION OF INOCULATION DOSE

A standard dose of *A. suum* eggs for inoculation was prepared as follows. A dose of 50µl egg suspension containing 10 eggs μl^{-1} was drawn from the egg suspension during gentle magnetic stirring (IKA-mini-MR at setting 1) and gentle agitation by hand, into a 10-100µl Gilson pipetman® fitted with a disposable tip. The pipette contents were emptied into a 1.5ml Eppendorff tube, and this was diluted to 100µl by the addition of 50µl of 0.1 N H_2SO_4, thus giving a prepared dose of 500 *A. suum* eggs suspended in 100µl (5 eggs μl^{-1}) per Eppendorff tube. A technique to evaluate the number of eggs in the prepared dosages was used to ensure a constant dose size (Slotved *et al.*, 1997a). A random sample of prepared doses was removed for counting. The number of eggs containing larvae was counted under x10 magnification by dispensing (using a Gilson pipetman® 10-100 µl) ten by 10 µl samples from an Eppendorff tube. This allowed the calculation of the number of eggs in a 100µl aliquot dose. This procedure was repeated 6 times, thus six mouse doses of 500 infective *A. suum* eggs were counted after being dispensed to the Eppendorff tubes. The tip was changed between each replication. Using this method the viability of the batch of eggs can be determined (Slotved *et al.*, 1997a) and thus there was standardisation of the number of eggs in an inoculation dose (see **appendix A.1**). The concentration of eggs in the stock solution was constantly monitored particularly before each experimental procedure. Dilutions and concentrations of the initial egg dose were performed as necessary for subsequent procedures.

INOCULATION OF THE MICE WITH *A. SUUM*

Mouse inoculation was performed between 8am and 10am by intragastric intubation with the corresponding dose of 500 eggs. The egg dose was within recommended non-lethal levels, suggested by Johnstone *et al.* (1978) and Eriksen (1981). Prior to inoculation (time zero) the mice were weighed using a Satorius portable balance. Mice were inoculated by restraining them by the scruff of the neck and the tail, gently. The inoculation apparatus, which consisted of a 1ml graduated syringe (Terumo 1ml syringe) containing 100µl of egg suspension with a gastric intubation needle, was inserted into the mouth of the mouse. The tube was delicately guided down the oesophagus and into the stomach. The plunger was depressed, releasing the dose into the stomach and the tube was retracted. Between each

mouse inoculation, the syringe and needle were washed in tap water. The mouse cages were covered subsequent to inoculation of each individual group, with sterile filter hoods for 24 hours to prevent possible contamination between cages with *A. suum* ova in faecal matter or air-borne allergen particles that may impact on the observations. After 24 hours the hoods were removed and the mouse cages changed to prevent coprophagia influencing larval burdens and temporal patterns of migration.

POST-MORTEM PROCEDURE

The migration pattern and the number of *A. suum* larvae in the liver and lungs were determined from days 3 to 9 p.i.. Groups of 5 mice from each strain were euthanased at p.i. intervals of 3, 4, 5, 6, 7, 8 and 9 days by cervical dislocation and their bodies immediately processed as follows. These days have been identified as the days within which larval migration in mouse liver and lungs reach their peak (Mitchell *et al.,* 1976; Johnstone *et al.,* 1978; and Slotved *et al.,* 1997a).

The following experimental data was noted:

- Experimental number
- Mouse weight pre- (day 0) and post-infection
- Date and time of post-mortem
- Number of days post-inoculation
- Strain
- Clinical signs (migratory tracks in the liver, white spots in the liver, weight of the spleen, migratory tracks in the lungs e.g. greyness caused by eosinophilia, and other macroscopic observations were all noted)

The euthanased mice were placed on dissection trays. The animal was placed on its back (dorsal) with its limbs outstretched and attached to the tray (**Fig. 2.2.3**). With a pair of scissors an incision was made in the skin immediately above the anus and the cut was expanded forward to the neck while ensuring not to damage the underlying organs. The sternum was incised to expose the lungs. The spleen was removed and quickly weighed on a four point Satorius balance (**appendix A.3**). The whole of the small intestine was displaced to the right hand side and carefully lifted to expose the mesenteric lymph nodes in the second loop of the intestine (**fig. 2.2.3**). These were removed, weighed on a four point balance (**appendix A.3**). The liver and lungs were examined *in situ* for macroscopic lesions or discolouration. Once this was completed, these organs were removed from the

mice. The liver was removed whole and placed in a flat bottomed glass vial with a drop of warm saline (37.5°C) and processed as follows. The lungs were removed in two sections (the left and right hand side lung were dealt with as separate organs) (**fig. 2.2.3**) and again they were placed in a flat bottom glass vial with a drop of warm saline and processed. This procedure was repeated for each individual mouse.

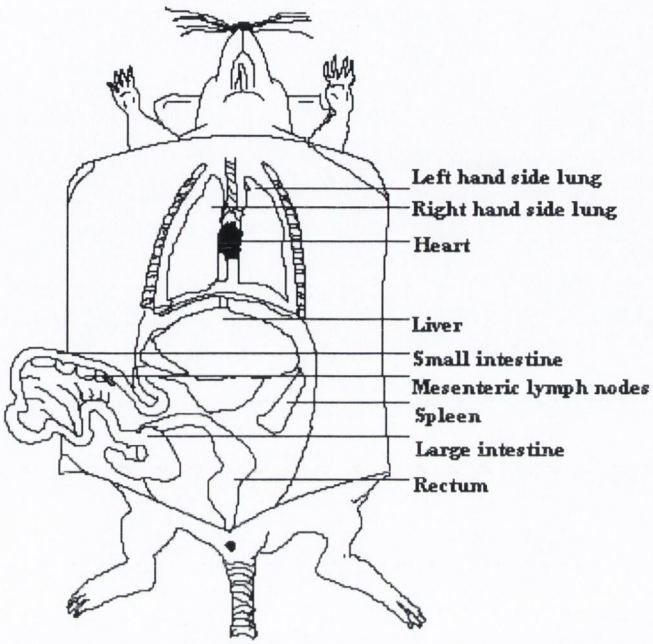


Figure 2.2.3: The location of organs for post-mortem procedures (original figure).

THE MODIFIED BAERMANN METHOD *(adapted from Eriksen, 1981)*

Using a fine scissors, the lungs and liver were macerated in the glass vials in preparation for the Baermann process. The modified Baermann apparatus was constructed using a mesh which was made by a double layer of gauze (sterile absorbent type 13 light gauze [Boots Pharmacy]) fixed with a rubber band (size 10) to the bottom of a plastic beaker (20ml universal sample tubes) from which the base was removed. This gauze-tube apparatus was suspended in a 50 ml centrifuge tube with the gauzed end inserted into the tube (the sample tube should reach down to the 35 ml mark on the centrifuge tube)*. 0.9% saline was added to this apparatus up to the 35 ml mark*. The centrifuge tube apparatus was suspended up to the 10 ml mark in a water bath at 37.5°C, overnight prior to post-mortem*. On the day of post-mortem the liver and lungs were macerated using fine scissors. The macerated organ was placed on top of the gauze net and the vials that had held the macerated organs were rinsed with 0.9% warm saline, which was then passed through the gauze to ensure the sample was completely submerged and all macerated tissue was collected. The centrifuge-tube apparatus was capped to prevent loss of liquid (**fig.**

2.2.4). This apparatus was kept at a constant 37.5°C in the water bath for 24 hours* to encourage the larvae to migrate from the colder tissue into the warmer saline.

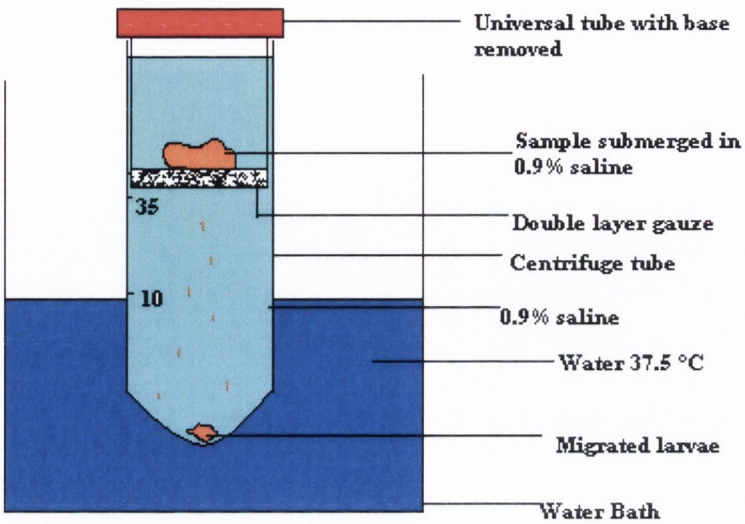


Figure 2.2.4: Modified Baermann apparatus (original figure).

RECOVERY OF WORMS

After overnight incubation the beaker-gauze apparatus was removed from the centrifuge tubes and the tubes capped*. These were then centrifuged (Hettich Universal 32) for 5 minutes at 3000 rpm (1000g)*. The centrifuge was allowed to stop after each cycle without mechanical braking (Johnstone *et al.,* 1978). After centrifugation, the solution (supernatant) was extracted carefully, so as not to disturb the pellet, using an aspirator, until 10 ml of solution with the pellet beneath remained. 10 ml of 6% formalin was added and the solution was allowed to settle for 24 hours at room temperature. This was then re-centrifuged for 5 minutes at 3000 rpm and reduced to 4 ml*.

*Adaptations to the modified Baermann method (Eriksen, 1981)

In the case of lungs (but not livers), the organ remaining in the gauze mesh was placed in 50 ml pepsin after the Baermann process (0.25% in 0.1 N HCl 34 mM NaCl) overnight at 38°C and non-migrating (or trapped) larvae numbers were determined (Mitchell *et al.,* 1976). It was believed that the large size of larvae at 7 days may impede their passage through filter material (Johnstone *et al.,* 1978). In was suggested that non-migrating larvae accounted for about 10% of the total larval burden (Mitchell *et al.,* 1976). After the digestion process these samples were treated as outlined above with centrifugation and preservation in formalin. This digest process was not repeated in subsequent examinations.

LARVAL COUNTS

The remaining 4 ml solution and pellet in the centrifuge tube was agitated using a vortex mixer (Vortex Genie 2). Five, 200µl samples were then placed on a slide and the number of larvae were counted with a phase contrast stereomicroscope (Olympus BX-41) at x40 magnification. The sum of the larvae counted in the 5 aliquots was then calculated using the appropriate calculations.

STATISTICAL ANALYSIS

Larval recovery was assessed for normality visually and statistically. Values for means, standard deviations, ranges, skewness, kurtosis and standard errors were obtained for each sub-set of data. The influence of the various factors on larval burden and size were analysed by means of analysis of variance (ANOVA in SPSS 12.0.1), using 1- 2- or 3-way ANOVAs depending on the number of explanatory factors and covariates involved. Full-factorial models were simplified by step-wise deletion of non significant terms beginning with the 3-way interactions, and minimum sufficient models in which only significant terms remained, were used to assess significance of fitted factors. In some cases least squared difference (LSD) post-hoc tests were applied to tease out the major sources of variation within factors. A paired t-test examined the changes in weight between the pre- and post-infection period. Statistical analysis was carried out at a confidence limit of 95% ($\alpha = 0.05$).

2.3 RESULTS

A COMPARISON OF THE TWO STRAINS OF MICE UNDER INVESTIGATION, C57BL/6j AND BALB/c, ON DAYS 3-9 POST-INOCULATION

Larval counts in the liver changed significantly with day in both mouse strains (**fig. 2.3.1**; 2-way ANOVA with day and strain as factors, model R^2=0.75, main effect of day $F_{6,54}$=22.0, P<0.0001), and the strains differed significantly in their larval burdens (main effect of strain $F_{1,54}$=4.5, P=0.038 and 2-way interaction strain * time $F_{6,54}$=2.3, P=0.05), with larvae moving into the livers of C57BL/6j at a faster rate than in BALB/c. This was particularly evident by the larger worm burdens on day 3 p.i., although by day 4 p.i. the mean larval burdens were similar in both strains. After this, worm burdens stabilised in BALB/c mice but continued to rise in C57BL/6j mice until day 5, after which there was a steady decline to zero at day 9 p.i. in both strains (**fig. 2.3.1**). The highest percentage recovery of the initial inoculum from the liver was 42.2% in C57BL/6j on day 5 and 24.4% in BALB/c on day 4 (**table 2.3.3**).

The reduction in larval numbers in the liver coincided with the movement of larvae into the lungs on days 5-6 p.i. in both strains, and again this was reflected in significant changes in worm burdens with time (**fig. 2.3.1**; 2-way ANOVA with strain and day as factors on lung worm burdens, model R^2=0.74, main effect of day $F_{6,54}$=9.0, P<0.0001). Overall, in both strains, there was a reduction in the number of larvae that successfully migrated to this organ in comparison to the numbers observed in the liver. By day 7 p.i., in C57BL/6j, the majority of larvae were found in the lungs (**table 2.3.3**; 14.7% of the inoculated dose). However, in respect of lung worm burdens there was a more marked difference between strains compared with liver counts (main effect of strain, $F_{1,54}$=15.8, P=0.0002 and 2-way interaction strain*day, $F_{6,54}$=3.58, P=0.005). The greatest difference in the mean number of larvae recovered between the two strains was observed in the lungs (≥67.4%) on days 6 and 7 p.i.. This is in contrast to the liver, which although displaying higher larval numbers showed less of a percentage difference in worm burdens on the days of peak recovery (=43.1%).

Table 2.3.1: Mean number of larvae (± S.E.M.) recovered from the organs of C57BL/6j mice on each post-mortem day and the number of larvae recovered in total.

Day	n	Liver	Left lung	Right lung	Lung total	Total
3	5	83 ±23.8	0.8 ±0.8	1.6 ±1.6	2.4 ±2.4	**85.4 ±24**
4	5	124 ±17.3	0 ±0	4.8 ±1.5	4.8 ±1.5	**128.8 ±17.3**
5	5	211 ±37.4	6.4 ±2.7	13.6 ±4.8	20 ±7.5	**231 ±43.6**
6	5	40 ±9.1	20 ±5.9	52 ±13.3	72 ±18.1	**112 ±25.5**
7	5	5 ±2.2	28.8 ±6.9	44.8 ±12.2	73.6 ±18.5	**78.6 ±19.6**
8	5	3 ±1.2	16 ±5.2	32.8 ±8.5	48.8 ±12.6	**51.8 ±13.5**
9	4	0 ±0	11 ±1.9	18 ±7.4	29 ±7.6	**29 ±7.6**

Table 2.3.2: Mean number of larvae (± S.E.M.) recovered from the organs of BALB/c mice on each post-mortem day and the number of larvae recovered in total.

Day	n	Liver	Left lung	Right lung	Lung total	Total
3	5	11 ±9.8	0 ±0	1.6 ±1.6	1.6 ±1.6	**12.6 ±9.5**
4	5	122 ±28.4	1.6 ±1	4.8 ±3.9	6.4 ±3.5	**128.4 ±30**
5	4	120 ±44.1	4 ±2.3	7 ±4.4	11 ±6.2	**131 ±49.8**
6	5	54 ±19.7	8.8 ±3.2	6.4 ±1.6	15.2 ±2.7	**69.2 ±20.9**
7	5	5 ±3.9	9.6 ±2.7	14.4 ±5.2	24 ±6.7	**29 ±6.9**
8	5	0 ±0	13.6 ±4.8	19.2 ±2.7	32.8 ±5.9	**32.8 ±5.9**
9	5	1 ±1	8 ±1.3	16.8 ±3.9	24.8 ±4.3	**25.8 ±5**

Table 2.3.3: Percentage of *A. suum* larvae recovered from the original inoculum from each strain on each post-mortem day

Strain	Days p.i.	n	Liver	Left lung	Right lung	Lung total	Total
C57BL/6	3	**5**	16.6	0.16	0.32	0.48	**17.1**
	4	**5**	24.8	0.00	0.96	0.96	**25.8**
	5	**5**	42.2	1.28	2.72	4	**46.2**
	6	**5**	8	4.00	10.4	14.4	**22.4**
	7	**5**	1	5.76	8.96	14.7	**15.7**
	8	**5**	0.6	3.20	6.56	9.8	**10.4**
	9	**4**	0	2.20	3.6	5.8	**5.8**
BALB/c	3	**5**	2.2	0.00	0.32	0.32	**2.5**
	4	**5**	24.4	0.32	0.96	1.3	**25.7**
	5	**4**	24	0.80	1.4	2.2	**26.2**
	6	**5**	10.8	1.76	1.28	3.04	**13.8**
	7	**5**	1	1.92	2.88	4.8	**5.8**
	8	**5**	0	2.72	3.84	6.6	**6.6**
	9	**5**	0.2	1.60	3.36	5.0	**5.2**

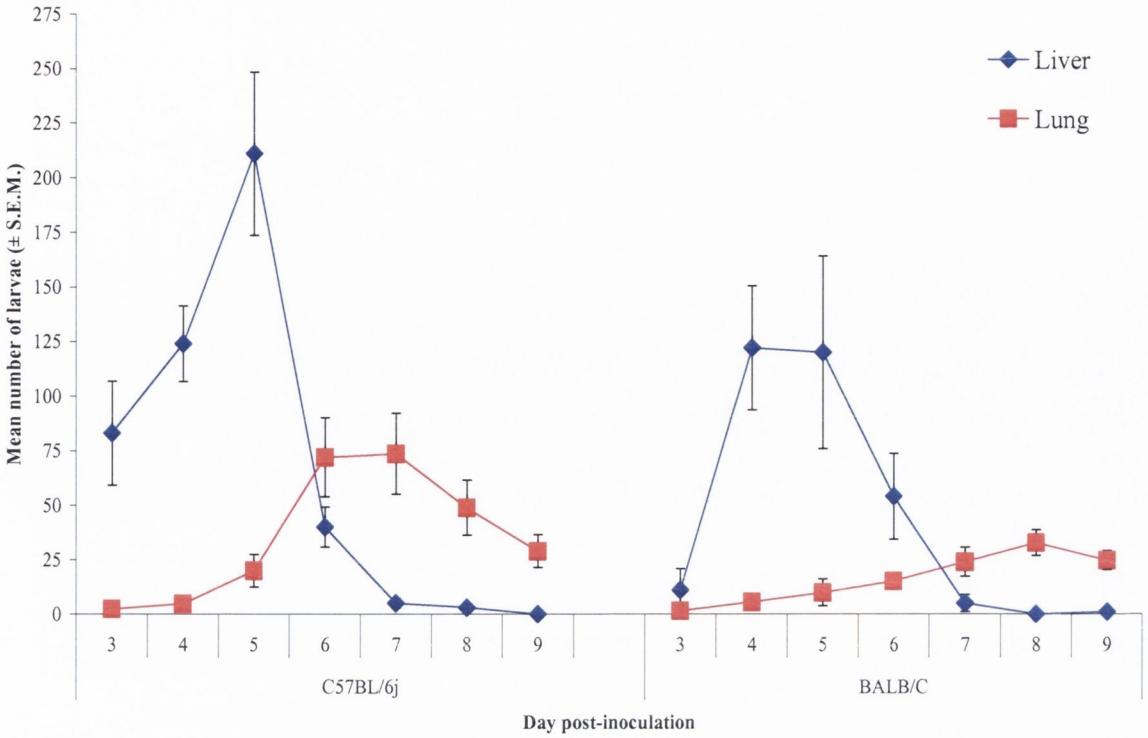


Figure 2.3.1: Mean number of larvae (±S.E.M.) in the liver and lung (total) on days 3-9 p.i. in the mouse strains C57BL/6j and BALB/c.

THE DISTRIBUTION OF *A. SUUM* LARVAE IN THE TISSUES OF C57BL/6j MICE OBSERVED DAILY FROM DAYS 3-9 POST-INOCULATION

Larvae were recovered from the liver, and the left and right hand side lung to determine the pattern of migration and the differences in larval burdens between two strains of mice during the infection period. Larvae were evident in both the liver and lungs of C57BL/6j mice on day 3. On day 3 p.i., C57BL/6j mice were observed to have high numbers of larvae in the liver. There was continuous movement of larvae into the liver up to and including day 5 (**fig. 2.3.1**). The highest larval burden was recorded on day 5 p.i. after which there was a significant reduction in the numbers of migrating larvae recovered on day 6 (**table 2.3.1**) until a gradual descent towards zero on days 7-9 (**fig. 2.3.1**). The decline of larvae in the liver corresponded to a significant increase in the number of larvae observed in the lungs between day 5 and 6 (**fig. 2.3.1**). Analysis of this data showed that the organ (liver and lungs) of recovery was a highly significant factor in determining larval burdens (2-way ANOVA with day and organ as explanatory factors, model R^2_{adj}=0.72, main effect of organ $F_{1,54}$=13.2, P=0.0006) with the liver harbouring more worms early in infection and the lungs on the latter days. Larval burdens also differed significantly with

day (main effect of day $F_{6,54}$=8.6, P≤0.0001). The sigmificant interaction between these two factors suggested that larvae moved into the organs at different rates (**fig. 2.3.1**; interaction between day*organ $F_{6,54}$=20.1, P≤0.0001).

The number of larvae successfully migrating into the lungs never attained the same levels as those penetrating the liver (**fig. 2.3.1**). The number of larvae recovered from the liver had the largest impact on the overall number of larvae recovered during early stage infection (days 3-5). In contrast, the lungs influenced the overall burden of larvae during the later stages of infection (**table 2.3.1**). An interesting observation was made by considering the number of larvae reaching the left and right hand side of the lung separately. Consistently more larvae were recovered from the right hand lung on all post-mortem days than from the left hand side (**table 2.3.1**; 2-way ANOVA with day and side of organ (left and right hand lung) as explanatory factors, model R^2_{adj}=0.51, main effect of organ $F_{1,54}$=11.2, P=0.0013). Furthermore, larvae accumulated at different rates in the two sides of the lung (main effect of day $F_{6,54}$=10.3, P≤0.0001) however, there was no interaction between the two factors. The maximum larval accumulation was observed in the lungs on day 7 p.i. (73.6 ±18.5) and then there was a gradual decrease in the number of larvae (**fig. 2.3.1**). No larvae were observed in the lung tissue digests and thus trapped larvae did not impact on larval burdens.

THE DISTRIBUTION OF *A. SUUM* LARVAE IN THE TISSUES OF BALB/C MICE OBSERVED DAILY FROM DAYS 3-9 POST-INOCULATION

The pattern of migration of larvae in BALB/c mice was very similar to that of C57BL/6j mice, except that larvae did not accumulate to the same extent, during the liver and lung phases, in BALB/c. The accumulation was delayed in the liver of BALB/c mice with large numbers arriving in this organ on day 4 (**fig. 2.3.1**). However, by day 4 the number of larvae increased rapidly and there were equivalent larval burdens in the livers of both strains (**fig. 2.3.1**). The larval count remained high on day 5, although, the number of larvae recovered from the liver of BALB/c mice did not accumulate a maximum worm burden comparable to that of the strain C57BL/6j (**fig. 2.3.1**). Again, there was considerable loss of larvae from the liver on day 6 and a gradual descent to zero from days 7-9 and this corresponded to a steady increase in the lungs (**fig. 2.3.1**). The organ (liver and lungs) of recovery was a highly significant factor in determining larval burdens (2-way ANOVA with day and organ as factors, model R^2_{adj}=0.58, main effect of organ $F_{1,54}$=13.8,

P=0.0005) with consistently more larvae recovered from the liver on the early days of infection and then a shift in the organ of recovery on the latter days and larvae accrued in the lungs. Larval burden was significantly influenced by the day of recovery (main effect of day $F_{6,54}$=6.0, $P \leq 0.0001$) and the interaction between these two factors was highly significant (interaction between day*organ $F_{6,54}$=9.4, $P \leq 0.0001$) and this corresponded to the difference in accumulation of larvae during the infection period between the two organs.

The number of larvae observed in the lungs remained low throughout the experimental procedure in contrast to that of C57BL/6j mice (**table 2.3.2**). The accumulation of larvae in the lungs was very gradual and did not show a dramatic increase as observed in C57BL/6j. The maximum number of larvae observed in this organ was on day 8. On day 9 the number of larvae in the lungs declined (**fig. 2.3.1**). Larvae significantly preferentially migrated to the right hand side rather than the left hand side of the lung (**table 2.3.2**; 2-way ANOVA with day and side of organ as factors, model R^2_{adj}=0.34, main effect of organ $F_{1,54}$=4.4, P=0.040) and the day was highly significant factor ($F_{6,54}$=6.5, $P \leq 0.0001$).

CHANGES IN MOUSE BODY WEIGHT ASSOCIATED WITH LARVAL MIGRATION

Mouse body weight data showed that there was variation in weight between the two strains associated with time between the initial (pre-infection) weight on day 0 and the weight of mice at post-mortem (**appendix A.2**) with mouse groups consistently losing weight over the infection period but this was particularly evident in the more susceptible strain C57BL/6j with consistent weight loss on each post-mortem day (**fig. 2.3.2**). A paired t-test analysing the difference in weight pre- and post-infection revealed that there was a significant reduction between the pre-infection and post-mortem weight for C57BL/6j mice (t=4.2, df=66, P=0.0002) but this was insignificant for BALB/c mice (t=1.5, df=66, P=0.134) and this may be due in part to the large discrepancy between individual weight observed in this strain (**fig. 2.3.2**).

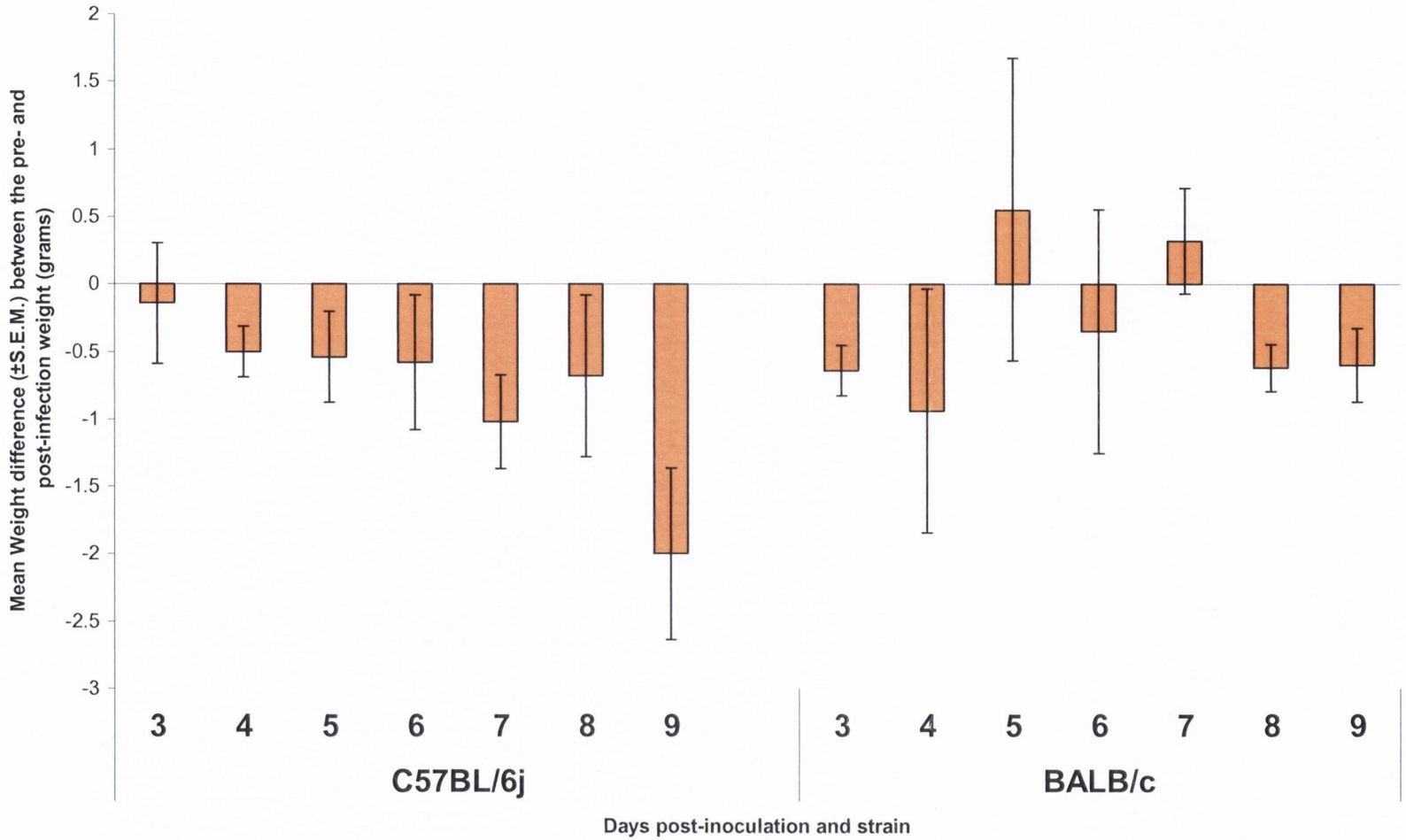


Figure 2.3.2: Mean body weight difference (grams) (±S.E.M.) between pre-infection (time zero) and the day of post-mortem in both the C57BL/6j and BALB/c mouse strains on each post-mortem day.

2.4 DISCUSSION

A proposed criterion for the investigation of susceptibility/resistance to the larval stages of *A. suum* was the examination of larval burdens in the lungs of mice on the latter days of infection (Crandall & Arean, 1965; Mitchell *et al.*, 1976; Johnstone *et al.*, 1978; Eriksen, 1981). However, the variation in larval burdens associated with mouse strains, experimental protocol and the day of observation suggested that a standardised mouse model was required that could be used with consistency and provide comparative results between experimental groups. For example, Mitchell *et al.* (1976) established that inbred strains of mice differ markedly in their susceptibility to early infection as measured by larval burden in the lung on day 7 post-infection. However, these authors noted a marked difference in the infectivity of different batches of eggs taken from female worms at abattoirs and thus it was difficult to compare between experimental groups in terms of relative susceptibility. In addition, Mitchell *et al.* (1976) inoculated mice with infective eggs of different dose size which was later shown to be an extrinsic factor that can affect the observed larval burden (Johnstone *et al.*, 1978). This group demonstrated a logarithmical increase in larval burdens, from 100 to 6000 administered eggs, but at higher doses the recovery rates were on a plateau of the dose/recovery curve in C57BL/6 mice (Johnstone *et al.*, 1978). Due to the susceptible nature of C57BL/6 mice this threshold may be lower than in resistant strains. Thus the administration of variable doses by Mitchell *et al.* (1976) between strains does not allow the strains to be directly compared. The present study confirmed that the two reference strains of inbred mice did differ significantly in the number of worms they harbour in their lungs on day 7, although the resistance of BALB/c was not as great as observed by Mitchell *et al.* (1976) (**table 2.4.1**). The observations of the current study demonstrated differences in the pattern of infection over time and variation in the peak numbers of lung larvae between the strains demonstrated the importance of observing larval migration over a number of days rather than contrasting larval burdens on a single post-mortem day. The improved methodology employed and the homogeneity of egg batches and inoculation doses resulted in consistent larval recoveries in the current study. Finally, the percentage of the initial inoculum recovered using a refined Baermann technique resulted in higher larval recoveries in the two reference strains in contrast to earlier published studies (**table 2.4.1**).

A number of extrinsic factors and intrinsic host factors were believed to impact on the pulmonary larval burdens of mice. Bindseil (1969a/b) suggested that host age influenced

the migration pattern of *A. suum* larvae and the observed patterns were highly variable in mice older than 6 weeks and based upon these observations he used 3-week old mice for *A. suum* studies. Similarly, Johnstone *et al.* (1978) evaluated C57BL/6 mice as a model for the study of resistance to infection because it was previously shown to be relatively susceptible to primary infection and immune to challenge (Mitchell *et al.*, 1976). The comparison of larval recovery methods (Baermann, tissue digest, and spin methods) by Johnstone *et al.* (1978) led to the conclusion that certain extrinsic parameters influenced the number of *A. suum* larvae recovered. These parameters were dose administered (more larvae were recovered as the dose of ova administered increased but at higher doses recovery rates plateau), organ of recovery (liver and lungs), and host age. Johnstone *et al.* (1978) in contrasting larval recoveries from 3 and 8 week old mice showed higher mean numbers of larvae from the older mice at each of a number of different dosages. This was believed to be the result of factors including physiological changes in maturing mice, passive immunity from the dam, and the crowding effect due to smaller body size. These two groups (Bindseil, 1969a/b; Johnstone *et al.*, 1978) showed opposing results and this may be due to an improved inoculation and recovery technique and also the use of inbred mouse strains in the latter study. This reflected the need to provide a consistent model that accounted for these issues and other possible extrinsic factors.

Table 2.4.1: A comparative examination of the successful recoveries of larvae (maximum burden recovered) in previous investigations and the current procedure using mice of a similar age and comparable recovery techniques.

	Mitchell *et al.* (1976)† C57BL/6	**Johnstone *et al.* (1978)‡ C57BL/6**	**Current investigation with C57BL/6j§**	**Mitchell *et al.* (1976)† BALB/c**	**Slotved *et al.* (1997b)* BALB/c**	**Current investigation with BALB/c§**
Number of larvae recovered	314, 145 and 116	92.7 and 224	73.6	7	106.7 and 58.4	32.8
Percentage of dose recovered	31.4% 14.5% and 0.73%	3.5% and 7.5%	14.7%	0.04%	4.3% and 2.3%	6.6%

† From three administered doses of 1000, 1000, and 16000 for C57BL/6j and 16000 for BALB/c
‡ From two doses of 3000 eggs
§ Administered dose of 500 embryonated eggs
* Quoted Figures from two recovery techniques: Agar gel method and Baermann method, and an administered dose of 2500

It was also apparent that the published methods for both inoculation of eggs and larval recovery were not always reliable. The reduction of the inconsistency caused by eggs was the first objective of the current research by providing eggs that were fully infective to experimental animals. Previous investigation showed that temperature influenced the vitality and infectivity of *A. suum* eggs and those eggs that developed at inadequate temperatures were less infective than their counterparts matured at ideal temperatures (Stevenson, 1979). Although higher temperatures (e.g. 31.1°C, (Stevenson, 1979)) may give the maximum rate of development, these larvae may have a poor performance when inoculated into the host. Similar effects are observed at lower temperatures (Cram, 1924). Furthermore, Eriksen (1981) demonstrated that the infectivity of a batch of *A. suum* eggs changed during storage. The infectivity shown after 2 months incubation increased after 4 months incubation. In addition, Eriksen (1990) observed that the rate of development to infective stage in eggs was very slow when the egg concentration was high in a given suspension. Egg concentrations of <25 egg/µl were the optimum concentration for embryonation. This has implications for past results as they may have had varying egg concentrations which inhibited egg development. Some batches may have had under-developed eggs which were not infective or eggs that had not stabilised in terms of their potential infectivity. Earlier studies provided mice with doses as high as 100 000 eggs, often this was administered in food and the recovery rates were as low as 0.3% (Jenkins, 1968). The poor recovery from these earlier studies raised questions in relation to their reliability.

Another potential source of variation that was thought to contribute to undeveloped eggs was based on whether those eggs harvested directly from worm uteri exhibit a normal course of embryonation and achieve full infectivity. It was believed that such eggs were not fully developed with regard to the outer composition of the outer albuminous shell layer and possibly other characteristics acquired during normal oviposition and subsequent passage trough the intestinal tract of the host. Oksanen *et al.* (1990) concluded that eggs isolated from worm uteri and subsequently embryonated in 0.1 N H_2SO_4 were fully comparable with normal eggs. To ensure that these batches were comparable this group used eggs known to be from the same females, i.e. the eggs had originated from the same worms either normally passed through faeces or dissected from worms. Therefore it was concluded that eggs dissected from worms was an acceptable source of *A. suum* eggs. Further to this, there was a question regarding the infectivity of eggs obtained from worms expelled by anthelmintic treatment. It was concluded that the ovicidal activity of

albendazole *in vivo* inhibits subsequent development of *A. suum* eggs *in vitro*, and therefore albendazole was not suitable to obtain worms for egg embryonation to produce experimental inoculums (Boes *et al.*, 1998a). Again this has implications for studies that may have utilised eggs obtained from worms expelled by such anthelmintic treatment.

To control for the possible variation caused by egg batches experienced by other research groups, the following aspects relate to the eggs used in the present investigation. The eggs administered came from "an isolate" of *A. suum* removed from pigs at a Danish slaughterhouse, therefore no anthelmintics were employed. The eggs were removed from the cranial 2-4 cm of the uterus and stored in 0.1 N H_2SO_4 at a concentration of <25egg/µl at room temperature (Eriksen, personal communication). This batch of eggs was employed in all subsequent experiments to ensure that the egg batch did not account for the observed variation in larval burdens between mouse strains or experimental groups. The eggs had been stored for a period of approximately one year by which time they would have been fully infective (Eriksen, 1981). Furthermore, inoculation doses were administered by gavage directly into the stomach and this eliminated the uncertainty in infective dose experienced by earlier studies when dosages were fed via food to the mice. To ensure the consistency of the administered inoculation dose, it was considered prudent to examine a number of doses to guarantee the uniformity of the doses dispensed to each individual mouse (Slotved *et al.*, 1997a). The results of this demonstrated that the inoculation dose was not a factor with the mean administered dose (± S.E.) being 493.5 (± 3.8) eggs per dose (**appendix A.1**). Thus the observed variation between the strains was not likely to be the result of heterogeneity in the administered dose of infective eggs but was likely to be the result of inherent differences between the inbred mouse strains although other extrinsic factors will be examined such as strain and the size of the initial inoculum before this can be determined conclusively.

The method of recovery of larvae from host tissues can also result in large variations in mean larval burden. The most frequently used method for larval recovery was the modified Baermann method of Eriksn (1981). However there was no standard protocol described for this method that provided consistency. Johnstone *et al.* (1978) compared three methods of recovery and determined that the centrifuge method (spin method) was superior for recovering tissue larvae compared to both the Baermann method and the tissue digest method. This group believed that the large size of larvae on day 7 p.i. impeded their passage through filter material; thereby resulting in the lower larval numbers observed

using the Baermann method. A number of recent studies compared the existing modified Baermann method (Eriksen, 1981) with an agar-gel technique for larval recovery from pigs (Slotved *et al.*, 1996; Murrell *et al.*, 1997b; Slotved *et al.*, 1997b) and mice (Slotved *et al.*, 1997a). The methods of macerating the tissue prior to recovery were also examined (Slotved *et al.*, 1997a). These authors suggested that the Baermann method results in large variation due to the mincing of the tissue. They found that the agar gel method which involved incubation at 37 °C yielded significantly higher results than the Baermann method for mice (**table 2.5.1**). Although this was not the case for pig tissue, it did reduce the sample counting time by a factor of 5-10 compared to the Baermann method for porcine hepatic tissue but there was no change in pulmonary counts. The agar method was preferred particularly for use with intestinal tissue of pigs as larvae were easy to see among the tissue debris.

However, the Baermann method was chosen for a number of reasons in the current investigation. Firstly, the method can be used to handle large numbers of tissues simultaneously. Secondly, it recovers viable larvae, unlike the tissue digest and the spin methods which included a tissue digest. Therefore the Baermann method does not include larvae that were immobilised or killed during migration as only the number of successfully migrating larvae were of importance in terms of susceptibility. Finally, it was an easy and inexpensive method that gave consistent results. The method of larval recovery described within was a refined Baermann method and drew on combined aspects from the modified Baermann technique (Eriksen, 1981), the agar-gel method (Slotved *et al.*, 1997a/b), and the spin method (Johnstone *et al.*, 1978) with some features from previous personal experience. This refined Baermann technique involved an incubation period of 24 hours in a water bath at 37 °C and also a centrifugal spin. To ensure larvae were capable of passing through the gauze material an increased pore size was used. This method, as described in the materials and methods section, proved to be a valuable method that gave comparable larval recoveries to those observed by Slotved *et al.* (1997a) using the agar-gel method for recovering larvae from pulmonary tissue.. Slotved *et al.* (1997a) used the strain BALB/c and using the Baermann method and the agar gel method achieved recoveries of 2.3% and 4.3%, respectively. The modified Baermann technique employed in this chapter with BALB/c resulted in an increased number of larvae recovered from the initial inoculum of 6.6% (**table 2.4.1**) on day 8 p.i. (although these have not been compared directly). It is significant that the results of the current study are analogous to that of Slotved *et al.* (1997a) using BALB/c mice. Both these studies revealed that BALB/c were not as resistant

as suggested by Mitchell *et al.* (1976). The proposal that up to 10% of the larvae in the tissue were trapped in the lung tissue or could not pass through the gauze was also examined (Mitchell *et al.*, 1976; Johnstone *et al.*, 1978). The results of a tissue digest on the remains of the sample after Baermannisation found no larvae in the tissues suggesting that all larvae capable of migrating had passed through the gauze material.

Previous investigations observed that the majority of the larvae capable of migrating were recorded in the liver on day 4/5 p.i. and there was a shift in the organ of highest recovery to the lungs on day 7 p.i. (Sprent & Chen, 1948; Sprent, 1962; Douvres & Tromba, 1971; Eriksen, 1981; Prokopič & Figallová, 1982a; Wade *et al.*, 1987; Fagerholm *et al.*, 2000). The results of the current experiment corroborated this as the maximum accumulation of larvae was on day 5 in the liver of C57BL/6j and day 4/5 in BALB/c mice (**fig. 2.3.1**). Day 6 corresponded to a shift in the location of larvae with fewer larvae recovered in the liver and a surge of larvae into the lungs. On day 7 there was an accumulation of larvae in the lungs of C57BL/6j (**fig. 2.3.1**). This maximum accumulation of larvae is not observed until day 8 p.i. in BALB/c mice. The slower movement of larvae into the lungs of BALB/c mice highlights the importance of observing larval migration over a period of times and not at one time point. It is recommended that larval migration is observed on days 6 and 8 as well as day 7, to ensure that peaks of accumulation either side of day 7 are not missed when comparing new strains of mice.

A variety of reasons have been proposed for variations in kinetics of migration between mice of different strains that are sex-matched and of comparable age. There are three possible alternatives for the observed differences in the pattern of migration. One suggestion for a delayed pattern in reaching the liver may represent the time taken by larvae to locate a suitable venule or lacteal, which is probably dependent on chance, to transport them to this organ (Jenkins, 1968). Another alternative was that larvae are continuing to come from other sites into the lungs at later time points or the possibility that larvae were migrating rapidly in one strain over another (Mitchell *et al.*, 1976). Finally, the possibility exists that there are delays in larvae reaching the various sites, due to the probability that there may be different routes available and hence the variation in peak days between the strains (Murrell *et al.*, 1997a). These are all sufficient explanations to describe the differences in the day of accumulation between the mouse strains, however, to date these factors have not been explored.

The analysis of the liver and lungs was also utilised to determine the stages at which larvae were eliminated from the host and also the point at which relative susceptibilities were most apparent. Taffs (1968) considered the liver to be an important site for the destruction of migrating larvae. Mitchell *et al.* (1976) suggested that resistance by challenge inoculation or relatively resistant strains of mice did not appear to be effected at the level of entry of larvae through the intestinal wall or migration to the liver but rather during the period in residence in the liver or *en route* to the lungs. Studies of the time course showed that the period of residence in the liver or migration to the lungs were the stages in the life cycle in which larvae were eliminated by the host's responses (Sprent, 1962; Douvres & Tromba, 1971; Eriksen, 1981). However these studies often experienced large degrees of inconsistency in the number of larvae recovered in these organs due to the heterogeneity in the infectivity of batches of eggs and therefore the results could only be compared between groups within each experiment. The results of this chapter confirmed these observations as the larval numbers continually decreased at each phase of migration but the greatest reduction in those larvae that successfully migrated appears to be between the liver and the lungs. The majority of the initial administered inoculum was recovered in the liver of both strains and this percentage of the administered inoculum recovered was greatly reduced in the lungs of both strains (**table 2.3.3**). This suggested that relative susceptibility to this parasite is best evaluated by larval lung burdens. Many eggs and larvae may be lost prior to migration from the intestine, it has been proposed that this may not be a host feature but failure in relation to the juvenile worms to hatch or successfully locate the appropriate tissue for migration (Slotved *et al.*, 1998). Prokopič & Figallová (1982) observed that during the first 24 hours, as many as 277 eggs could be detected in one gram of faeces from an initial inoculum of 2000 *A. suum* eggs. Crompton & Pawlowski (1985) further suggested that some loss of larvae may be attributable to a failure of migrating worms to locate the organs through which they normally migrate. However, these may be losses resulting from intrinsic parasite features rather than differences in susceptibility of the infected hosts.

The continued movement of larvae into the liver up until day 5 raises questions about the location of the larvae prior to this. Early migration of *A. suum* during the first 12 hour after egg inoculation has previously been reported in mice (Slotved *et al.*, 1998). This group found that the number of larvae decreased to a mean of 1 by 12 hours p.i. in the large intestine and that by this time a majority of the larvae could be recovered in the liver, although this was only 15% of the inoculated dose. The majority of larvae not recovered at

this time point may be accounted for by those remaining in the intestine, those still migrating between the intestinal wall and the liver and the lungs and the number of eggs that passed through the gastrointestinal tract (Slotved *et al.*, 1998). However, the rapid movement of larvae from the GI tract and the long delay in larvae accumulating in the liver has not yet been explained by evidence of migration to other organs or larvae accumulating in tissues other than those they normally reside in.

An unexpected observation was the significant differences observed between the right and left hand side lung in both strains. The right lung had consistently more larvae on all post-mortem days. Soutiere *et al.* (2004) examined structural differences in alveolar size among inbred strains of mice which had previously been identified to have other physiological differences in lung-pressure volumes. They assessed the relative size or number of alveoli in three strains; C3H/HeJ, C57BL/6j and A/J and related this to lung volume differences. In this group of mice there were significant differences in mean chord length of alveoli with C57BL/6j containing the smallest. So there appears to be a significant genetic difference in lung structure among different mouse strains. A similar approach may be applied to investigate the differences in the larval burdens between the left and right hand side lung.

There has been no definitive description of a model for the investigation of the factors resulting in predisposition to early *A. suum* infection using mice of varying phenotype in relation to their susceptibility to larval *A. suum*. This investigation gives confidence that the developed experimental procedures can yield consistent pulmonary larval *Ascaris* burdens between two inbred strains of mice. The natural progression of these results was to explore the variation in larval burden between strains and to examine the extrinsic factors proposed by Johnstone *et al.* (1978) that may affect the observed larval burdens. This model system will further enable exploration of the intrinsic mechanisms underlying the observed susceptibility and resistance to early *Ascaris* infection, an aspect of the host-parasite relationship that has proved very difficult to investigate in human and porcine hosts for financial, logistical and ethical reasons.

CHAPTER 3

INVESTIGATION OF INBRED MOUSE STRAINS AND THE PHENOTYPIC PRESENTATION OF SUSCEPTIBILITY TO THE PARASITE *ASCARIS SUUM*

3.1 INTRODUCTION

Studies have shown that host genetics is an important determinant of the intensity of infection and morbidity caused by human helminths (Behnke *et al.*, 2003). *Ascaris* show an aggregated distribution in their hosts, and although recent studies have suggested an underlying genetic basis to this phenomenon in humans (Williams-Blangero *et al.*, 2002). These may be expressed as behavioural, immunological and physiological factors which play key roles in causing predisposition (McSharry *et al.*, 1999; Holland & Boes, 2002). The mechanistic basis of predisposition is not understood, but it may be initiated during the early phase of infection and could represent a disparity in the immune processes between individuals with varying genotypes or innate host defences during the migratory stage.

In the intestine there are a range of gene products that regulate the response to parasitic infection and differences between hosts responses (fast or strong versus slow or weak) could be explained by allelic variation in crucial genes associated with the gene cascade that accompanies the immune response and/or genes encoding constitutively expressed receptor/signalling molecules (Behnke *et al.*, 2003). MHC genes have been recognised for some time as having a crucial role in controlling the immune response (Kennedy, 1989), and there is evidence that the non-MHC genes are equally, if not more, important (Behnke *et al.*, 2003). These genes do not necessarily correspond to the genes involved in susceptibility to migrating larvae. The host response to larvae may determine the intensity of the potential adult worm burdens. Immunological evidence suggests that the host response to migrating *Ascaris* can be detrimental to the development of a patent infection or to the resident adult worms (Jungersen *et al.*, 1999a; Miquel *et al.*, 2005).

The starting point for studies of host genetics is the observation of phenotypic differences between individuals. This is not possible for the early stage infection with *Ascaris* in humans and is time-consuming when investigated in pigs and thus the mouse has been proposed as a suitable candidate, as inbred strains are known to vary in their susceptibility to infection during this period (Mitchell *et al.*, 1976). The aim of this procedure was to examine the relative susceptibilities of a range of inbred mouse strains commonly employed in laboratories, to gastrointestinal helminths. By ensuring that the conditions of exposure in individuals are similar, controlling intrinsic factors such as host age and sex and that the extrinsic factors are also kept constant, then differences in susceptibility can be attributed to inherent differences between the mouse genotypes.

3.2 MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The early pattern of migration in the lungs (days 6-9 p.i.) of nine strains of mice inoculated with 500 *A. suum* ova (see chapter 2 for inoculation procedure) was investigated. Male mice were purchased from Harlan, UK at 7 weeks of age and were 8 weeks old upon commencement of the initial inoculations. One hundred and eighty inbred mice (20 each of; A/J, BALB/c, C57BL/6j, C3H/HeN, CBA/Ca, DBA/2, NIH, SJL and SWR) were purchased for this procedure (**fig. 3.2.1**). This panel of mouse strains was chosen, incorporating some of those used by earlier workers but focusing particularly on the strains that have been highlighted for the mouse genome projects. Mice were housed and maintained in conditions referred to in chapter 2.

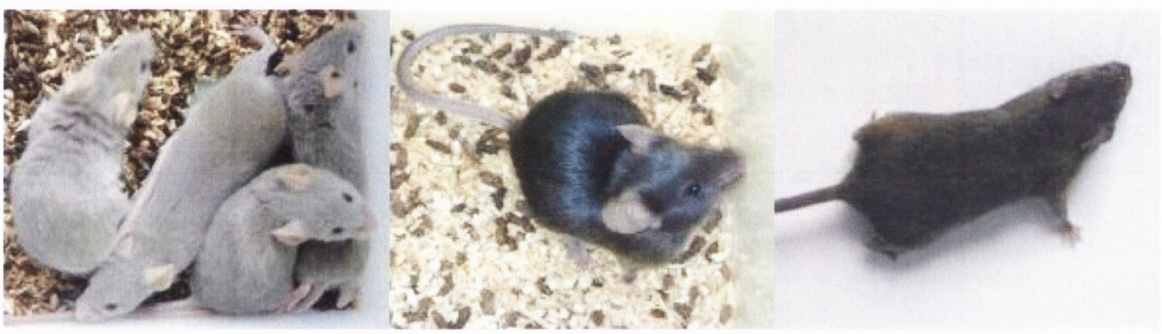


Figure 3.2.1: Three inbred mouse strains employed for this experimental procedure (DBA/2, C57BL/6j and CBA/Ca)

POST-MORTEM PROCEDURES

Groups of 5 mice from each strain were euthanased at 6, 7, 8 and 9 days p.i. by cervical dislocation and their bodies immediately processed. These days have been identified as the days within which larval migration in the lungs reach their peak (see chapter 2, Mitchell *et al.*, 1976; Johnstone *et al.*, 1978). Only the lungs (divided into the left and right hand side) were removed for examination of larval burdens. The spleen was weighed (**appendix A.5**). The post-mortem procedure, the recorded information on mouse weight and other experimental data, and the recovery process were performed on all samples as described in the previous chapter (see chapter 2). The liver was examined *in situ* for macroscopic lesions or discolouration. The liver was removed whole, weighed (**appendix A.5**) and then discarded.

STATISTICAL ANALYSIS

All statistical analysis was performed using two pc based statistical packages, SPSS® 12.0.1 and Datadesk® 6.0. Graphical summaries were designed in Microsoft® Excel©. Larval recovery was assessed for normality. Similar values relating to mean etc. were obtained as in chapter 2. The influence of time, strain and other relevant factors on larval burden was analysed by means of ANOVA, using 1- 2- or 3-way ANOVAs. In some cases least squared difference (LSD) post-hoc tests were applied. Statistical analysis was carried out at a confidence limit of 95% ($\alpha = 0.05$).

3.3 RESULTS

GENERAL OBSERVATIONS

The design of the experimental procedure was to determine the phenotypic variation in larval accumulation in the lungs of mice associated with strain and to identify for future work, two strains that show the greatest contrast of resistance/susceptible phenotype. The results show that for the reference strain C57BL/6j, the mean number of larvae recovered and the migratory kinetics in the lungs on the four days examined were indistinguishable from those observed in chapter 2. Across all strains, worm burdens in the lungs changed significantly with day (**fig. 3.3.1**; 2-way ANOVA with day and strain as explanatory factors, model R^2adj=0.61, main effect of day, $F_{3,140}$=14.2, $P \leq 0.0001$). As can be seen in **fig. 3.3.1** the strains differed markedly in lung worm burdens throughout the period after infection (main effect of strain, $F_{8,140}$=17.95, $P \leq 0.0001$) but in general the pattern of changes in worm burdens with time did not differ significantly between strains (2-way interaction strain*day $F_{24,140}$=1.5, P=N.S.), indicating consistency in the daily changes in worm burdens across mouse strains.

Post-hoc analysis identified those strains that differed significantly from each other. Thus, C57BL/6j differed significantly from all strains ($P \leq 0.0001$) on day 7 p.i. and was classified as the most susceptible strain. NIH, SJL and BALB/c mice, in which mean larval recoveries on day 7 ranged from 6-8%, were significantly different from the 6 other strains, but not each other and were classified as intermediate susceptible strains (**table 3.3.1**). BALB/c mice did not differentiate as distinctly as the other two strains from the remaining groups. This was associated with the early movement, on day 6, of larvae into the lungs of this strain and the rapid descent to zero thereafter. Finally A/J, C3H/HeN, CBA/Ca, DBA/2 and SWR, with worm burdens of less than 4%, were not significantly different from each other but were significantly different to the other two groupings of intermediate susceptible and most susceptible (C57BL/6j), and were classified as resistant strains.

The greatest significant difference between strains (1-way ANOVA, confined to day 7 pi, model R^2=0.61, effect of strain, $F_{8,34}$=6.8, $P \leq 0.0001$) was observed on day 7 post-inoculation. This was also the day on which larvae accumulated to their maximum number in the lungs of the susceptible C57BL/6j mouse strain and the day on which this strain differed most markedly from the other strains. In four of the other strains, A/J, SJL,

C3H/HeN and CBA/Ca worm burdens were also maximum on day 7 pi, but not in DBA/2, SWR, NIH and BALB/c mice. However, while in the latter strains worm recoveries were higher on day 6, the difference between day 6 and day 7 counts was marginal.

Table 3.3.1: Results of a *post-hoc* least significant difference (LSD) test between the strains following the two-way ANOVA for the number of larvae recovered in the lungs at post-mortem for all strains (only significant results are shown).

Strain	Strain	Probability	Strain	Strain	probability
C57BL/6j-	NIH	0.0002	**NIH-**	A/J	≤0.0001
	SJL	≤0.0001		C3H/HeN	≤0.0001
	BALB/c	≤0.0001		CBA/Ca	≤0.0001
	SWR	≤0.0001		DBA/2	≤0.0001
	A/J	≤0.0001		SWR	≤0.0001
	C3H/HeN	≤0.0001		A/J	0.0013
	CBA/Ca	≤0.0001	**SJL-**	C3H/HeN	0.0168
	DBA/2	≤0.0001		CBA/Ca	0.0015
BALB/c-	A/J	0.0070		DBA/2	0.0061
	CBA/Ca	0.0081		SWR	0.0407
	DBA/2	0.0263			

The most marked overall difference was detected between CBA/Ca and C57BL/6j (*post-hoc* test, P= 0.000014), which despite the large difference in numbers of larvae recovered on each day, showed the same pattern of worm migration during the 4-day period (**fig. 3.3.2**). The percentage difference in the mean number of larvae recovered from these two strains on the four days was also highly consistent ranging between 69-81%. These recoveries contrasted with the next most resistant strain C3H/HeN, the recovery of larvae from this strain was also consistent but with a lower percentage difference relative to C57BL/6j mice, 59.5%-70.2%. It must be noted that although DBA/2 mice appeared to be most resistant on day 7 p.i., accumulation of larvae occurred in the lungs on day 6 in this strain (**fig. 3.3.1**). While A/J mice appeared to be highly resistant overall, there was a great deal of variation among individuals of this strain with a range of values on the day of peak recovery, from 0-40 larvae, contrasting with CBA/Ca mice where the range of individual burdens was considerably more restricted (12-28 larvae).

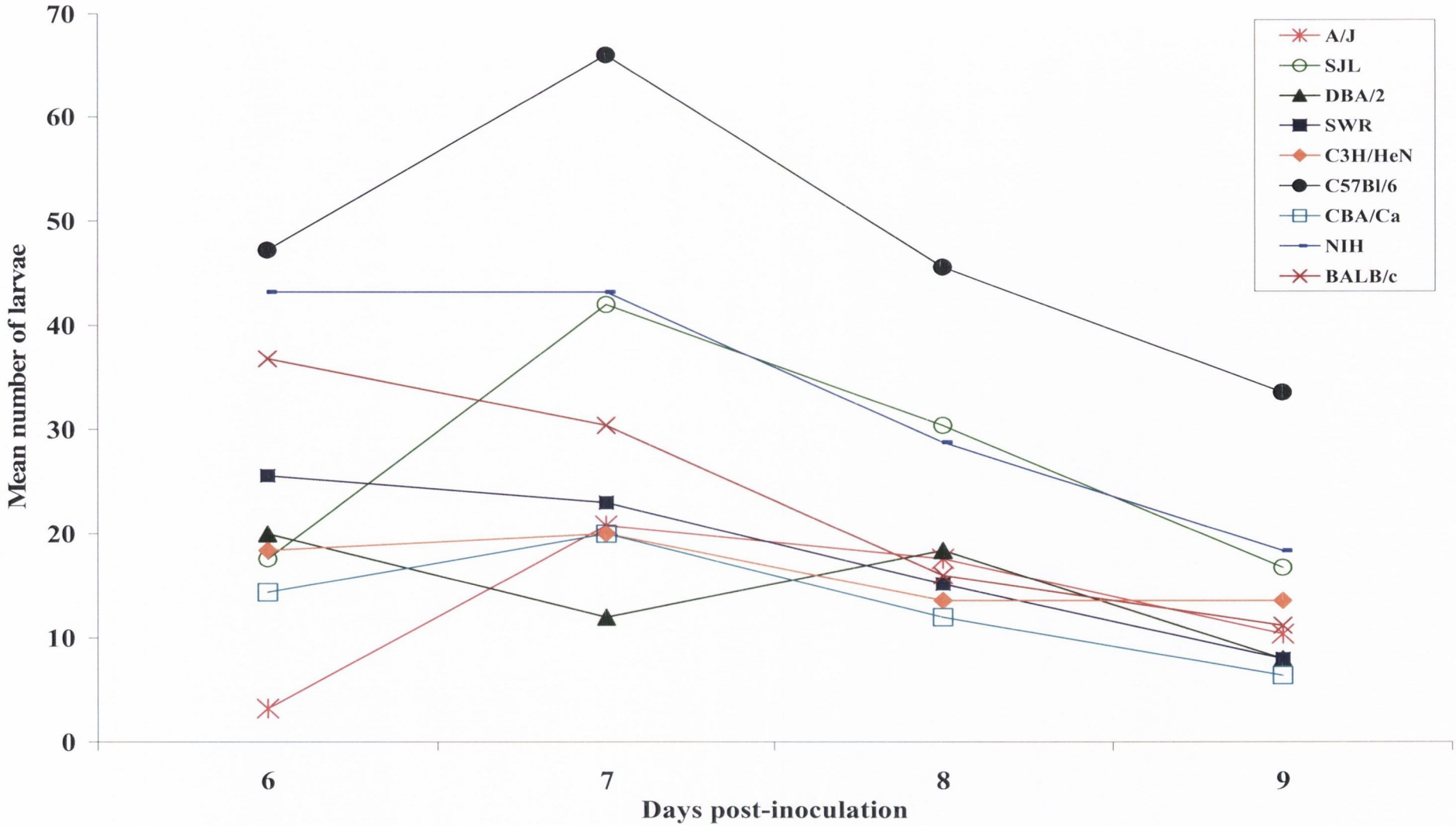


Figure 3.3.1: The mean number of larvae recovered in the lungs (total) of the nine strains of mice on days 6-9 post-inoculation.

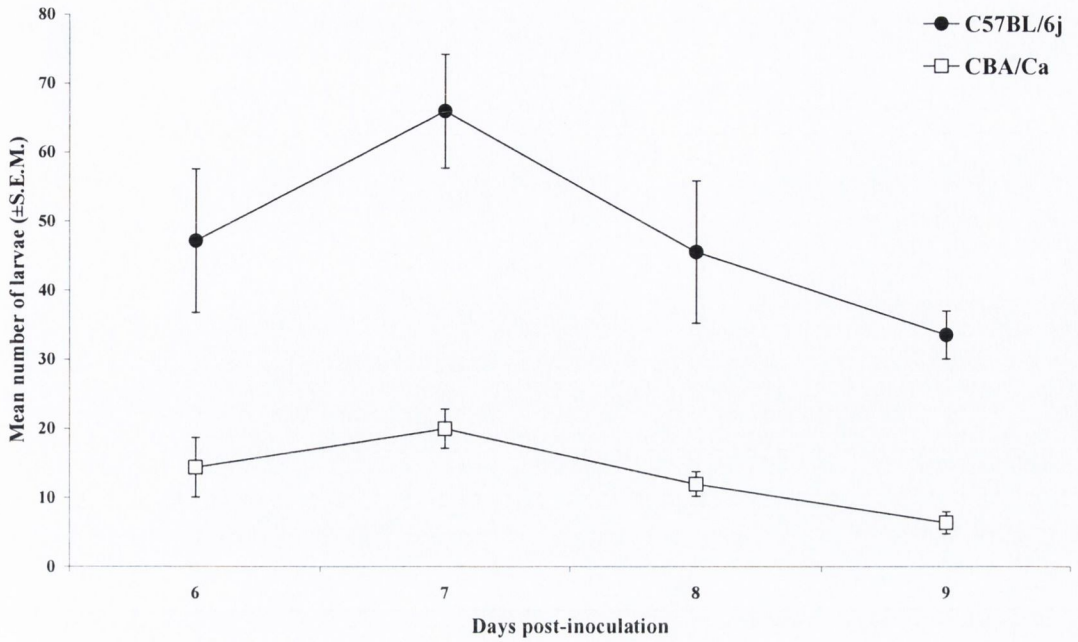


Figure 3.3.2: Mean number of larvae recovered (±S.E.M.) in the lungs in the strains C57BL/6j and CBA/Ca on days 6-9 post-inoculation.

THE DISTRIBUTION OF *A. SUUM* LARVAE IN THE TISSUES OF INDIVIDUAL INBRED MOUSE STRAINS OBSERVED DAILY FROM DAYS 6-9 POST-INOCULATION

C57BL/6j mice

This experiment, as well as determining strain variation, also provided a comparison of the level of consistency between experimental procedures and determined whether C57BL/6j mice were in fact the most susceptible strain (**fig. 3.3.1**). Larvae were present in high numbers on all days of post-mortem and showed a similar pattern of accumulation into the lungs as previously observed (**fig. 3.3.3**) accumulating in the lungs on day 6 p.i. and peaking on day 7 p.i. (**table 3.3.2**). Thereafter there was a decrease in the number of larvae until the last post-mortem day observed (day 9). This pattern was similar to that observed in chapter 2; however, the initial movement of larvae into the lungs on day 6 was slower than that previously observed but the later days were analogous (**fig. 3.3.3**). The percentage of the initial inoculum recovered on day 7 p.i. was similar between the two experiments, 14% (see chapter 2) in comparison to 13.2% in the current investigation.

Analysis within the strain C57BL/6j revealed that day was a significant factor in the number of larvae recovered from the lungs (2-way ANOVA with side of organ and day as

factors, model R^2_{adj}=0.406, main effect of day $F_{3,32}$=2.9, P=0.05). A *post-hoc* analysis revealed, however, that only day 7 differed to day 9 in terms of larval burden (P=0.0064). This reflected the gradual descent in larval burden after the peak accumulation (**fig. 3.3.1**). There was a significant difference in the number of larvae recovered from the left and right hand side lung of this strain (main effect of organ $F_{1,32}$=20.3, $P \leq 0.0001$), with more larvae consistently recovered in the right in contrast to the left side (**table 3.3.2**). This difference between the sides of the lung was initially very small on day 6 as the larvae are still migrating into the lungs but increases by day 7 and remains considerably higher over the latter three days (**table 3.3.2**).

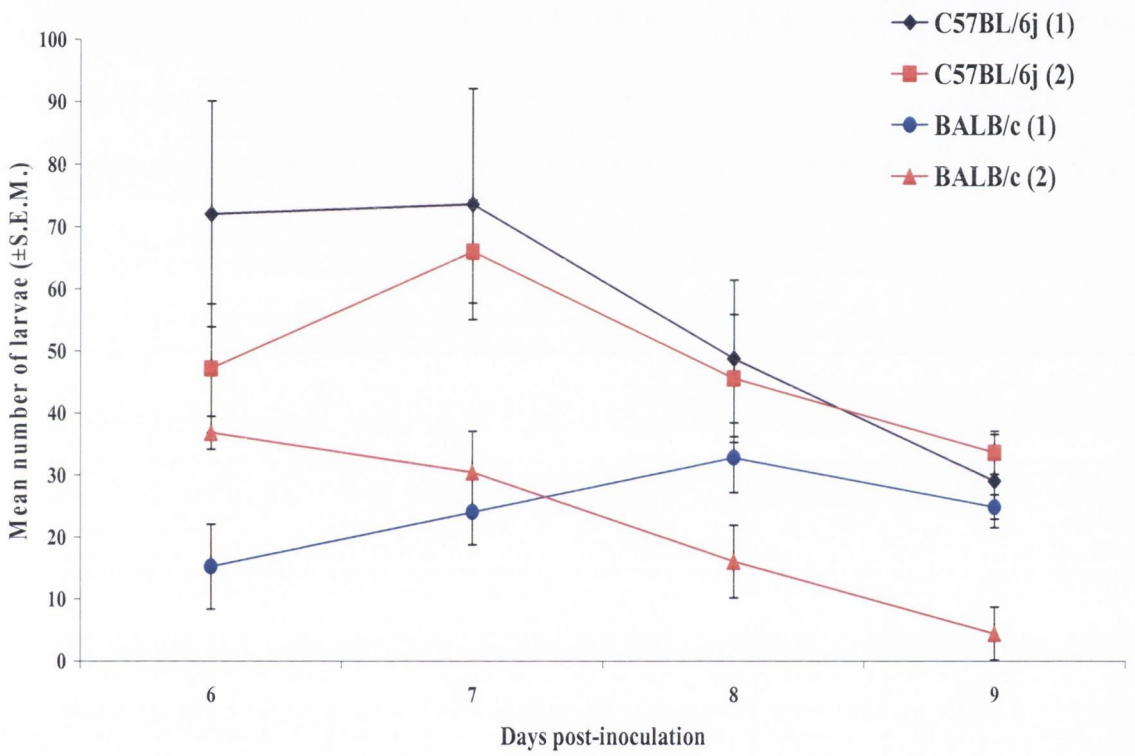


Figure 3.3.3: Mean number (±S.E.M.) of larvae recovered from the lungs of the two strains of mice (C57BL/6j and BALB/c) on days 6-9 post-inoculation in two separate experimental procedures {chapter 2 (1) and chapter 3 (2)} with an initial dose of 500 *A. suum* eggs.

NIH mice

NIH mice were classified in this study as members of the intermediate susceptible group based on *post-hoc* analysis and the percentage of the initial inoculum recovered. The percentage of the initial inoculum recovered on the day of maximum larval burden was 8.6% (**table 3.3.3**). Both day 6 and day 7 mean larval burdens were identical suggesting more rapid movement of larvae into the lungs of NIH mice than C57BL/6j mice (**fig.**

3.3.1). The number of larvae recovered declined quickly after day 7 and was comparable to the resistant strains on the latter days of infection (18.4 ±2.99). This was reflected in the post-hoc analysis as day 9 was significantly different from both day 6 and day 7 (P=0.011 for both days). The number of larvae was significantly different in the right and left hand side lung (**table 3.3.2**; 2-way ANOVA with day and side of organ, model R^2_{adj}=0.18, main effect of organ $F_{1,32}$=4.7, P=0.038). There was no significant interaction between organ*day ($F_{3,32}$=0.12, P=0.95) and thus larvae accumulated preferentially in the right hand side consistently on the four days examined. There was a significant effect of day on larval burden within the strain NIH (main effect of day $F_{3,32}$=3.5, P=0.026).

SJL mice

The mouse strain SJL were allocated into the intermediate susceptible group as the percentage number of larvae recovered from the initial inoculum on the day of maximum accumulation was 8.4% (±2.18%) (**table 3.3.3**) and there was no significant difference between this strain and the other members of the intermediate grouping when analysed by an LSD *post-hoc* test (**table 3.3.1**). The movement of larvae into the lungs of these mice was initially very slow and the larval burden on day 6 (**fig. 3.3.2**) was similar to that of the resistant strains (17.6 ±2.04). On day 7 the number of larvae increased dramatically and the number of larvae in the lungs was equivalent to that of NIH (42 ±10.9). There was a significant day effect on the larval burden observed during the four day period (**fig. 3.3.1**; 2-way ANOVA with day and side of organ as factors, model R^2_{adj}=0.25, main effect of day $F_{3,32}$=5.7, P=0.003). The results of the *post-hoc* analysis showed that day 7 was significantly different from both day 6 (P=0.0017) and day 9 (P=0.0012) but did not differ to day 8, illustrating the slow movement into this organ on day 6 and the decline in larval numbers following the peak of accumulation. The result of the analysis of the effect of recovery of larvae from the left and right hand side lung showed no variation in accumulation between the two sides (**table 3.3.2**), although on the latter days the arithmetic mean is greater on the right hand side.

Table 3.3.2: Mean number of larvae (±S.E.M.) recovered from the left and right hand side of the lung and the total number recovered from the lungs on each post-mortem day in each inbred mouse strain.

Strain	n	Day	Left lung	Right lung	Lung total
C57BL/6j	5	**6**	20.8 (±4.08)	26.4 (±7.22)	47.2 (±10.38)
	5	**7**	18.4 (±5.15)	47.6 (±4.58)	66.0 (±8.25)
	5	**8**	12.8 (±3.88)	32.8 (±8.52)	45.6 (±10.32)
	5	**9**	8.80 (±2.94)	24.8 (±5.85)	33.6 (±3.49)
NIH	5	**6**	16.8 (±5.28)	26.4 (±2.99)	43.2 (±7.74)
	4	**7**	19.2 (±5.85)	24.0 (±7.16)	43.2 (±8.33)
	5	**8**	10.4 (±4.31)	18.4 (±3.25)	28.8 (±7.53)
	5	**9**	6.40 (±0.98)	12.0 (±3.79)	18.4 (±2.99)
SJL	5	**6**	9.60 (±2.71)	8.00 (±2.19)	17.6 (±2.04)
	4	**7**	21.0 (±3.42)	21.0 (±7.90)	42.0 (±10.89)
	5	**8**	12.0 (±2.19)	18.4 (±3.71)	30.4 (±4.66)
	5	**9**	7.20 (±2.65)	9.60 (±0.98)	16.8 (±3.44)
BALB/c	5	**6**	19.2 (±4.27)	17.6 (±5.46)	36.8 (±6.86)
	5	**7**	14.4 (±3.71)	16.0 (±4.00)	30.4 (±5.31)
	5	**8**	5.60 (±2.71)	10.4 (±3.49)	16.0 (±5.66)
	5	**9**	4.00 (±1.26)	7.20 (±1.50)	11.2 (±1.96)
SWR	5	**6**	8.80 (±2.65)	16.8 (±2.94)	25.6 (±2.04)
	4	**7**	7.00 (±2.52)	16.0 (±4.90)	23.0 (±7.00)
	5	**8**	4.00 (±1.26)	11.2 (±4.08)	15.2 (±2.94)
	5	**9**	4.80 (±2.33)	3.20 (±1.50)	8.00 (±2.19)
A/J	5	**6**	2.40 (±0.98)	0.80 (±0.80)	3.20 (±1.50)
	5	**7**	10.4 (±3.25)	10.4 (±3.71)	20.8 (±6.86)
	5	**8**	8.00 (±1.26)	9.60 (±3.49)	17.6 (±2.40)
	5	**9**	3.20 (±1.50)	7.20 (±3.20)	10.4 (±4.12)
C3H/HeN	5	**6**	11.2 (±5.99)	7.20 (±4.45)	18.4 (±9.93)
	5	**7**	4.80 (±2.33)	15.2 (±3.88)	20.0 (±2.83)
	5	**8**	7.20 (±2.33)	6.40 (±2.40)	13.6 (±4.12)
	5	**9**	4.80 (±2.33)	8.80 (±2.65)	13.6 (±3.25)
CBA/Ca	5	**6**	2.40 (±0.98)	12.0 (±4.73)	14.4 (±4.31)
	5	**7**	11.2 (±3.67)	8.80 (±2.94)	20.0 (±2.83)
	5	**8**	6.40 (±1.60)	5.60 (±0.98)	12.0 (±1.79)
	5	**9**	2.40 (±0.98)	4.00 (±1.26)	6.40 (±1.60)
DBA/2	4	**6**	9.00 (±4.12)	11.0 (±1.91)	20.0 (±4.90)
	5	**7**	3.20 (±1.50)	8.80 (±3.44)	12.0 (±4.56)
	5	**8**	5.60 (±2.99)	12.8 (±3.44)	18.4 (±4.31)
	4	**9**	3.00 (±1.91)	5.00 (±1.91)	8.00 (±1.63)

BALB/c mice

BALB/c mice were placed in the intermediate susceptible group with a peak larval burden of 36.8 (±6.86) or 7.4% of the administered dose (**table 3.3.3**). BALB/c mice were utilised previously in chapter 2 and again showed to be only relatively resistant compared to C57BL/6j (**fig. 3.3.2**). They were significantly different from the majority of the resistant group except C3H/HeN and SWR (**table 3.3.3**). The accumulation into the lungs occurred at an earlier stage than previously observed, occurring on day 6 p.i. compared to day 8 previously (**fig. 3.3.3**). Thereafter larval numbers in the lungs declined until the last observation. Day was a significant factor determining larval burden in the lungs of BALB/c mice (2-way ANOVA with day and organ as factors, model R^2_{adj}=0.23, main effect of day $F_{3,32}$=5.7, P=0.0031). Again day 9 and day 7 were significantly different following the *post-hoc* analysis (P=0.011). Day 6, the day of maximum accumulation, was also significantly different to days 8 (P=0.0063) and day 9 (P=0.0011), however, it was not significantly different to day 7. Although the day of accumulation varied between the two experiments, the number of larvae successfully migrating to this organ was very similar irrespective of day. The peak burden observed on day 8 was 32.8 ±5.9 (or 6.6% of the administered dose was recovered) in chapter 2. In the current chapter the number of larvae that accumulated at the peak in the lungs occurred on day 6 and was 36.8 ±6.86. There was no significant variation in the number of larvae recovered between the left and right hand side lung and no real preference was shown for either side (**table 3.3.2**).

SWR mice

The migratory pattern of the strain SWR resembled that of BALB/c with larvae accumulating on day 6 and declining thereafter (**fig. 3.3.1**). There was a steady reduction in the larval burden to 8 (±2.19) on day 9 p.i.. Following *post-hoc* analysis, this strain differed significantly from NIH and SJL in the intermediate susceptible group but not BALB/c (**table 3.3.1**). However, the number of larvae recovered expressed as a percentage of the initial inoculum was not as high as that observed in the three strains of the intermediate susceptible group (**table 3.3.3**). Thus SWR mice were classified as a resistant strain. The larval burden in the lungs of SWR mice was significantly influenced by day (2-way ANOVA with day and side of organ as factors, model R^2_{adj}=0.30, main effect of day $F_{3,30}$=3.9, P=0.019). Day 9 showed a significant difference in the larval burden of mice from both day 6 (P=0.004) and day 7 (P=0.018). There was a significant difference between the number of larvae accumulating in the left and right hand side lung in this strain (main effect of side of organ, day $F_{1,30}$=7.5, P=0.01). As with the previous strains,

significantly more larvae accumulated in the right in contrast to the left hand side of the lung (**table 3.3.2**).

A/J mice

The strain A/J was designated as a resistant strain with a maximum larval burden of 20.8 (±6.86) on day 7 post-inoculation (**fig. 3.3.1**). This was 4.2% of the initial number of ova administered recovered from the lungs of this strain (**table 3.3.3**). A/J mice had the slowest movement of larvae into the lungs of all strains examined with a larval burden of only 3.2 on day 6 (**table 3.3.2**). The majority of the larvae appeared rapidly in the lung on day 7 after which the larval burden slowly declined on the latter days (**fig. 3.3.1**). Day was a significant factor in the recovery of larvae (2-way ANOVA with day and side of organ as factors, model R^2_{adj}=0.18, main effect of day $F_{3,30}$=4.7, P=0.008) with day 6 showing a significant difference to both day 7 (P=0.0016) and day 8 (P=0.0082). There was no significant difference between the left and right hand side lung in this strain and on the majority of the days the two sides of the lung harbour similar numbers of larvae. On day 9 the right hand side lung showed considerably more larvae than the left hand side (**table 3.3.2**) perhaps suggesting a disproportionate movement of larvae away from the left hand side compared to the right side of the lung.

C3H/HeN mice

The strain C3H/HeN was a highly resistant strain and *post-hoc* analysis and the percentage of the initial inoculum administered recovered from the lungs on the peak day confirmed this classification (**table 3.3.1**). The maximum accumulation of larvae was observed on day 7 p.i. (**Fig. 3.3.1** and **table 3.3.3**) and accounted for 4% of the administered dose. The pulmonary larval burden was maintained during the infection period and there was no statistical difference in larval numbers between the post-mortem days. The number of larvae decreased to 13.6 on day 8 and remained at this level on day 9 (**table 3.3.2**). There was no significant difference in the number of larvae recovered from either side of the lung and each side varies in its dominance in terms of the larval accumulation (**table 3.3.2**).

Table 3.3.3: The percentage of larvae recovered (±S.E.M.) from the initial inoculum dose of 500 *A. suum* eggs in each strain on each post-mortem day in the total lung.

Strain	n	Percentage recovered in the lung (total)	Days p.i.	Strain	n	Percentage recovered in the lung (total)
C57BL/6j	5	9.44 (±2.08)	**6**	**A/J**	5	0.64 (±0.30)
	5	13.2 (±1.65)	**7**		5	4.16 (±1.37)
	5	9.12 (±2.06)	**8**		5	3.52 (±0.48)
	5	6.72 (±0.70)	**9**		5	2.08 (±0.82)
NIH	5	8.64 (±1.55)	**6**	**C3H/HeN**	5	3.68 (±1.99)
	5	8.64 (±1.67)	**7**		5	4.00 (±0.57)
	5	5.76 (±1.51)	**8**		5	2.72 (±0.82)
	5	3.68 (±0.60)	**9**		5	2.72 (±0.65)
SJL	5	3.52 (±0.41)	**6**	**CBA/Ca**	5	2.88 (±0.86)
	4	8.40 (±2.18)	**7**		5	4.00 (±0.57)
	5	6.08 (±0.93)	**8**		5	2.40 (±0.36)
	5	3.36 (±0.69)	**9**		5	1.28 (±0.32)
BALB/c	5	7.36 (±1.37)	**6**	**DBA/2**	4	4.00 (±0.98)
	5	6.08 (±1.06)	**7**		5	2.40 (±0.91)
	5	3.20 (±1.13)	**8**		5	3.68 (±0.86)
	5	2.24 (±0.39)	**9**		4	1.60 (±0.33)
SWR	5	5.12 (±0.41)	**6**			
	4	4.60 (±1.40)	**7**			
	5	3.04 (±0.59)	**8**			
	5	1.60 (±0.44)	**9**			

CBA/Ca mice

This strain was among the most resistant of the strains examined and had a similar pattern of movement of larvae in comparison to C57BL/6j with larvae accumulating in the lungs on day 7 (20 ±2.83 or 4% of the administered inoculation recovered) and then migrating away from this organ on days 8 and 9 (**fig. 3.3.2** and **table 3.3.3**). The percentage difference in the number of larvae recovered between this strain and C57BL/6j ranged from 69-81% during the infection period with CBA/Ca mice harbouring constantly lower larval numbers. The percentage difference was calculated as: (Total number of larvae recovered from C57BL/6j- Total number of larvae recovered from CBA/Ca)/Total number of recovered from C57BL/6j. There was no significant effect of day on the observed number of larvae in the lungs (2-way ANOVA with day and side of organ as factors, model R^2_{adj}=0.18, main effect of day $F_{3,32}$=2.5, P=N.S.) and this was due to the low number of larvae recovered on each post-mortem day. However, the importance of day 7 was revealed by the results of the *post-hoc* analysis which showed that day 7 was significantly different to day 9 p.i. (P=0.012). There was no significant difference in the accumulation of larvae between the two sides of the lung (**table 3.3.2**).

DBA/2 mice

The strain DBA/2 was another member of the resistant group of mice. However, this strain was unique in the movement of larvae through the lung tissue. DBA/2 showed two peak days either side of day 7 (**fig. 3.3.1**). On day 6 the number of larvae accumulating in the lungs was 20 and this was analogous to the two strains discussed previously. Larval numbers began to decline in the lungs on day 7 with a mean larval burden of 12 (±4.56), however, worm numbers increased again on day 8 to 18.4 (±4.31) and then descended once again on day 9 (**table 3.3.2**) after which it would presumably continue to decrease. The percentage recovery (4 ±0.98%) (**table 3.3.3**) and the results of the *post-hoc* analysis (**table 3.3.1**) placed this strain within the resistant category. The day 7 group of DBA/2 mice appeared to have some underlying divergence to the rest of the mice of this strain. The day 7 group of DBA/2 show a reduction in weight between the pre-infection weight and the post-mortem weight, an observation that was reversed in the mice euthanased on the other p.i. days within this strain. There was no day effect on larval burden observed in this strain (2-way ANOVA with day and side of organ as factors, model R^2_{adj}=0.11, main effect of day $F_{3,32}$=0.17, P=N.S.) suggesting that the day 7 discrepancy was marginal. There was a significant effect of organ on larval burdens (main effect of organ $F_{1,32}$=4.1, P=0.05) and larvae accumulated on the right hand side of the lung in comparison to the left during the four day period (**table 3.3.2**).

THE EFFECT OF LARVAL BURDEN ON MOUSE BODY WEIGHT

Mouse body weight data showed that there was variation in weight between the strains and over time from the initial (pre-infection) weight on day 0 and the weight of mice at post-mortem (**appendix A.4**). A paired t-test analysing the difference in weight pre- and post-infection revealed that there was a significant reduction between these time points (t=-6.046, df=175, P≤0.0001, 95% CI= (-0.633) - (-0.322)). C57BL/6j mice lost weight during the infection period particularly on day 8 and day 9 compared to all the other strains observed (**fig. 3.3.4**) and this was correlated with the higher number of larvae in the tissue of these mice on all p.i. days. The strain CBA/Ca exhibited initial weight loss on days 6 and 7 but on day 7 and 8 the individuals of this strain showed an overall weight gain. BALB/c mice displayed a great deal of variation in weight gain during the period of investigation. NIH mice were consistently the heaviest strain of mice both pre- and post infection. Mice were not weight matched when separated into inoculation groups.

As an additional precaution an analysis was undertaken that took host body weight into consideration. This was performed to exclude host weight as an intrinsic factor that could potentially influence larval burdens. The expression of the lung worm burden as the number of worms per gram of body weight (total worm burden from the lungs/ mouse body weight; **fig. 3.3.5**) accounted for the body weight of mice as a factor in the number of larvae recovered from this tissue ensuring that heavier mice did not harbour more worms. The two-way ANOVA for the number of larvae recovered per gram of mouse body weight (model R^2=0.59) revealed that the main effects of strain ($F_{8,140}$=15.0, $P\leq$0.0001) and day ($F_{3,140}$=14.7, $P\leq$0.0001) were highly significant explanatory factors for variation in larval burdens but in this case there was a marginally significant 2-way interaction (strain*day, $F_{24,140}$=1.61, $P\leq$0.0468). Interestingly, *post-hoc* analysis confirmed a comparable outcome to the result when strains were compared in terms of total worm burden, with the relative ranking of mouse strains identical to that concluded on the basis of total worm burdens (**table 3.3.4**).

Finally, an assessment was made on whether there were any significant differences in body weight between the mouse strains that might have confounded the analysis. The analysis revealed that the body weight of mouse strains varied significantly (2-way ANOVA, with strains and time as factors, on mouse weight, main effect of strain, $F_{8,140}$=31.9, $P\leq$0.0001), but not with time (day p.i.) and the 2-way interaction between strain and day was not significant. *Post hoc* analysis showed that one strain differed from all of the other strains; NIH mice were significantly heavier than all the other strains (**table 3.3.5**). Thus no relationship emerged that related host body weight to parasite burdens. For example BALB/c and NIH differed significantly in their body weight (**table 3.3.5**) but not in the number of worms recovered per gram of body weight (**table 3.3.4**).

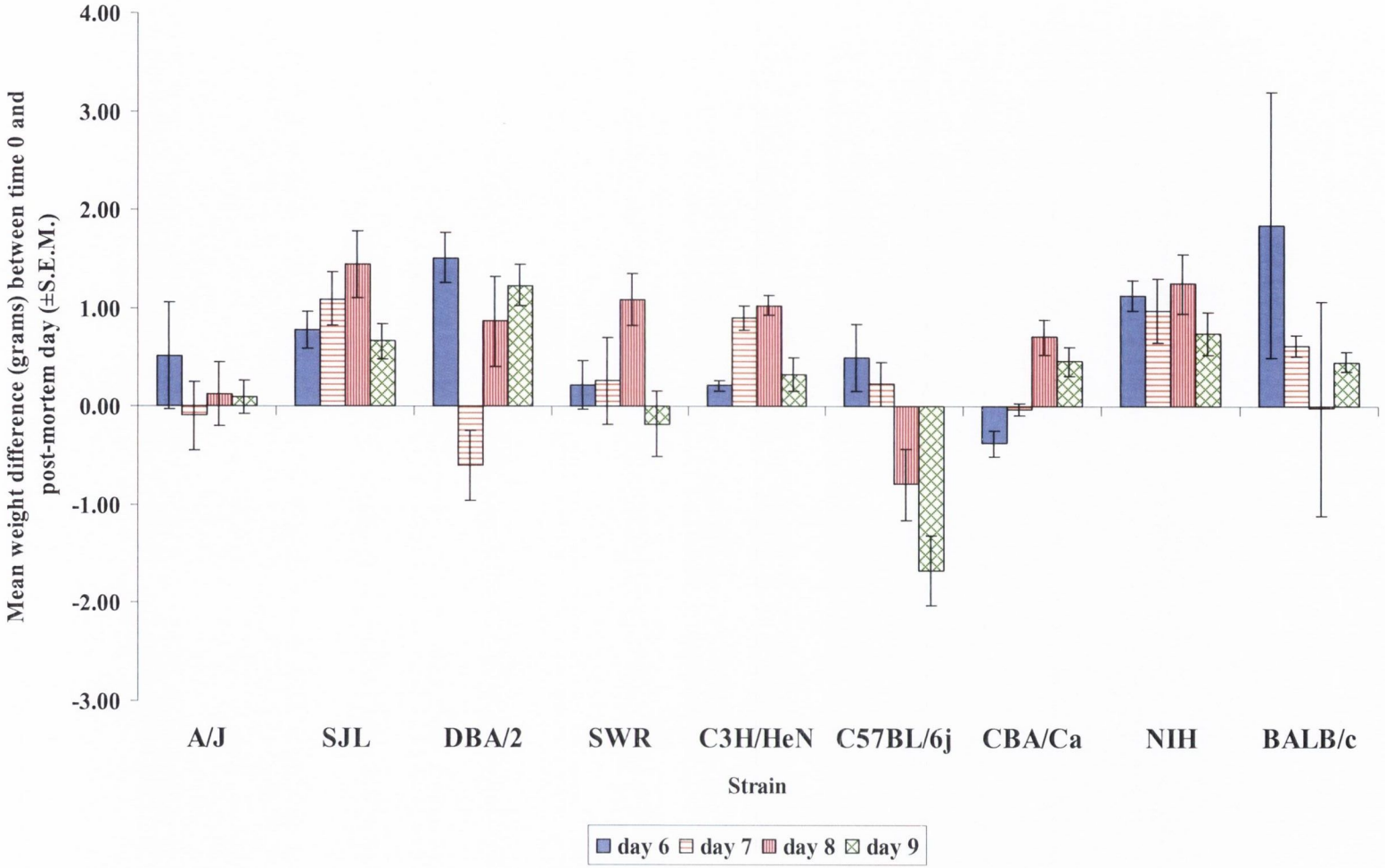


Figure 3.3.4: Mean body weight difference (grams) between pre-infection (time zero) and the day of post-mortem (±S.E.M.) of each of the nine mouse strains on each post-mortem day.

Table 3.3.4: Results of a *post-hoc* least significant difference (LSD) test between the strains following the two-way ANOVA examining the effects of day and strain on the number of larvae recovered per gram of mouse body weigh at post-mortem for all strains (only significant results are shown).

Strain	Strain	Probability	Strain	Strain	Probability
C57BL/6j-	NIH	≤0.0001	**NIH-**	A/J	≤0.0001
	SJL	≤0.0001		C3H/HeN	0.0009
	BALB/c	≤0.0001		CBA/Ca	0.0002
	SWR	≤0.0001		DBA/2	0.0010
	A/J	≤0.0001		SWR	0.0204
	C3H/HeN	≤0.0001	**SJL-**	A/J	0.0015
	CBA/Ca	≤0.0001		C3H/HeN	0.0148
	DBA/2	≤0.0001		CBA/Ca	0.0042
BALB/c-	CBA/Ca	0.0068		DBA/2	0.0148
	DBA/2	0.0231			

Table 3.3.5: Results of a *post-hoc* least significant difference (LSD) test between the strains following the two-way ANOVA examining the effects of day and strain on the post-mortem body weight of each strain on each post-mortem day (only significant results shown)

Strain	Strain	Probability	Strain	Strain	probability
C57BL/6j-	A/J	0.0001	**NIH-**	A/J	≤0.0001
	BALB/c	≤0.0001		BALB/c	≤0.0001
	CBA/Ca	≤0.0001		C3H/HeN	≤0.0001
	DBA/2	≤0.0001		CBA/Ca	≤0.0001
	SWR	≤0.0001		DBA/2	≤0.0001
C3H/HeN-	A/J	0.0133		SWR	≤0.0001
	BALB/c	0.0034		C57BL/6j	≤0.0001
	CBA/Ca	≤0.0001		SJL	≤0.0001
	NIH	≤0.0001	**SJL-**	DBA/2	0.0007
	SWR	≤0.0001		CBA/Ca	≤0.0001
	DBA/2	0.0003		SWR	0.0004
A/J-	CBA/Ca	0.0353	**SWR-**	DBA/2	0.0227
	SWR	0.0004		BALB/c	0.0018

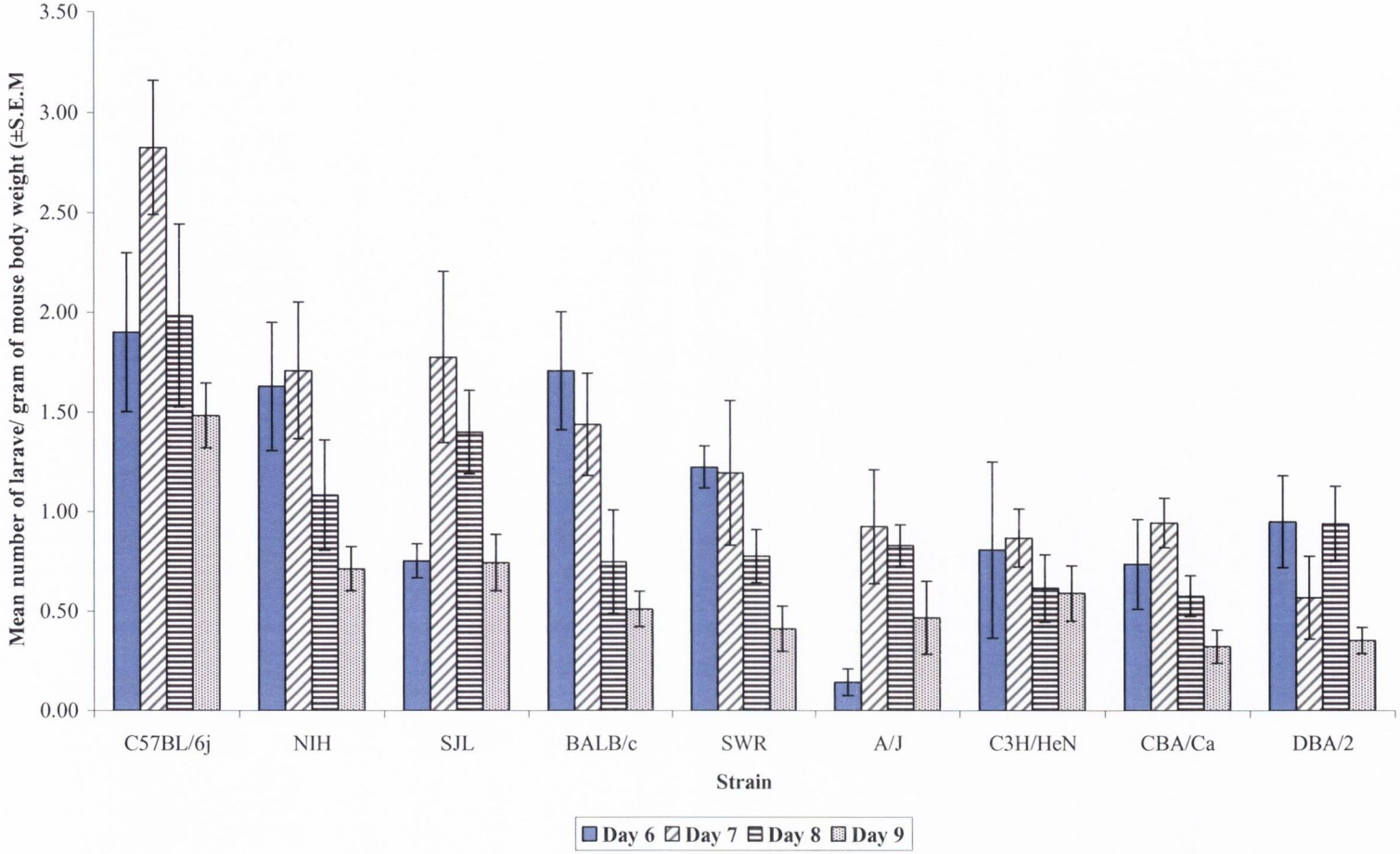


Figure 3.3.5: Mean number of larvae recovered per gram of mouse body weight (±S.E.M.) on each post-mortem day in each strain of mouse.

3.4 DISCUSSION

Epidemiological studies of a number of parasite species have shown that the intensity of infection is a heritable phenotype of the host (Quinnell, 2003). The genotype of mice influences the level of resistance developed against GI nematodes, such as *Trichuris muris*, this is manifest as differences between strains of inbred mice in their ability to survive a lethal dose of infection, to depress the development and reproduction of the parasite, to develop protection against reinfection and to mount protective humoral and cellular responses (Mitchell *et al.*, 1982). The present study provided strong evidence that inbred strains of mice differed markedly in their susceptibility to early *A. suum* infection as measured by larval burden in the lung on day 7 post-infection. C57BL/6j mice were uniquely the most susceptible strain and exhibited consistency in larval burden in the lung between two infection protocols. Other inbred strains fell into two categories – the intermediate susceptible group (NIH, BALB/c and SJL) and the resistant group (A/J, C3H/HeN, CBA/Ca, DBA/2 and SWR). CBA/Ca mice were selected as the most resistant group based upon the highly significant difference in larval numbers between the two strains on day 7 over the four-day period of assessment.

The results illustrated obvious differences in the relative susceptibilities to *A. suum* in the various strains of mice examined. Earlier work by Mitchell *et al.* (1976) demonstrated variation in susceptibility to *Ascaris* in the lungs of inbred strains of mice, C57BL/6 mice were identified as being the most susceptible, but disparity in infectivity of batches of eggs and doses resulted in lack of comparability between groups. For example, the average recovery of larvae from the lungs of C57BL/6j varied from 14.5% to 0.73% in two separate experiments with varying dosage administered. Johnstone *et al.* (1978) reported large variation in the number of larvae recovered from the lungs of C57BL/6j mice as a result of using two different protocols for the recovery of larvae (8.2% to 5.3% between two procedures). Interestingly, the larval burdens observed here in the BALB/c mice are comparable to the recoveries achieved by the more recent work of Slotved *et al.* (1997a) using improved inoculation/recovery procedures, with higher percentages of the initial inoculum recovered (4.3% [(Slotved *et al.*, 1997a)] and 7.3% and 6.6% in the current experiments). It is pertinent that their recoveries of worms did not correspond to the observations of Mitchell *et al.* (1976) who concluded that BALB/c were a highly resistant strain, with a mean recovery of 7 (± 1) or 0.04% of the administered dose. The methodology employed in the present study with resultant consistency between separate

experiments in worm recoveries from uniformly treated mice, and consistent between-strain variation, as well as a significantly higher proportions of larvae being recovered compared to previous studies (Johnstone *et al.,* 1978; Slotved *et al.,* 1997a, 1998), all represented marked improvements on earlier published studies. Thus there is confidence in the observations of heterogeneity in the susceptibility to *A. suum* in different strains of mice in the present study. This reflects inherent differences between the mouse genotypes and from this two contrasting strains in relation to their pulmonary larval burden have been successfully identified.

Although there was some disparity in the movement of larvae into the lungs of a number of strains (for example; larvae recovered from BALB/c and DBA/2 mice show a different pattern than C57BL/6j mice), this variation can in part be accounted for by the smaller number of larvae recovered in these strains compared with the most susceptible C57BL/6j mice. These apparent departures in the sequence of events in larval migration in the lungs may be artefacts of lower worm burdens and possible counting errors that give disproportional variation when larval numbers are low. Small variations in the number of larvae counted in resistant strains of mice can have a disproportionate effect on the mean number of larvae recovered. However, this does not impact on the significance of the analysis. The maximum accumulation in the susceptible strain C57BL/6j was consistently observed on day 7 and as such this day post infection was advocated as the best day for assessing differences in susceptibility between strains of mice. Variation of the pattern of migration of larvae into the lungs was discussed in chapter 2, and it was suggested that this can be the result of an array of possible migratory routes available to the larvae as they begin the journey from the liver to the lungs and larvae become temporarily lost or having indirect paths of migration to this organ. Evidence supporting this theory was revealed by the slower movement of larvae into the lungs of C57BL/6j mice on day 6 in the current chapter in comparison to chapter 2, but the similar worm burdens on day 7 revealed that ultimately the same number of larvae will accumulate in the lungs. It was proposed that day 6 larval burdens were highly variable and influenced by the expeditious rate at which larvae could locate the lungs but larval burdens were consistent on day 7 between the two experimental procedures and so represented a population of larvae that could successfully migrate to this tissue from the liver.

Crucially, differences in the larval burden were not attributable to host body weight (a possible confounding factor), since heavier mice were not more susceptible to increased

larval burdens. The three groups- susceptible, intermediate susceptible, and resistant- were not altered following the analysis of the number of larvae per gram of mouse body weight, although the strains did differ significantly in their body weights, thus showing that weight was not an explanatory factor. This aspect was analysed as a precaution to ensure that host weight did not act as a hidden intrinsic factor that could compound the examination of susceptibility when considered on the basis of total worm burden. Johnstone *et al.* (1978) examined 3-week and 8-week old C57BL/6 mice as a model for the study of resistance to *A. suum* infection. They found that significantly more larvae accumulated in the livers of older mice administered a dosage of 3000 ova. Factors that could have resulted in this disparity included, physiological changes in maturing mice, passive immunity from the dam, and the crowding effect due to smaller body size. Johnstone *et al.* (1978) found that these figures were not significantly different when the number of larvae was converted to a per weight basis. In the case of the lungs, however, significantly more larvae reached the lungs of 8-week old mice by day 7 p.i. and this remained significant even when corrected for differences in body weight.

Perhaps the most intriguing question to emerge from these experiments is why C57BL/6 mice are so much more susceptible than other strains of mice. There have been a number of proposals for the divergence of host responses to the presence of parasites, particularly GI nematodes such as those outlined previously regarding differences in genotype resulting in heterogeneity of the immune response. The mechanism underlying the greater susceptibility of C57BL/6j mice to infection with *Ascaris* is still uncertain at this stage but it is likely to be genetic and consist of innate or molecular/structural/ physiological differences between the strains with one set of alleles enabling *Ascaris* to migrate and survive in large numbers and the other impeding this migration. The early variation in the larval burden observed in the liver between C57BL/6j and BALB/c in chapter two and the differences in the timing of the accumulation in this organ suggest that larvae may be lost early in infection. One possibility is a difference in larval penetration of the large intestine between strains. Higher rates of gut motility could decrease the possibility of successful hatching and penetration as the effective transit time in the intestine would be reduced and the result could be insufficient time for larvae to hatch in as large numbers. Similarly, individuals that mount an effective innate response to the presence of invading pathogens may result in the thickening of the intestinal mucosa preventing successful penetration. Equally it is conceivable that more larvae are entrapped in the livers of the resistant strain, or that their onward migration from the liver is less successful than in the susceptible

strain. The latter is likely to be dependent on host cues that are exploited by the parasite to guide their homing to the pulmonary tissues, and it may be that the relevant signals vary between mouse strains. It is also possible that on arrival in the lungs more larvae fail to establish in resistant mice, or are expelled possibly through the bronchial airways by coughing. Thus the difference in phenotype between the mice may be attributable to any of a number of strategic points along the normal developmental and migratory route of this parasite.

There may be some suggestion that the turnover of the larval population can vary between strains and thus can result in artefactual strain differences. However, this is unlikely to explain the consistent differences observed between the strains and the consistent day and number of larvae recovered from the lung tissue of C57BL/6j mice. Larval migration was observed over a number of days and during this period C57BL/6j mice had heavier larval burdens on all days in contrast to the o ther strains. The re is a distinctive pattern of accumulation and then a dispersion of worms from the lungs. The population does not fluctuate randomly which would be expected if larvae were not actively migrating but rather migrating directly through the tissues due to the host circulatory system. Although the transit time through the intestine is relatively fast (all larvae disappear from the intestine 24 hours p.i.) (Slotved *et al.*, 1998), previous studies (Sprent & Chen, 1948; Sprent, 1962; Douvres & Tromba, 1971; Eriksen, 1981; Prokopič & Figallová, 1982a; Wade *et al.*, 1987; Fagerholm *et al.*, 2000) and the current study have demonstrated larvae can be expected to take between 3-4 days to reach the liver and reach the lungs in large numbers by day 7 or 8. Thus suggesting that they are not passively migrating but actively migrating through the tissues and that there is a predetermined signal to the movment through these tissues. However, it is accepted that a relatively small number of individuals may directly migrate through the host viscera and quickly return to the intestine.

The kinetics with which different strains of inbred mice resolve infection with *Trichinella spiralis* was shown to correlate with their ability to mount mucosal mast cell responses in the intestinal mucosa. When mast cell precursor frequencies were compared in BALB/c mice and C57BL/10 strains of mice that, respectively, respond rapidly and slowly to infection, this frequency was significantly greater in BALB/c mice, as were other indicators (Brown *et al.*, 2003). Interestingly, recent data showed that C57BL/10 mice do not express the gene for Intelectin-2, which is believed to play an important role in resistance to GI nematodes such as *T. spiralis* (Pemberton *et al.*, 2004). The authors

suggested that the presence of intelectin–2 in resistant BALB/c mice, its absence from the susceptible C57BL/10 strain and the kinetics of its up-regulation during *T. spiralis* infection were all consistent with this novel lectin serving a protective role in the innate immune response to parasite infection. Again this corresponds to the intestinal stages of infection and not the period of visceral migration or the period of penetration through the intestinal wall. However, this up-regulation was observed as early as day 3 of infection and suggests that it may be a component of the early innate response to nematode infection. The lack of intelectin-2 in C57BL/6 and C57BL/10 mice is not the sole contributor to the impaired ability to reject this nematode as there are a number of differences in the loci between these strains and BALB/c but plays an important role in innate immunity. The presence of this gene could influence the initial success of parasites, such as *A. suum* larvae, through the intestinal wall, for example.

Finally the resultant difference may be a physiological distinction between strains that impedes larval migration in one strain over another. For example this may relate to a disparity between strains in their lung structure. Soutiere *et al.* (2004) examined structural differences in alveolar size among inbred strains of mice which are known to have significant differences in lung pressure-volume relations. They assessed whether the relative size or number of alveoli in the C3H/HeJ, C57BL/6j and A/J strains are related to these lung volume differences. Mean chord lengths were significantly different among the three strains, with the largest alveoli found in the C3H/HeJ mice, the smallest in the C57BL/6j mice and A/J mice were intermediate. These results show a clear genetic difference in lung structure among the different strains. Similarly, the observed preference for a particular part of the lung by the parasite could be due to restrictions in the lung between the two sides and as such is not a parasite factor but influenced by the physiology of the host.

There was an obvious disparity between mouse strains in their ability to prevent larval migration through the host tissues. The majority of the strains were intermediate/resistant in terms of their day 7 larval burden, however, C57BL/6j were outstandingly the most susceptible strain and this appears to be the result of some fundamental intrinsic host factor that allows the successful migration or survival of *A. suum* through the tissues of these mice in contrast to their inbred counterparts. It would appear that resistance is the normal state for mouse strains and that susceptibility is an atypical genotype and results in C57BL/6j mice harbouring more *A. suum* larvae in their tissues. This is suggested as a

possible mimic of the natural aggregation pattern in both pigs and humans. This aggregation pattern has been well-defined for adult *Ascaris* but has yet to be confirmed for the migratory stages. There is now confidence in the experimental procedures that they can yield consistency in *Ascaris* larval lung burdens between two inbred strains of mice. The results of the current investigation provide two strains of mouse, the susceptible C57BL/6j and the resistant CBA/Ca strain, which will enable exploration of the mechanisms underlying the observed susceptibility and resistance to early *Ascaris* infection. Furthermore, successful identification of the gene or genes underlying the different (resistant/susceptible) phenotypes of CBA/Ca and C57BL/6j would not only provide an avenue to the homologous genes but also for working out the mechanisms involved in pigs and humans in the future.

CHAPTER 4

A. THE INFLUENCE OF DOSE ON LARVAL BURDENS IN TWO MOUSE STRAINS (C57BL/6j AND CBA/Ca) THAT SHOW DIVERGENT PHENOTYPIC EXPRESSION IN THEIR SUSCEPTIBILITY TO *A. SUUM*

B. THE IMPACT OF TIME, DOSE AND STRAIN OF MOUSE ON LARVAL GROWTH DURING THE PULMONARY PHASE OF MIGRATION

A. THE INFLUENCE OF DOSE ON LARVAL BURDENS IN TWO MOUSE STRAINS (C57BL/6j AND CBA/Ca) THAT SHOW DIVERGENT PHENOTYPIC EXPRESSION IN THEIR SUSCEPTIBILITY TO *A. SUUM*

4.1A INTRODUCTION

An examination of the distribution of *A. suum* in naturally infected pigs showed that the worms recovered were heavily overdispersed (Boes *et al.*, 1998a). A similar trend of overdispersion was also observed in people infected with *A. lumbricoides* (Crompton & Tulley, 1987; Guyatt *et al.*, 1990; Thein Hlaing, 1993; O'Lorcain & Holland, 2000). The experimental inoculation of pigs with single or multiple doses of *A. suum* ova have also resulted in this highly aggregated frequency distribution (Eriksen *et al.*, 1992; Roepstorff *et al.*, 1997; Boes *et al.*, 1998a). Moreover, individual pigs appeared to be predisposed to either heavy or light intensities of infection irrespective of the initial number of ova they are exposed to (Boes *et al.*, 1998a). The differences between hosts in innate susceptibilities or immunological competencies can only be detected when exposure is controlled.

Knowledge of the intensity of *Ascaris* infection is important in terms of morbidity and mortality (Pawlowski & Davis, 1989). The aggregation of intense infection of adult *Ascaris* in a relatively small proportion of infected individuals has implications for selective approaches to chemotherapeutic control, the distribution of morbidity within the host population, and the dynamics of transmission (Holland & Boes, 2002). Individuals predisposed to intense infections, may be at high risk from the complications associated with both adult and larval *Ascaris*. The possibility exists that contact with larger numbers of infective stages may alter susceptibility and result, for all individuals, in a positive correlation between infective dose and the number of migrating larvae or adult worm burdens that establish. Thus the morbidity associated with this parasite becomes possibly more life-threatening and serious morbidity may be observed in greater numbers of individuals irregardless of their immune competency.

An array of complications are allied to larval tissue migration and they include respiratory symptoms (Vogel & Minning, 1942) and signs such as pneumonitis, substernal pain, fever, rash and eosinophilia (Loeffler, 1956) which have been associated with hypersensitivity to larval antigens (Cooper *et al.*, 2000; Palmer *et al.*, 2002). A marked histopathological response occurs in the liver of pigs and is an important consequence of porcine ascariasis

(Ronéus, 1966). This liver damage has also been shown to occur in abnormal hosts (Eriksen, 1981). The longer the exposure to infective eggs, the greater the number of "whitespots" that will develop, but the number declines with continuing exposure indicating the induction of a dose dependent host reaction. The potential for liver damage by migrating *A. lumbricoides* has not been substantiated in humans. A positive dose-dependent effect on larval numbers associated with increased infection levels could inflame these related diseases.

The substantial genetic component demonstrated in the previous chapter in relation to *A. suum* showed that inbred mouse strains vary in their susceptibility to this parasite. However, individual exposure to high or low numbers of eggs may have an influence on the observed larval burdens (Johnstone *et al.*, 1978). There have been conflicting results from previous authors as to the effect altering initial inoculum size has on larval worm burdens (Galvin, 1968; Andersen *et al.*, 1973; Mitchell *et al.*, 1976; Johnstone *et al.*, 1978; Slotved *et al.*, 1996). Altering dose size may change the relative susceptibility of a host strain. This chapter explored the degree to which migration success is influenced by the dose of larvae administered and hence the size of the migrating population of worms. Finally, the effects of strain, day post-infection and dose on worm length were assessed.

4.2A MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

One hundred and eighty inbred male mice (90 each of; C57BL/6j, and CBA/Ca) were purchased from Harlan, UK at 7 weeks of age and were 8 weeks on the day of the initial inoculations. Animals were maintained as described in chapter 2.

INOCULATION OF THE MICE WITH *A. SUUM*

The concentration of the stock suspension (10 eggs μl^{-1} in 0.1 N H2SO4) of eggs was performed in the following way. A dose of 3000 *A. suum* eggs, for example, for the inoculation of mice was prepared as follows. 3000 eggs in a 300µl aliquot were removed from the stock solution which was then placed in an Eppendorff tube. This was allowed to settle overnight and the supernatant was removed carefully leaving 100µl of solution with eggs beneath. All dosage groups were administered in 100µl of egg suspension. The siphoned fluid was examined by light microscopy to ensure no eggs were removed. This process was utilised to ensure that the mice received a constant egg suspension dose (100µl) that would not be too large for their stomachs and to ensure that all mice received an equal volume of egg suspension irregardless of the required egg dose size. Mice were inoculated (as in chapter 2) with one of four single inoculum doses (3000, 1000, 500, or 100) and two doses of 1000 ova administered over consecutive days in split doses (250 x 4 and 500 x 2). The split dosages were administered at day 0 and then on consecutive days.

POST-MORTEM PROCEDURE

Mice were weighed pre-infection (day 0) and post-infection on the day of post-mortem and the weight of the spleen were also recorded at post-mortem. Other experimental data was also noted (see chapter 2). The migration pattern and the number of *A. suum* in the lungs were determined from days 6 to 8 p.i.. Groups of five mice were euthanased daily. The post-mortem days of the split dosage groups were calculated from the initial inoculum on day 0. Only the lungs, divided into the left and right hand side, were examined for larval burden. This process was repeated for each individual mouse and the post-mortem procedure and recovery of larval *Ascaris* were performed as described in the previous chapters (see chapter 2). The objective of this experiment was to determine the influence of dose on the larval burden not only within a strain, but also between two different strains, and to provide further data on the consistency of the protocol.

MEASUREMENT OF LARVAL BODY LENGTH

During larval counts, larvae were photographed using an Olympus camedia digital camera C5050 (5.0 megapixels with a F1.6 lens) at x10 magnification. These images were transferred to a computer (OS windows XP) and each individual was measured using the programme ImageJ 1.32j. The imageJ programme was calibrated using a graduated slide and graticule of known measurements and then the pixel distances of the programme were translated to μm (See **fig. 4.2A.1a**). Larvae were measured by drawing a segmented line through the centre of the body from the anterior to the posterior end (See **figure 4.2A.1b**).

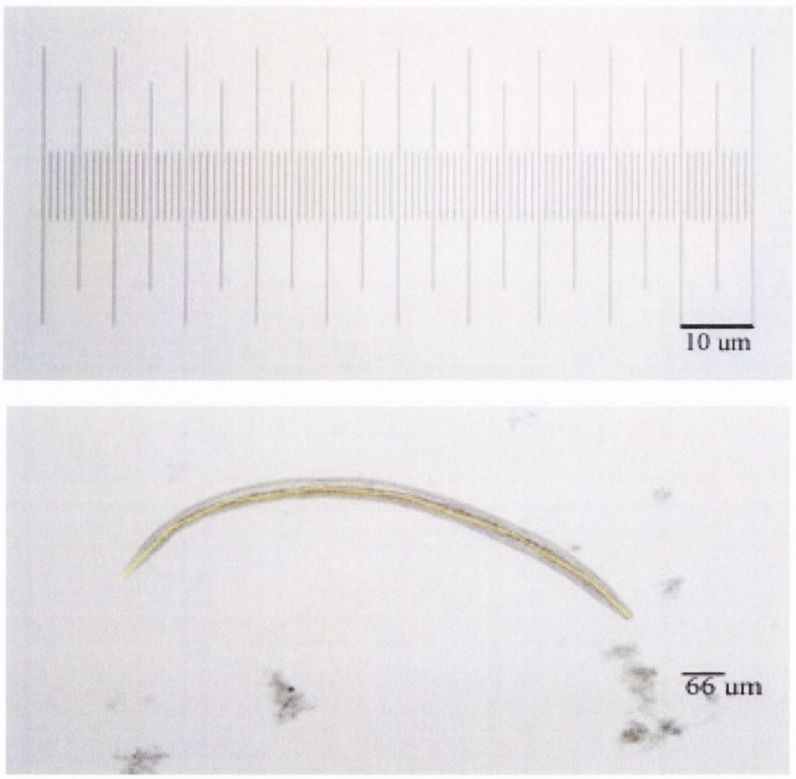


Figure 4.2A.1: (**a**) Photomicrograph of calibration slide used to convert the actual lengths observed under the x10 magnification of phase contrast microscope to the number of pixels in imageJ (**b**) Image taken from Image J programme of larva length measurements using a segmented line (yellow band) through the centre of the larva to determine body length.

STATISTICAL ANALYSIS

Statistitical analysis of larval recovery was similar to previous chapters. Continuous variables were examined by Spearman's rank correlation. Similar statistical analysis was performed on the larval length data. The influence of the various factors on larval burden and size were analysed by means of analysis of variance. Statistical analysis was carried out at a confidence limit of 95% (α= 0.05).

GENERAL OBERVATIONS

The outcome of this experiment was analysed in two stages. First the dose response with single pulse infections (100, 500, 1000 and 3000 larvae) administered to C57BL/6j and CBA/Ca mice was analysed. As previously, there was a highly significant difference between the strains with time and dose taken into consideration (**fig. 4.3A.1**, 2-way ANOVA with strain*time + dose as a covariate, model R^2= 0.70, main effect of strain $F_{1,112}$=30.4, $P \leq 0.001$). As in both of the earlier chapters, the arithmetic mean worm burdens were highest on day 7 in C57BL/6j mice at all four doses. This was also the case in the lower three doses (100, 500 and 1000 eggs) of CBA/Ca mice. There was an exception in CBA/Ca mice inoculated with 3000 eggs with the highest level of infection observed on day 6 (**fig. 4.3A. 2**). Unexpectedly, there was a positive dose dependent increase in the percentage of the initial inoculum recovered, on the day of peak accumulation, with increasing dose size (**fig. 4.3A.1**). In C57BL/6j mice percentage recoveries rose from 7.2% in mice given just 100 larvae to 21.6% in those given 3000. Similarly in CBA/Ca mice there was also a trend of increasing worm recoveries from 2.4% to 7.2%. However, the percentage difference in worm burdens between C57BL/6j and CBA/Ca mice was independent of doses of larvae administered, and ranged from 66.6-78.9% (**fig. 4.3A.1**; Spearman's rank correlation on day 7 p.i., R_S= 0.179, n=20, P=0.45). Although the percentage of the initial inoculum recovered increased within the strains, the percentage difference between the two strains remained constant.

The second stage of the analysis was confined to mice that had received 1000 eggs but either as a single dose, as two doses or as four doses (**table 4.3A.3**). The split dose regime preserved the variation between the two strains carrying significantly different worm burdens on different days after infection, especially on day 6 rather than on day 7. In the split dose groups, the pattern of recovery differed to the single inoculum groups with the maximum burdens observed on day 6 and decreasing thereafter. The percentage difference was 69% (500 x 2 dose group) and 76% (250 x 4) on this day, which is a similar percentage difference when compared to the single inoculum groups. Only in the single pulse infection was the peak accumulation of larvae in the lungs evident on day 7 in C57BL/6 mice. There was also a highly significant effect of time ($F_{2,70}$=6.8, P=0.002), with worm burdens generally declining across the three day period of the experiment.

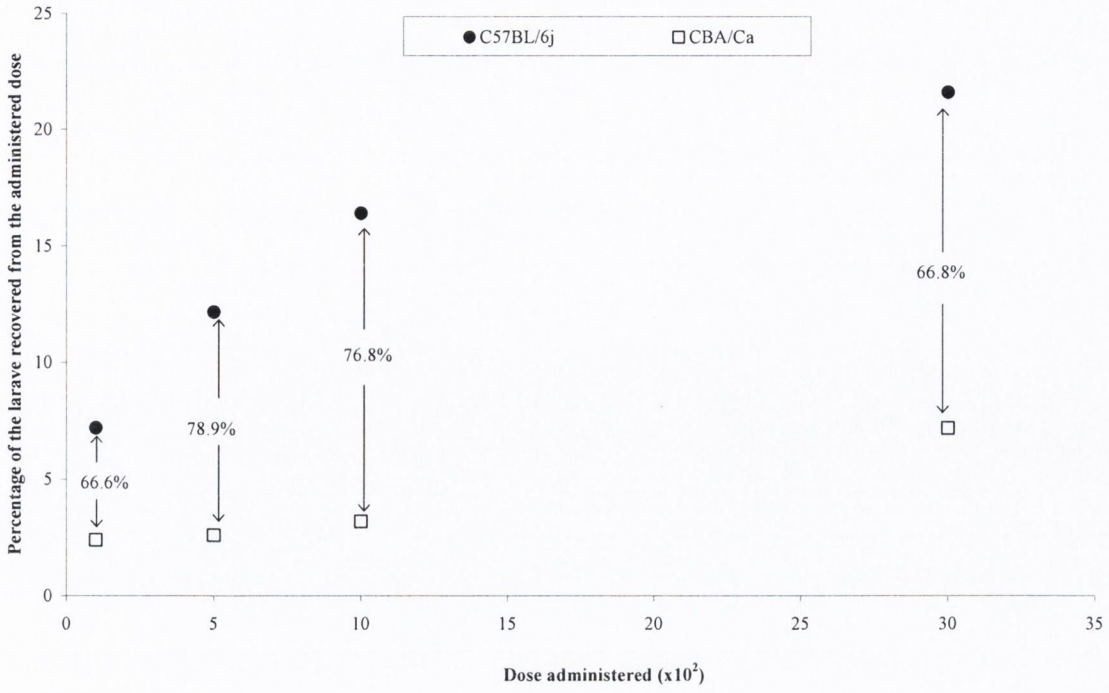


Figure 4.3A.1: The percentage of larvae recovered on day 7 p.i. from the initial inoculation doses of 100, 500, 1000 and 3000 from the lungs of the mouse strain C57BL/6j (•) and the strain CBA/Ca (□) and the percentage difference. (Percentage difference between the two strains was calculated as: {Total number of larvae recovered from C57BL/6j- Total number of larvae recovered from CBA/Ca}/Total number of recovered from C57BL/6j).

A. SUUM EGGS ADMINISTERED IN SINGLE INOCULUMS

Mice administered with 3000 *A. suum* ova

The larval burden of the two strains of mice (C57BL/6j and CBA/Ca) were analysed at each dose level to determine the significance of the results observed for each group for each inoculum both within the strains and between the strains. In terms of the pattern of larval migration, the strain C57BL/6j once again showed a similar pattern of migration to previous results (See **fig. 4.3A.2**). The percentage of the initial inoculum recovered from this organ on day 7 p.i. was 21.6% (**table 4.3A.2**). This percentage recovery was considerably greater than the mean percentage recovered in the previous chapters with mice inoculated with 500 *A. suum* ova (14.7 and 13.2%, respectively). There was variation in the number of larvae recovered between the left and right hand side lung of C57BL/6j mice, with consistently more larvae recovered from the right hand side (**table 4.3A.2**; 2-

way ANOVA with side of organ and day as factors and no interactions, model R^2_{adj}=0.30, main effect of organ $F_{1,26}$=14.5, P=0.0006) but there was no significant effect of day on the number of larvae recovered (main effect of day $F_{2,26}$=0.92, P=0.76). This predilection of larvae for a particular side of the lung was the same on all post-mortem days (**table 4.3A.2**). The term organ*day was removed from the model as it had no significant effect on the observed worm numbers, and it was removed from all further analysis relating to variation in the side of the organ preferred by larvae. The body weight of the strain C57BL/6j decreased throughout the three days post-mortem from the pre-infection body weight at this egg dosage level (**fig. 4.3A.4a**).

The mean worm burden of CBA/Ca mice administered with 3000 ova was highest on day 6 and decreased thereafter. The percentage of the initial inoculum recovered on day 7 p.i. was 7.2% (**table 4.3A.2**). Once again there was a divergence in the accumulation of worms into the two sides of the lungs with larvae preferentially migrating in the right hand side (**table 4.3A.2**; 2-way ANOVA with organ side and day as factors and no interactions, model R^2_{adj}=0.48, main effect of organ side $F_{1,26}$=24.8, $P\leq$0.0001) but there was no significant effect of day on larval recovery. Therefore there was no significant difference between day 6 and 7. The pre-infection weight of CBA/Ca mice was heavier than that of C57BL/6j mice. There was an initial weight gain up to day 6 and CBA/Ca mice lost weight on the latter days of infection (**fig. 4.3A.4b**). The strain CBA/Ca had significantly less larvae in the lungs during the period of infection in comparison to C57BL/6j (**table 4.3A.2**, full factorial 2-way ANOVA, model R^2_{adj}=0.49, main effect of strain, $F_{1,24}$=26.1, $P\leq$0.0001). Thus the relationship between these two phenotypes was maintained at the higher dose level. The percentage difference between the two strains on day 7 was 66.8% (**fig. 4.3A.1**). There was no significant effect observed with day or the interaction between strain and day either (**fig. 4.3A.2**).

Mice administered with 1000 *A. suum* ova

Larval accumulation in the lungs of C57BL/6j followed the prescribed migratory pattern with maximum accumulation on day 7 (**fig. 4.3A.2**) at this dosage level. The percentage of the initial inoculum recovered was lower than the previous dose (**table 4.3A.2**). An analysis of the effects of the factors of time (day) and side of organ (left and right hand side lung) revealed a significant influence of these explanatory factors on worm burdens within this strain (2-way ANOVA with day and organ and no interaction, model R^2_{adj}=0.51, main effect of side of organ, $F_{1,26}$=17.6, P=0.0002) with a higher percentage of

the initial inoculum accumulating in the right side compared to the left side of this organ, particularly on day 7 p.i. (**table 4.3A.2**). There was a significant effect of day on the observed larval burden (main effect of day, $F_{2,26}$=7.85, P=0.0021). The body weight of mice in this group increased on day 6 in contrast to an overall reduction on day 7 and 8 (**fig. 4.3A.4a**), during which period the larval burden was highest.

In contrast to the higher inoculum group, the percentage of larvae recovered from the initial inoculum was reduced in CBA/Ca mice at this infection level also and this was proportional to the decrease observed in C57BL/6j mice, thus the percentage difference between the two strains was maintained (**fig. 4.3A.1**). The pattern of migration was consistent with previous results with the culmination of larvae on day 7 and lower larval numbers observed on the days either side in CBA/Ca mice (**fig. 4.3A.2**). The predilection towards the right hand side lung was still statistically evident (2-way ANOVA with organ and day as factors, model R^2_{adj}=0.13, main effect of organ $F_{2,26}$=6.69, P=0.016) as the larval burdens were still large enough to highlight the discrepancy between the two sides of this organ (**table 4.3A.2**). CBA/Ca mice inoculated with 1000 ova tended to increase body weight between the pre- and post-infection measurements of body weight at this reduced dose level on all days post-infection (**fig. 4.3A.4b**). Strain was highly significant factor determining the larval burdens at this dosage level with CBA/Ca mice harbouring fewer larvae than C57BL/6j (2-way ANOVA with strain*day, model R^2_{adj}=0.63, main effect of strain $F_{1,24}$=33.8, $P\leq$0.0001) but day also had a significant effect on the variation in larval burdens (main effect of day, $F_{2,24}$=5.8, P=0.0086). The pattern of recovery differs between the two strains (**fig. 4.3A.2**; 2-way interaction strain*day interaction $F_{2,24}$=4.4, P=0.024).

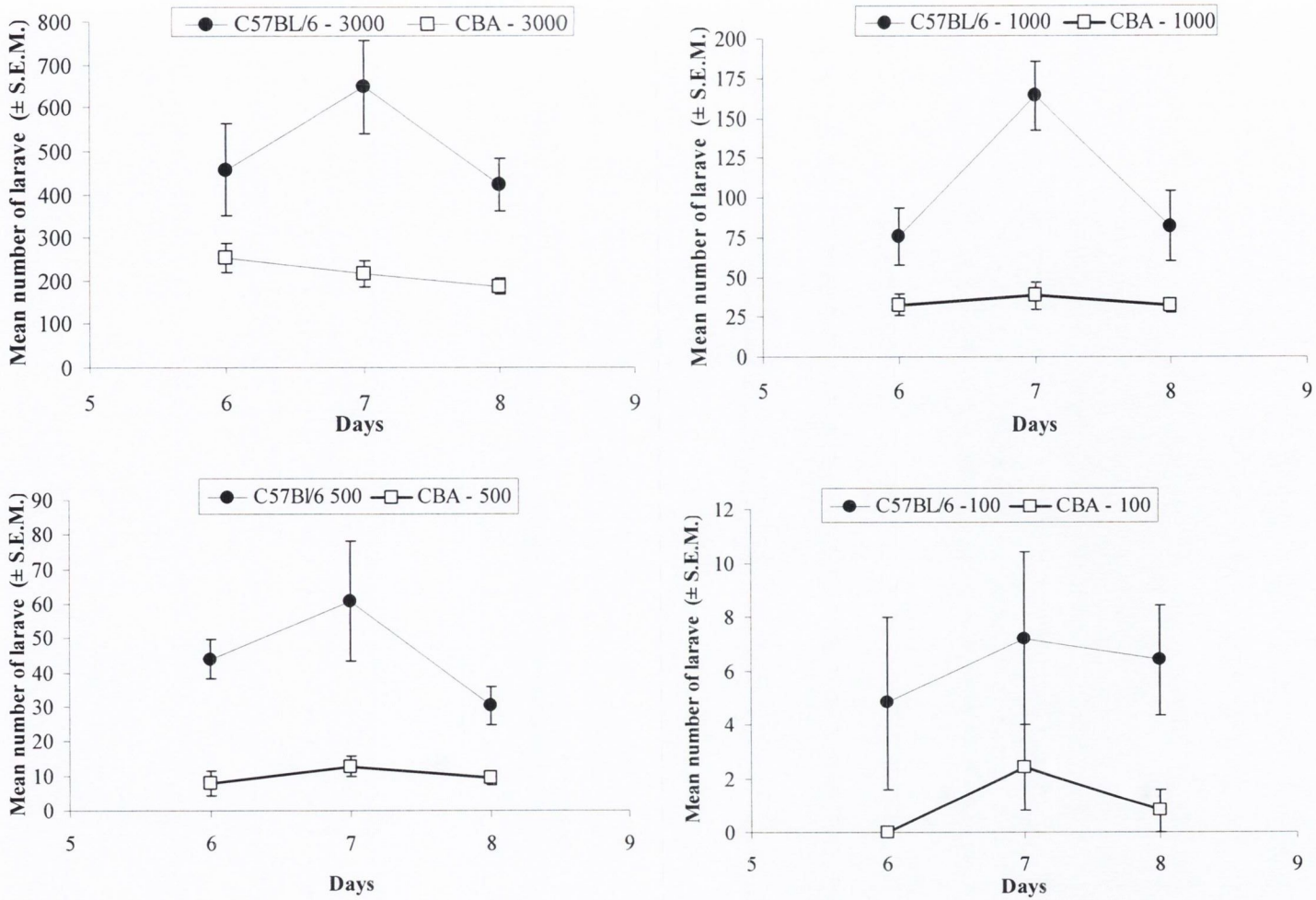


Figure 4.3A.2: The mean number (±S.E.M.) of larvae recovered from the lungs of both C57BL/6j and CBA/Ca mice for each of the single pulse infections (3000, 1000, 500 and 100 *A. suum* ova) on the three days of post-mortem (days 6-8).

Mice administered with 500 *A. suum* ova

The result of this dosage group offered comparative data between the present study and the preceding chapters with regards to larval recovery in the lungs. **Figure 4.3A.3** contrasts these results to those of the previous chapters in the lungs of C57BL/6j. These observations suggested that, throughout the three experimental procedures, larval burdens remained constant and the arithmetic mean worm burden was consistently highest on day 7. Again there was a constant percentage difference between C57BL/6j and CBA/Ca (**fig. 4.3A.1**). This was also comparable to the results of the previous chapters with a percentage difference of 67.4% and 69.7%, respectively. An interesting comparison between the procedures was the number of larvae per gram of mouse body weight as the pre-infection mouse body weight varied between experimental groups (**table 4.3A.1**). Although the average body weight of mice varied between the experimental procedures, the number of larvae per gram revealed that all groups had very similar numbers of larvae irrespective of the average body weight of the group on day 7 (**table 4.3A.1**). The analysis of preferential accumulation in the lung of C57BL/6j mice showed that once again larvae favoured the right hand side particularly on day 7 (**table 4.3A.2**; 2-way ANOVA with no interactions, model R^2_{adj}=0.298, main effect of side of organ $F_{1,26}$=9.879, P=0.0041) The reduction in larval burden was also represented in a change in the weight gain/loss pattern. In this group the majority of mice gained weight on the initial days, but this weight gain was reduced on the latter day (**fig. 4.3A.4a**).

CBA/Ca mice also demonstrated an analogous number of larvae in contrast to the previous investigations (**fig. 4.3A.3**). There was no significant difference in the observed worm burden between the two sides of the lung (2-way ANOVA with no interactions, model R^2_{adj}=0.34, main effect of organ, $F_{1,26}$=2.1, P=N.S.) and this supported the suggestion that at lower worm burdens the preferential accumulation can be difficult to determine. Although this was not statistically significant, the predisposition for the right hand side of the lung was still marked (**table 4.3A.2**). Finally, the increase on all post-mortem days between the pre- and post-infection body weight, (**fig. 4.3A.4b**) highlights the impact of larval burden on body weight. The results that the number of successfully migrating larvae has consequences for body weight were increasingly evident as the size of the dose was reduced (**fig. 4.3A.4**) and thus mice with lower burdens were less likely to show weight loss. The most important explanatory factor that described worm burden was the effect of strain with C57BL/6j mice once again harbouring greater pulmonary larval numbers (**fig. 4.3A.2**; full factorial 2-way ANOVA with strain and day as factors on worm burdens,

model R^2_{adj}=0.52, Min effect of strain $F_{1,24}$=28.4, $P \leq 0.0001$). Again, the day and the strain*day interaction did not influence the model but the inclusion of these factors was the best descriptive model of the overall change in worm burdens.

Table 4.3A.1: The mean number of larvae recovered from the lungs per gram of mouse body weight (± S.E.M.) from each strain; C57BL/6j and CBA/Ca on day 6-9 post-inoculation in three separate experimental procedures (Chapter 2 (1); Chapter 3 (2); Chapter 4 (3)) from an initial inoculation dose of 500 *A. suum* eggs.

Strain	Day 6	Day 7	Day 8	Day 9
C57BL/6j (1)	3.11 (±0.25)	3.21 (±0.39)	2.19 (±0.29)	1.25 (±0.22)
C57BL/6j (2)	1.90 (±0.40)	2.83 (±0.33)	1.98 (±0.46)	1.48 (±0.16)
C57BL/6j (3)	2.51 (±0.35)	3.41 (±0.96)	1.87 (±0.36)	
CBA/Ca (2)	0.74 (±0.22)	0.95 (±0.12)	0.58 (±0.10)	0.32 (±0.08)
CBA/Ca (3)	0.35 (±0.15)	0.55 (±0.13)	0.42 (±0.08)	

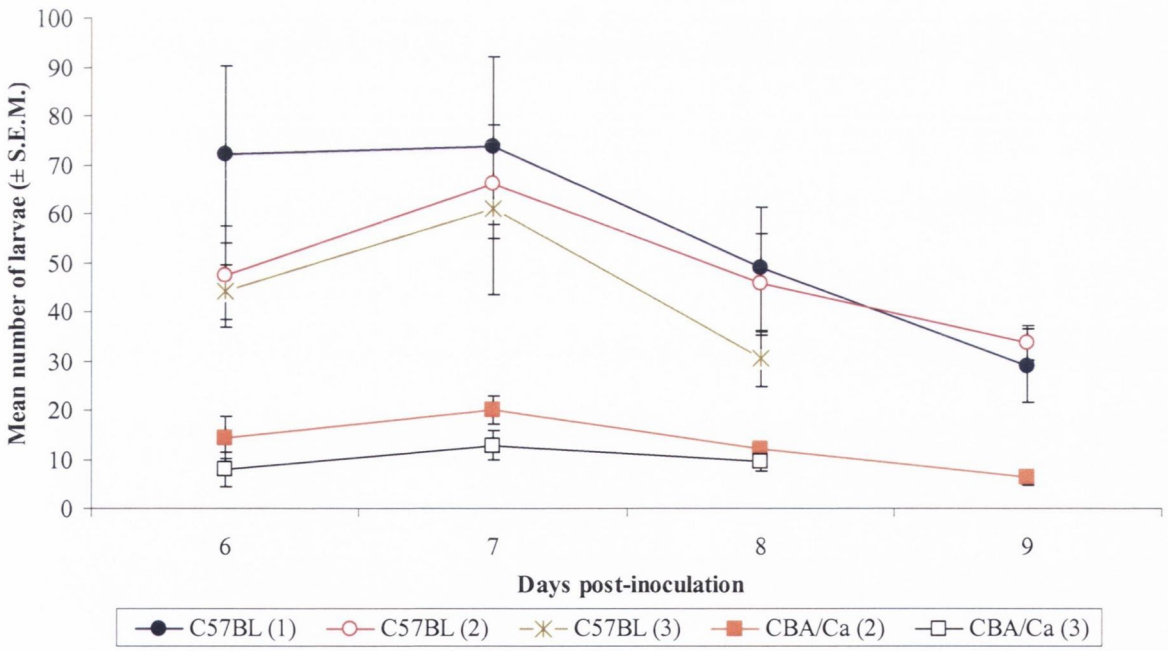


Figure 4.3A.3: The mean number of larvae (±S.E.M.) recovered from the lungs of the two strains of mice (C57BL/6j and CBA/Ca) on days 6-9 post-inoculation in three separate experimental procedures (Chapter 2 (1); Chapter 3 (2); Chapter 4 (3)) inoculated with an initial dose of 500 *A. suum* eggs.

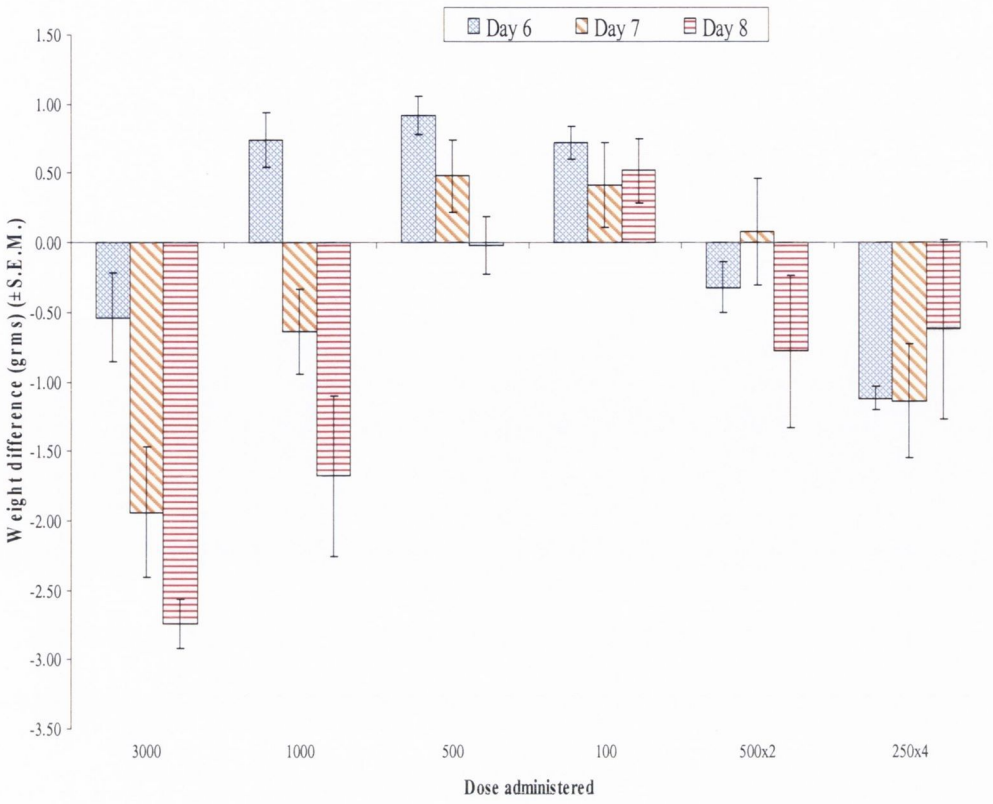
Mice administered with 100 *A. suum* ova

The final single pulse dose group was mice administered with 100 eggs. The trend in the reduction of the percentage of the initial inoculum recovered from the lungs in both mouse strains at this infection level was continued; however, the percentage difference between the two strains remained the same on the day of maximum accumulation (**fig. 4.3A.1**). The number of larvae that were successfully recovered from the lungs of C57BL/6j was once again highest on day 7 (**fig. 4.3A.2**). There was no significant effect on larval burdens from the side of the lung that larvae were recovered from and no day effect (**table 4.3A.2**). Interestingly, there was weight gain in all the subgroups of mice at the 100 dose level (**fig. 4.3A.4a**) suggesting that there was no effect of the lower larval burden on the condition of mice.

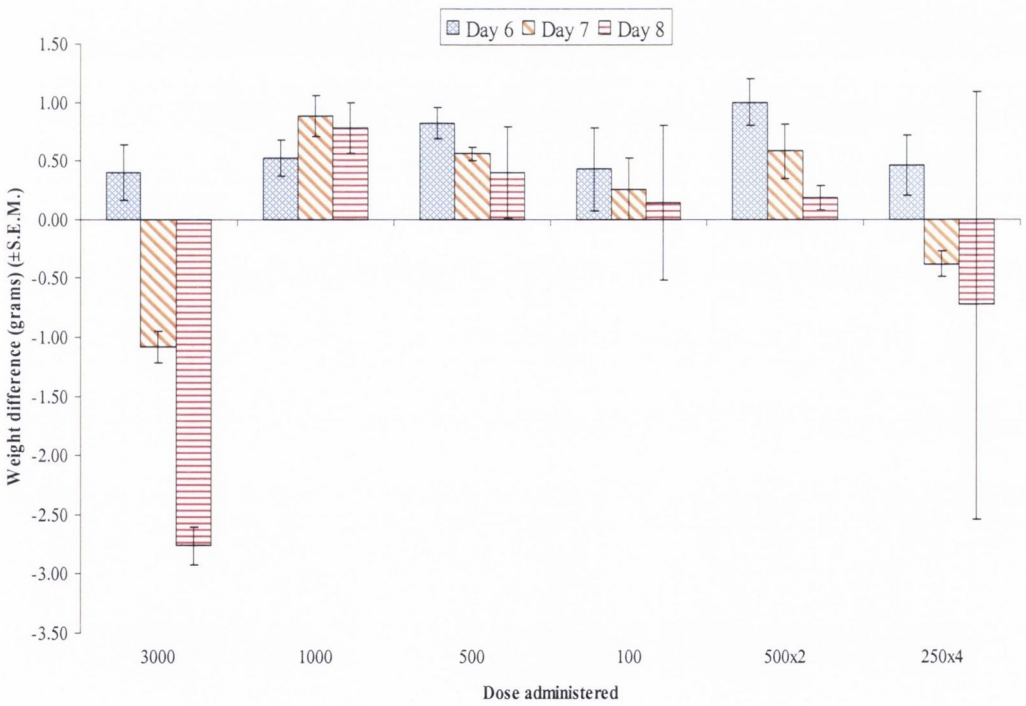
The strain CBA/Ca had significantly less larvae in their lungs in contrast to the susceptible group at this dosage level (**fig. 4.3A.2**). The effect of side of the lung was insignificant in CBA/Ca mice. However, the disparity between the two sides was still evident when the arithmetric mean number of larvae in the two sides was examined (**table 4.3A.2**). There was a gain in the average body weight of mice on all post-mortem days for CBA/Ca mice (**fig. 4.3A.4b**). Although the number of worms in the lungs at this stage was very low, the effect of strain remained highly significant (full factorial 2-way with strain and day as factors, model R^2_{adj}=0.214, main effect of strain $F_{1,23}$=7.121, P=0.0137). There was no effect of day and the interaction between these two factors on larval burdens.

Table 4.3A.2: A comparison of the number of worms recovered (±S.E.M.) from the left and right hand side of the lung in both mouse strains (C57BL/6j and CBA/Ca) for each of the single pulse infections (3000, 1000, 500 and 100 *A. suum* ova) on the three post-mortem days (days 6-8). Also shown is the percentage of the initial inoculum recovered from the total lung (±S.E.M.).

Strain	Day	n	Dose	Left lung	Right lung	Lung total	Percentage recovered from the lung
C57BL/6j	6	5	**3000**	159.2 ±41.1	297.6 ±74.5	456.8 ±106.4	15.2 ±3.5
	7	5		185.6 ±37.6	461.6 ±74.3	647.2 ±108.4	21.6 ±3.6
	8	5		152.8 ±21.3	269.4 ±42.9	422.2 ±60.1	14.1 ±2.0
CBA/Ca	6	5		86.40 ±14.2	165.60 ±27.3	252.0 ±33.9	8.4 ±1.1
	7	5		76.00 ±7.8	139.20 ±25.2	215.2 ±30.3	7.2 ±1.0
	8	5		60.00 ±9.5	127.20 ±12.8	187.2 ±9.6	6.2 ±0.7
C57BL/6j	6	5	**1000**	27.2 ±7.09	48.8 ±11.6	76 ±18.0	7.6 ±1.8
	7	5		44.8 ±10.2	119.2 ±14.9	164 ±21.6	16.4 ±2.2
	8	5		25.6 ±6.9	56.8 ±15.6	82.4 ±22.2	9.0 ±1.6
CBA/Ca	6	5		13.6 ±4.8	19.2 ±3.4	32.8 ±6.6	3.3 ±0.7
	7	5		16.8 ±4.6	21.6 ±4.8	38.4 ±8.5	3.8 ±0.9
	8	5		8.8 ±1.5	23.2 ±3.2	32.0 ±4.2	3.2 ±0.4
C57BL/6j	6	5	**500**	12.8 ±4.5	31.2 ±5.3	44.0 ±5.7	8.8 ±1.1
	7	5		16.8 ±6.1	44.0 ±11.7	60.8 ±17.3	12.2 ±3.5
	8	5		12.8 ±2.3	17.6 ±4.5	30.4 ±5.6	6.1 ±1.1
CBA/Ca	6	5		4.0 ±2.5	4.0 ±1.8	8.0 ±3.6	1.6 ±0.7
	7	5		4.8 ±1.5	8.0 ±1.8	12.8 ±2.9	2.6 ±0.6
	8	5		3.2 ±1.5	6.4 ±1.6	9.6 ±2.0	1.9 ±0.4
C57BL/6j	6	5	**100**	3.2 ±3.2	1.6 ±1.6	4.8 ±3.2	4.8 ±3.2
	7	5		4.0 ±1.8	3.2 ±2.0	7.2 ±3.2	7.2 ±3.2
	8	5		1.6 ±1.0	4.8 ±1.5	6.4 ±2.4	6.4 ±2.0
CBA/Ca	6	4		0	0	0	0
	7	5		0	2.4 ±1.6	2.4 ±1.6	2.4 ±1.6
	8	5		0	0.8 ± 0.8	0.8 ±0.8	0.8 ±0.8



A. C57BL/6j



B. CBA/Ca

Figure 4.3A.4: Mean body weight difference (grams) (±S.E.M.) between pre-infection (time zero) and the day of post-mortem (±S.E.M.) in both the **(A)** C57BL/6j mouse strain and **(B)** CBA/Ca mouse strain, on each post-mortem day.

Administration of 1000 eggs in two doses of 500 eggs (500 x 2)

The dose 500 x 2 was administered on two consecutive days (day 0 and 1) and mice were euthanased on days 6, 7 and 8, determined from the initial inoculation. The pattern of accumulation of larvae in the strain C57BL/6j was different to previous observations with larvae accumulating on day 6 and decreasing thereafter (**fig. 4.3A.5a**). The maximum worm burden was lower than the single inoculum of 1000 eggs (**table 4.3A.3**). The percentage of the initial inoculum recovered from this regime was analogous to the 500 dose regime (**table 4.3A.2** and **table 4.3A.4**). Migratory success of larvae was reduced when mice were exposed in this way to a large dose administered over a number of days in smaller inoculums (12.2 ±3.14). A significant effect of the side of the lung on worm burden was observed in C57BL/6j mice, with larvae preferentially accumulating in the right hand side lung (**table 4.3A.4**: 2-way ANOVA with organ and day as factors on worm burden with no interactions, model R^2_{adj}= 0.29, main effect of organ $F_{1,26}$=6.17, P=0.0197). The lung worm burdens differed significantly, associated with day (main effect of day $F_{2,26}$=4.43, P=0.022). Mouse body weight decreased during the examination period (**fig. 4.3A.4a**).

The maximum percentage of the initial inoculum (3.84 ±0.69) recovered from CBA/Ca mice was equivalent to the single inoculum group of 1000 eggs (**table 4.3A.3**) with a similar pattern of migration (**fig. 4.3A.5a**). There was a significant distinction between the left and right lung with more larvae once again accumulating in the right hand side (**table 4.3A.4**: 2-way ANOVA with organ and day, model R^2_{adj}=0.20, main effect of organ, $F_{1,26}$=7.6, P=0.0107). The reduced worm burden was reflected in the overall gain in mouse body weights on all post-mortem days (**fig. 4.3A.4b**). There was a highly significant difference between the two mouse strains with day taken into consideration (**fig 4.3A.5a**; 2-way ANOVA with strain and day as factors, model R^2_{adj}=0.33, main effect of strain $F_{1,24}$=8.95, P=0.0063) with more larvae accumulating in the lungs of C57BL/6j. However, day was insignificant ($F_{2,24}$=3.2, P=0.06) and there was no significant interaction between strain and time. Again, there was a consistent effect on worm burdens between the strains and the percentage difference between the two strains on day 6 was 69.7%.

Administration of 1000 eggs in four doses of 250 eggs (250 x 4)

The dose of 250 x 4 eggs was administered on four consecutive days (day 0, 1, 2 and 3) and the mice were euthanased on days 6, 7 and 8, determined from the initial inoculum. As observed in the previous dose group, larvae accrued in the lungs of C57BL/6j mice on day 6 and declined thereafter (**fig. 4.3A.5b**). The percentage of the total inoculum recovered was greatly reduced in contrast to the single administration of 1000 ova showing a further effect on worm burdens when the dose is administered over an extended period (**table 4.3A.3**). This percentage of the total administered inoculum was similar to the recovery rate of the 100 egg dose administered as a single inoculum (**table 4.3A.2**). The side of the lung had no effect on larval burdens in C57BL/6j (2-way ANOVA with day and organ side as factors, model R^2_{adj}=0.305, main effect of organ $F_{1,24}$=2.7, P=N.S.) but there was a significant day effect ($F_{2,24}$=6.1, P=0.0073). However, the examination of the arithmetic means showed that there was an apparent predilection for the right hand side (**table 4.3A.4**). Weight loss was observed in this group over the three day period (**fig. 4.3A.4b**).

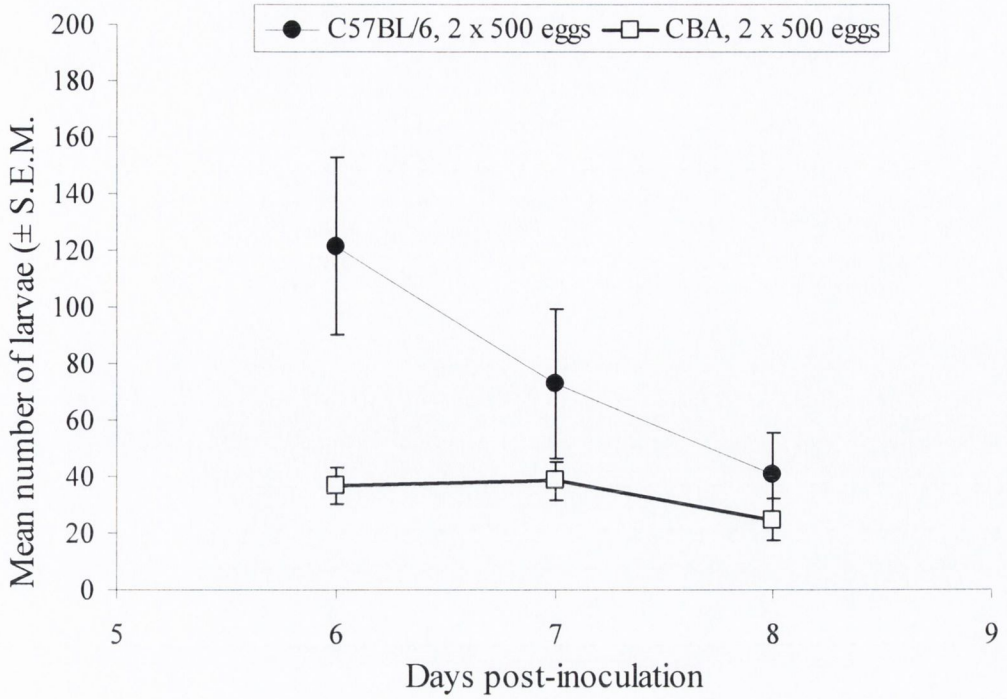
The strain CBA/Ca showed a reduced percentage of the initial inoculum recovered in contrast to the previous dosage group (**table 4.3A.3**). The pattern of migration did not differ to previous dosage groups in CBA/Ca with the highest larval burden observed in the lungs on day 7 (**fig. 4.3A.5b**). There was a significant effect of organ (left and right side of the lung) (**table 4.3A.4**: 2-way ANOVA with organ and day as factors, model R^2_{adj}=0.20, main effect of organ $F_{1,24}$=5.8, P=0.024) even though low numbers of worms were present. Finally, the latter days (7 and 8) in this group showed reductions in mouse body weight (**fig. 4.3A.4b**) but the high degree of variation on day 8 between individual mice influenced these results. This was due to the gain in weight of one individual of 1.9 grams while another individual lost 6 grams between the pre-infection (day 0) and post-infection day (day 8). The results of analysis between the strains revealed that strain of mouse was a significant factor on larval burden (**fig. 4.3A.4b**; 2-way ANOVA with strain*time as factors, model R^2_{adj}=0.501, main effect of strain $F_{1,22}$= 11.1, P=0.003) and larval burden changed significantly over the period of analysis (main effect of day, $F_{2,22}$=6.1, P=0.0078). The marginal effect of the strain*day interaction ($F_{2,22}$=3.5, P=0.048) suggested that larvae moved into the lungs of C57BL/6j faster than in CBA/Ca mice (**fig. 4.3A.5b**). The percentage difference between the two strains was maintained once again (75.95% on day 6) (**fig. 4.3A.5b**).

Table 4.3A.3: A comparison of the percentage of the initial inoculum of worms recovered (±S.E.M.) from the lungs of both strains of mice (C57BL/6j and CBA/Ca) administered with either a single inoculum of 1000 ova and those inoculated with a split dose of either 250 x 4 or 500 x 2 of *A. suum* ova during the infection period (day 6-8 p.i.).

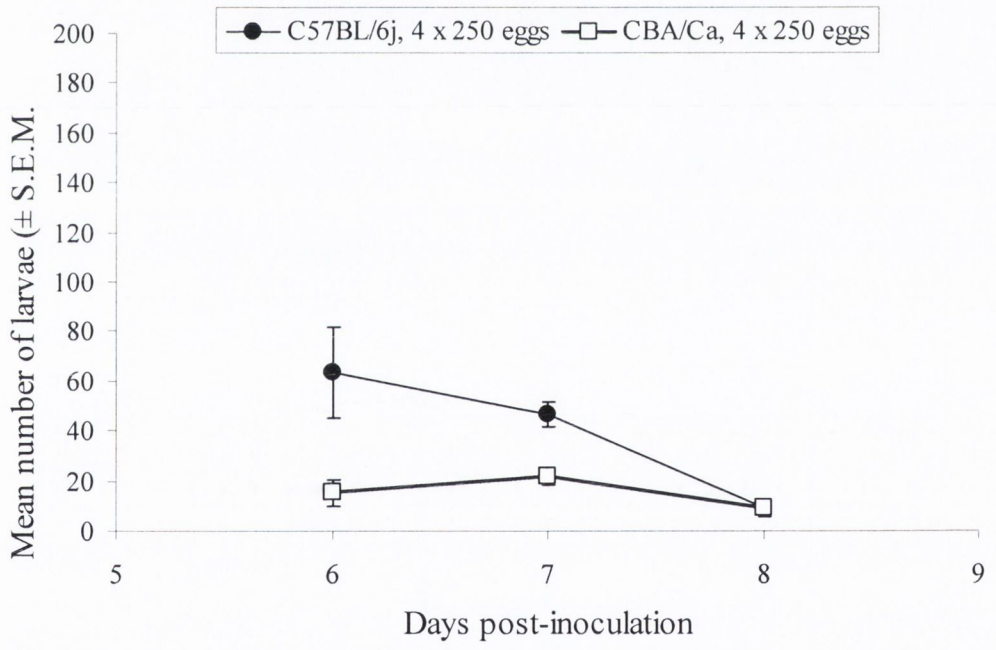
Strain	day	n	Lung total 1000	Lung total 500 x 2	Lung total 250 x4
C57BL/6j	6	5	7.6 ±1.8	12.16 ±3.14	6.32 ±1.82
	7	5	16.4 ±2.2	7.28 ±2.63	4.64 ±0.48
	8	5	8.96 ±1.6	4.08 ±1.47	0.90 ±0.34
CBA/Ca	6	5	3.3 ±0.7	3.68 ±0.67	1.52 ±0.54
	7	5	3.8 ±0.9	3.84 ±0.69	2.16 ±0.33
	8	5	3.2 ±0.4	2.48 ±0.73	0.90 ±0.19

Table 4.3A.4: Mean number of larvae (± S.E.M.) recovered on each post-mortem day from the organs of both C57BL/6j and CBA/Ca mice inoculated with 250 ova on four occasions and those inoculated with 500 ova on two days. Also shown is the percentage of the initial inoculum recovered from the total lung (±S.E.M.).

Strain	day	n	Dose	Left lung	Right lung	Lung total	Percentage recovered from the lung
C57BL/6j	6	5	**500 x 2**	38.4 ±10.63	83.2 ±22.75	122 ±31.4	**12.16 ±3.14**
	7	5		22.4 ±10.48	50.4 ±16.33	72.8 ±26.3	**7.28 ±2.63**
	8	5		15.2 ±6.25	25.6 ±9.77	40.8 ±14.7	**4.08 ±1.47**
CBA/Ca	6	5	**500 x 2**	12.8 ±2.94	24.0 ±4.20	36.8 ±6.74	**3.68 ±0.67**
	7	5		12.0 ±4.00	26.4 ± 5.46	38.4 ±6.88	**3.84 ±0.69**
	8	5		9.6 ±2.04	15.2 ±7.63	24.8 ±7.31	**2.48 ±0.73**
C57BL/6j	6	5	**250 x 4**	29.6 ±12.37	33.6 ±10.70	63.2 ±18.2	**6.32 ±1.82**
	7	5		12.0 ±1.26	34.4 ±5.31	46.4 ±4.82	**4.64 ±0.48**
	8	4		3.0 ±1.00	6.0 ±2.58	9.0 ±3.42	**0.90 ±0.34**
CBA/Ca	6	5	**250 x 4**	4.8 ±1.50	10.4 ±4.49	15.2 ±4.43	**1.52 ±0.54**
	7	5		9.6 ±0.98	12.0 ±2.83	21.6 ±3.25	**2.16 ±0.33**
	8	4		2.0 ±1.15	7.0 ±1.91	9.0 ±1.92	**0.90 ±0.19**



A.



B.

Figure 4.3A.5: The mean number (±S.E.M.) of larvae recovered from the lungs of both C57BL/6j and CBA/Ca mice administered with either a trickle dose of 250 x 4 (A) or 500 x 2 (B) *A. suum* ova during the infection period (days 6-8 p.i.).

The effect of single dose inoculums on larval numbers

A 2-way analysis was utilised to examine the effect of the three explanatory factors; time (day), strain and dose (single dose inoculums) as a covariate on the pulmonary worm burdens. There was a significant effect of strain on larval burdens using this model (**fig. 4.3A.1**, 2-way ANOVA with strain*time + dose as a covariate, model R^2= 0.70, main effect of strain $F_{1,112}$=30.4, $P \leq 0.0001$) but no significant interaction between strain and time. Because this experiment was restricted to just 3 days, and given the variation in recovery within sub groups of mice, overall worm burdens did not vary significantly with time (main effect of days $F_{2,112}$=2.4, P=N.S.).

A more accurate statistical examination involves the analysis of the percentage of the initial inoculum recovered. This reflects the precise relationship between the dose size and their effect on larval burden in the mouse lung and is not an artefact of increased numbers caused by higher dose regimes. Again a full factorial model was proposed using all the factors involved with dose as a covariate. This analysis once again demonstrated the strong effect of strain (2-way ANOVA with strain*time + dose as a covariate, model R^2_{adj}= 0.59, main effect of strain $F_{1,112}$=89.7, $P \leq 0.0001$). The variation associated with day remained significant also (main effect of day, $F_{2,112}$=7.1, P=0.0013) and there was a significant effect associated with the interaction between time and strain (interaction $F_{2,112}$=3.8, P=0.025) and this suggested that the percentage of the initial inoculum recovered varied between the strains on the days under investigation.

There was a highly significant positive relationship between the dose of eggs administered and the worm burdens in the combined data set with strain and time taken into account (Effect of dose $F_{1,114}$=242.1, β=0.129, t=15.6, $P<0.001$) and *post hoc* within each of the two strains when analysed separately (1-way ANOVAs with time as a factor day and dose as a covariate, main effect of dose, β=0.179, R^2_{adj}=0.848, t=13.98 C57BL/6j mice and β= 0.079, R^2_{adj}=0.903, t=18.68, for CBA/Ca and $P<0.0001$ in both cases). This relationship was maintained when the analysis was based on the percentage of the initial inoculum recovered at each dosage level within the strains (1-way ANOVAs with time as a factor day and dose as a covariate, main effect of dose, β=0.003, R^2_{adj}=0.389, t=5.3 C57BL/6j mice and β= 0.002, R^2_{adj}=0.615, t=9.7, for CBA/Ca and $P<0.0001$ in both cases). This positive effect of increasing dose on the percentage of the initial inoculum recovered on the

day of peak accumulation was an unexpected result (**fig. 4.3A.1**). Nevertheless, the percentage difference between C57BL/6j and CBA/Ca mice in terms of worm burdens was constant and dose-independent (**fig. 4.3A.1**; Spearman's rank correlation on day 7 p.i., R_S= 0.179, n=20, P=0.45). Thus the disparity between the strains was maintained across the range of infection doses examined, leading to the conclusion that dose does not influence the underlying mechanisms determining these two phenotypes but that dose results in increased worm loads.

The effect of single dose inoculum on mouse body weight

There was an apparent effect of body weight (gain/loss) between the single inoculum dosage groups with those mice infected with larger numbers of eggs showing greater weight loss on all post-mortem days. Mouse body weight was analysed for each strain with dose as a factor but not between the strains as inherent strain differences may influence the statistical analysis. Within the strain C57BL/6j, the higher doses (in particular 3000 and 1000) showed a loss in body weight during the three post-mortem days, while the lower dose groups displayed increased body weight (**appendix A.6**). This appeared to be correlated with the number of larvae inoculated and thus the increased number of larvae successfully migrating through the tissues (**fig. 4.3A.4a**; 2-way ANOVA with dose and day as factors, model R^2_{adj}=0.85, main effect of dose, $F_{3,48}$=37.2, $P\leq$0.0001). This was reflected in the *post-hoc* analysis for the C57BL/6j strain which reveals that the 3000 and 1000 dosage groups were significantly different in terms of changes in body weight from each other ($P\leq$0.0001). These groups were also significantly different in relation to the lower infection levels ($P\leq$0.0001). There was no significant difference between the weight of mice inoculated with 100 and 500 ova.

Due to the resistant nature of CBA/Ca mice only the highest dose group is expected to show weight losses, as lower dosage groups had significantly less migrating larvae in contrast to C57BL/6j mice at the same dosage level. Again statistical analysis showed a significant effect of dose and day on the observed weight changes in the lungs of CBA/Ca mice (**fig. 4.3A.4b**: 2-way ANOVA with dose and day as factors, model R^2_{adj}=0.699, main effect of dose, $F_{3,47}$=27.3, $P\leq$0.0001) and this reflected density dependent alterations on weight with greater weight loss in mice exposed to the heaviest inoculum. This was further substantiated by the *post-hoc* analysis with those mice inoculated with 3000 ova having significantly greater weight changes compared to the all other groups ($P\leq$0.0001) and there was no difference between the other three groups. There was also a significant time effect

($F_{2,47}$=9.8, P≤0.0001) and the interaction between these two factors ($F_{6,47}$=7.3, P≤0.0001). However, the effect of time was inconsistent with weights fluctuating up and down during the period of infection. There was no apparent directional pattern to the weight changes over time.

The effect of a split-dose regime of 1000 eggs on larval numbers

The strain difference was preserved irrespective of the number of inoculums administered to the groups i.e. 1000 dose administered over 1, 2 or 4 days (3-way ANOVA, with infection regime, strain and time as factors on worm burdens in the lungs, model R^2=0.547, main effect of strain, $F_{1,70}$=44.7, P≤0.0001), and the two strains were carrying significantly different worm burdens on different days after infection (2 way interaction strain*time, $F_{2,70}$=3.1, P=0.05) and this is reflected in the differences in accumulation between the strains. The 3 infection regimes clearly produced significantly different patterns of worm recovery overall, (main effect of regime, $F_{1,70}$=12.7, P≤0.0001) and on different days after infection (2-way interaction regime*time, $F_{4,70}$=3.17, P=0.019). The two strains of mice behaved differently (2-way interaction strain*regime, $F_{2,70}$=3.93, P=0.02 and 3-way interaction, $F_{4,70}$=2.8, P=0.032) with worm burdens being more consistent over the three day period in CBA/Ca mice compared to C57BL/6j, where worm burdens were considerably higher on day 7 (single dose of 1000 eggs) or day 6 (the other two regimes) compared to day 8. There was also a highly significant main effect of time ($F_{2,70}$=6.8, P=0.002), with worm burdens generally declining across the three day period of the experiment. There was a decrease in the percentage of the total administered inoculum recovered associated with increasing exposure to *A. suum* infective eggs (**table 4.3A.3**). Again there was a consistent percentage difference between the strains, irregardless of the infection regime. This suggests that the relative resistance of these strains is predefined and immunising them by a split dose regime has an equal effect.

4.4A DISCUSSION

The main finding of this chapter was that dose did not influence the relative susceptibility of the two strains. Therefore underlying intrinsic host factors appear to influence the observed phenotypes, as the difference in the migrating larval population between the two strains remained constant irrespective of the initial dose size. Within strain there was a correlation between the inoculum size and the mean number of larvae recovered. This was an inverse density dependent relationship between dose and the percentage of the initial inoculum that was recovered, as this increased with increasing inoculum size. This pattern was observed in both the susceptible and resistant strains of mouse, and was consistent with observations by Mitchell *et al.* (1976) and Johnstone *et al.* (1978) on *A. suum* in the lungs of inbred mice, but in other host-parasite systems it is more usual to observe a reduction in parasite establishment with increasing parasite density, particularly when considering the adult population. Mitchell *et al.* (1976) found that at all doses of embryonated eggs administered, C57BL/6 mice contained many more larvae in their lungs at day 7 than (BALB/c X CBA)F_1 mice but that in both groups the increase in dose resulted in an increase in the number of larvae isolated. This increase was not as great in the resistant group in contrast to the susceptible strain of mice which appeared to have a logarithmic increase in larval numbers associated with dose. These findings were analogous to the present observations with a more exponential increase in the susceptible strain in contrast to a gradual ascent in the resistant group.

It has been observed that the distribution of the individual worm burdens was consistently overdispersed in pig populations and aggregation occurred regardless of the nature, duration, frequency or dose level of the exposure (Roepstorff *et al.*, 1997; Boes *et al.*, 1998a). This suggested that an underlying genetic factor controlling susceptibility to infection was a significant factor in determining parasite burden. Therefore the susceptibility of an inbred mouse strain would be expected to be consistent irrespective of the initial dose administered. Roepstorff *et al.* (1997) investigated the experimental infection of pigs with *A. suum* using single inoculations with three doses of infective eggs. In pigs, the variation in administered dose showed no impact on the rate or success of migration and a trend towards an inverse relationship between dose and the adult worm burden (Roepstorff *et al.*, 1997). This provides further evidence of the inability of the host immune response during the early migratory phase of primary infections to respond to increased levels of *A. suum* in the natural host. Furthermore, pigs are a heterogenous

population which makes susceptibility/resistance difficult to examine consistently. Sample size will also be low due to maintenance constraints. This has implications when examining larval burdens in the population, as a small population may not represent the true parasite distribution. The benefit of employing the mouse model is that a strain may be either a high or low responder and they are consistent in the relative susceptibility to increasing dose size, consequently all individuals of a strain would show a corresponding increases in larval burden associated with larger inoculums.

There have been conflicting views in relation to the administration of different dosages and the successful establishment of *A. suum* in the intestine. The infection of pigs between 1-2 months old from *Ascaris* free swine stocks showed no disparity between three dose groups of; 5000, 1000 and 100 inoculated eggs (Galvin, 1968). Conversely, Andersen *et al.* (1973) found a negative dose dependent relationship between the inoculated egg dose and the numbers of worms found in the intestine. Piglets exposed to doses of 1000, 5000 and 10000 eggs did not establish significant worm burdens (0.013% of the administered dose), in contrast individuals inoculated with 500 ova had significantly higher worm burdens (2.9%), and the percentage of eggs that completed their migration and established as an intestinal worm population from animals administered with 50 eggs was 64%. However, Slotved *et al.* (1996) observed very little disparity between the dose of 1000 (65.6%) and 10000 (71.6%). Slotved *et al.* (1996), in contrast to Andersen *et al.* (1973), recovered significantly fewer larvae from animals inoculated with 100 ova (32%) and this was analogous to more recent findings described above in adult pigs (Roepstorff *et al.*, 1997).

The disparity between Andersen *et al.* (1973) and the more recent studies may have arisen from the use of piglets and in particular those born to sows that were previously infected with *A. suum*. This may be an age-related effect with innate immunity being transferred from the mother to the offspring. Immunoglobulins, parasite circulating antigens, immune cells, cytokines and other cell-related products can be transferred from infected mothers to their young. They can combine their effects to interact with the invading parasites, as well as inducing a continuing immune response to subsequent infections. The transfer of maternal antibodies can occur at a number of sites; such as the placental barrier (Carlier & Truyens, 1995) or via the milk of suckling young (Boes *et al.*, 1999) and across the intestinal barrier. The availability of certain immunoglobulins, for example IgG, in offspring has been estimated around six months after birth in humans. Contradictorily, maternal antibodies can also suppress the synthesis of immunoglobulins by newborn

piglets making them susceptible to infection (Carlier & Truyens, 1995). Recently, the results of experimental infections of *A. suum* in pigs has concluded that maternal exposure alters the distribution of worms in their offspring, in which the duration of exposure appeared to be an important influence (Boes *et al.*, 1999).

There have been more consistent observations in relation to the success of migrating larvae associated with increased inoculum size, particularly in mice. Johnstone *et al.* (1978) administered six different doses of *A. suum* eggs to inbred C57BL/6 mice. The doses administered were; 100, 500, 1500, 3000, 6000, or 9000 ova and larvae were recovered by the spin method from the liver of infected mice 2 days p.i. and from the lungs at 7 days p.i.. Larval numbers displayed logarithmic increases with increasing doses of eggs until 6000 or more eggs were administered at which point there was a plateau of the dose/recovery curve. Thus the dose of 3000 *A. suum* eggs was suggested to offer a satisfactory model for evaluating susceptibility to infection as it was non-lethal and gave larval recoveries on the logarithmic ascent. However, the standard error of this inoculum level was much greater than the lower dose groups. Similarly, mice inoculated with varying doses from 200-3200 infective ova were found to harbour most larvae in their lungs on day 8 when administered the higher dose- 3200 eggs (Massara *et al.*, 1990) although the percentage of the inoculum recovered was low (1.9%). These results corresponded to the observations of the present study with a positive dose-dependent increase in the percentage of larvae recovered from the initial inoculum within the two strains of mice examined.

One plausible explanation for this positive density dependent effect is related to the up-regulation of immune responses which are not associated with host protection against the migrating helminth population. The cascade of events in individuals capable of mounting a strong Th1 response may offer protective immunity to migrating larvae. Inhibition of this response, by migrating larvae or those individuals that are naturally predisposed to increased Th2 responses, produces an inappropriate response to the presence of migrating larvae, thus aiding the larvae in evasion of the immune defences. The Th2 response has been demonstrated to suppress the Th1-type immune responses (Mulcahy *et al.*, 2005). It has been shown, in the pig and mouse systems, that the up-regulation in non-effective T-type helper cells can inhibit appropriate responses and this was influenced by increased numbers of *A. suum* larvae or their antigenic products (Crandall *et al.*, 1978; Barta *et al.*, 1986; Macedo & Barbuto, 1988; Soares *et al.*, 1990; Rhee *et al.*, 1991; Soares *et al.*, 1992; Faquim-Mauro & Macedo, 1998; Faquim-Mauro *et al.*, 1999; Paterson *et al.*, 2002; Souza

et al., 2002; Oshiro *et al.*, 2005). This provides some explanation for the unexpected positive relationship between administered doses and increase the percentage of the initial inoculum recovered. The increased inoculum could lead to greater numbers of larvae migrating across the intestinal wall. This may have resulted in the down-regulation of the immune responses during the latter phase that would eliminate the parasite either due to increased parasitic immunomodulatory effects or suppression by the host of immune responses to prevent immunopathological damage caused by inflammatory responses as a consequence of larger worm burdens. The possible effect of this was a greater percentage of larvae surviving and arriving in the lungs with increasing dose size. This was supported by a parallel effect in both strains whether they were susceptible or resistant. However, there is an underlying factor within the hosts that maintains overall susceptibility/resistance as the relationship between the two strains was constant with a percentage difference ranging from 67-79% irrespective of dose.

The immunomodulatory effect may be related to suppression of the Th1 response to migrating larvae in favour of the inappropriate Th2-like response. Immunosuppressive proteins are present in the different larval stages of the *A. suum* lifecycle (Macedo & Barbuto, 1988; Soares *et al.*, 1990; Rhee *et al.*, 1991; Soares *et al.*, 1992; Faquim-Mauro & Macedo, 1998; Faquim-Mauro *et al.*, 1999; Paterson *et al.*, 2002; Souza *et al.*, 2002; Oshiro *et al.*, 2005). Adult *A. suum* body extract (Asc) is known to down-regulate the specific immune response of DBA/2 mice to ovalbumin and preferentially stimulates a Th2 response to its own components, which is responsible for the suppression of the specific Th1 response (Souza *et al.*, 2002). DBA/2 mice inoculated with the extracts of *A. suum* eggs (E-Asc) exhibited a dose dependent inhibition of hypersensitivity reactions and IgG1 antibody response to ovalbumin. Increased amounts of E-Asc resulted in reduced reactions in the model, and this indicated a significant effect on the Th-1 type cytokines. The highest dose of *A. suum* egg extract, which was most suppressive, induced more IL-4 and IL-10 (Th2 cytokines) and less IL-2 and IFN-γ (Th1 cytokines) whereas the opposing effect was observed for the lowest dose (Ferreira *et al.*, 1995). The extract appears to impair T-cell functions to antigens by inducing a Th2-like response. These observations were similar to the suppressive nature of adult extract (Macedo & Barbuto, 1988).

Challenge and trickle infections represent the study of parasite acquisition under more natural antigenic exposure. Mice have been shown to be resistant to an *Ascaris* challenge infection shortly after primary inoculation or when repeatedly inoculated at short intervals

(Mitchell *et al.*, 1976; Eriksen, 1981). Increased cellular responses were reported in the liver of re-inoculated mice and guinea pigs and in some instances these were associated with encapsulated larvae (Sprent & Chen, 1948; Taffs, 1965). Thus mice exposed to *A. suum* ova over a period of time would be expected to have an increased reaction to migratory stages of *A. suum* and this would be observed as fewer larvae successfully reaching the lungs during the latter stages. Larval burdens in the lung that established as the result of an inoculation divided over time (split doses) exhibited lower numbers compared to equivalent single doses and this was particularly pronounced in the C57BL/6j mice. The underlying reasons may relate to a differential immune response to gradual inoculation or the influence of stress due to increased handling. However it is also a possible artefact of the delayed arrival of larvae from other sites due to the staggered administration of infective ova. A disparity between the regimes that was considered was the effect of time as larvae inoculated on day 2 and 3 may not have had sufficient time to arrive in the lungs thus giving an artefactual disparity between the regimes. However, the effect of administering the split dose regimes did not change the relationship between the strains and C57BL/6j mice remained more susceptible than their resistant counterparts.

The increased exposure over a longer period by administering the 1000 dose over a four day period, although the total inoculum was identical, could stimulate an adaptive response thus reducing the successful penetration of larvae particularly through the liver. Eriksen *et al.* (1992) inoculated pigs with one of two trickle inoculations (either 500 or 25 ova twice weekly administered over a number of weeks) and suggested a dose-dependent effect associated with larval migration through the liver. Liver lesions ('white-spots') of pigs in the high-trickle group showed an initial increase in number but by week six there was a drastic reduction in this pathology. The numbers of 'white-spots' present on the liver is determined by the numbers of larvae successfully reaching the liver. In the absence of host reactions, there should theoretically be an initial increase in the numbers of spots up to a more or less constant level throughout the exposure period. This was the pattern observed in the low-trickle group until a much later stage. Thus a higher dose prevented larvae from reaching the liver. In contrast in the present study, in which the dose was administered over a shorter period, the effect of reducing the number of larvae in the primary inoculation and subsequent doses, in terms of the 250 egg group, resulted in less successful migration in both strains. By reducing the number of eggs the host was exposed to at one time point it could effectively minimise the Th2 response, resulting in a more appropriate Th1 response to migrating larvae and inducing a level of resistance. A contrasting effect was observed by

altering the dose in single pulse infections and increasing successful migration into the lungs of mice inoculated with greater numbers of eggs. This effect of administering a lower dose and thus reducing the antigenic exposure to the parasite has been observed in *Trichuris muris* in the mouse model. Low antigen doses favoured type 1 responses (susceptibility) and high antigen doses favour type 2 responses (resistance) in the GI tract of mice infected with adult *T. muris* (Bancroft *et al.*, 2001).

There was a significant influence on mouse body weight correlated with increased larval burdens during the period of migration in the lungs examined. Weight loss was most significant in the highest inoculation doses and in the lower infection protocols, weight gain was most common. Porcine ascariasis is sub clinical and infection is exhibited by reduced feed to gain ratios and liver condemnation at slaughter (Boes, 1999). A correlation between the numbers of patent worms and reduced weight gain has previously been reported. However, there has been no suggestion that pronounced weight changes are associated with migrating larvae (Hale *et al.*, 1985). Nevertheless, fenbendazole treatment during the liver migration phase improved feed conversion ratios by 22% and treatment during pulmonary migration improved these rates by 8% in comparison to controls (Stewart *et al.*, 1984). The underlying mechanisms resulting in this reduced growth/feed ratio in pigs may be due to parasite-induced physiological changes. Yang *et al.* (1990) observed a reduced gastrin level during the lung migration phase and increased cholecystokinin (CCK) levels after larvae had reached the intestines of pigs. This latter hormone appears to be directly linked to a satiation signal and causes reduced food intake. This would explain the decrease in weight associated with time. Animals that showed an overall weight gain showed diminished levels of weight gain over time, supporting these observations.

A strong positive correlation between the size of the inoculum and the mean worm recovery from the lungs was found in CBA/Ca and C57BL/6j mice. These results demonstrated that, using the developed protocols for infection and recovery, between-experiment variation in *A. suum* worm burdens is minimal, and C57BL/6j mice are highly susceptible to infection compared to other strains. This relationship between the phenotypes did not alter with dose size or exposure to *A. suum*. Overall, it was concluded that the 500 or 1000 single doses represented the optimum dose range for further experiments exploring the basis of the differential susceptibility between the two selected strains without generating unnecessarily heavy worm burdens in the mice and placing

undue infective burden upon the mice. Also these smaller doses show less variability within the strains than the larger inoculum. There is strong evidence to support a genetic effect underlying the predisposition to heavy larval burdens. The focus of the subsequent investigations will be to analyse intrinsic host differences that result in the disparity in larval burdens between strains.

B. The impact of time, dose and strain of mouse on larval growth during the pulmonary phase

4.1B INTRODUCTION

It is generally accepted that there are four moults in the development of *A. suum* (Maung, 1978). However there is some debate as to the location and timing of the moults. Previously it was reported that only the first moult was completed in the egg and the latter ecdysis occurred during migration in the liver and lungs and the final moult (fourth) occurred when larvae returned to the intestine. Maung (1978) first suggested that two moults occurred in the egg before hatching. Studies of the morphology of *A. suum* larvae developing in the egg during embryonation *in vitro* (at room temperature) showed that two moults took place within the egg (Geenen *et al.*, 1999) contrary to previous observations and that L_3 was the infective stage for mice. It appears that this stage remains dormant in the egg until conditions are suitable for hatching. Subsequently, after leaving the egg, the third stage larvae grow immensely (from 200 to 1200 μm during the first 7 days) but there is no moult in the liver and lungs between days 2 and 7 (Fagerholm *et al.*, 2000). The differentiation of the fourth-stage larvae appears to be initiated during the pulmonary phase and these larvae are differentiated by the centrids. The centrids are a pair of prominent lateral sensory structures with an asymmetric position at this stage which is analogous to the adult worm and are apparent under the 3rd stage cuticle (Fagerholm *et al.*, 2000). The final moult is completed in the small intestine.

Larval migration through the hostile environment that is the host's tissues appear to confer benefits to the parasites. Although the rate of attrition is high among the migrating population, those migrants that successfully evade the host immune responses and adapt to the altering physiology of the visceral environment appear to receive a selective advantage. Parasitic taxa that differ based on the presence or absence of a migratory phase of their lifecycle in the host show variation in body size and maturation. Helminths that migrate through the host tissues are associated with greater body size and generally an increased rate of maturation in contrast to their non-migrating counterparts (Read & Skorping, 1995). These factors result in nematodes that are bigger and thus are more fecund, both within species and across species and furthermore larger bodied species probably have longer reproductive life-spans (Read & Skorping, 1995).

Read & Skorping (1995) suggested a number of reasons for the faster development of larvae in tissues. Firstly they proposed that the chemical demands made on the larvae are less demanding than that experienced by GI nematodes which are under a constant barrage of chemicals designed to breakdown organic material which would also attack the parasites body. Secondly, the physical demands in the tissues are less severe for larval development, as the intestines are highly motile, the gut mucosa is constantly sloughed off, and the larval parasites, in constant threat of expulsion, can expend a lot of energy to prevent this that would otherwise be directed towards larger growth. Read & Skorping (1995) proposed that, at least at low worm burdens, the host may tolerate tissue migrans as a specific and localised immune response may result in greater trauma to the organs. This was further demonstrated by the intravenous administration of *A. suum* that bypassed the liver phase. Although the larvae do develop normally when using this method, development occurs at a slower rate (Jungersen *et al.*, 1999b) supporting the above hypothesis.

It was considered appropriate to examine the influence of host strain on larval fitness and ultimately larval body length which in turn influences the fecundity, body length and success of the adult worms in the intestine. This was achieved by comparing the development of larvae in the two inbred mouse strains and furthermore competition for space associated with increased numbers of larvae successfully migrating was examined by contrasting larvae recovered from mice inoculated with different dosages of infective larvae.

4.2B RESULTS

THE EFFECT OF STRAIN, TIME AND DOSE ON GROWTH OF LARVAE

An analysis of the body length of larvae and the factors resulting in variation in growth were investigated. The three explanatory factors used to determine larval growth were: strain of mouse, dose of larvae and day after infection. The infection protocol comprised of animals given single pulse infection and split dose infections and these were analysed separately. The mean lengths of larvae are illustrated in **table 4.2B.1**. The variation between the strains was insignificant and larval length was consistent between the strains. **Table 4.2B.2** is the pooled figures from both strains showing the average body length of larvae over the three days in the lungs and also the variation in length associated with increased larval burden, the dose effect. In the majority of the groups the mean body length of worms increased from day 6 until day 8 (**appendix B.1**). There were however a number of exceptions to this. The first was the decrease in the average length of worms between day 7 and day 8 in the 1000 dose group. This reduction in length was observed in both strains (**table 4.2B.1**). A comparable pattern was demonstrated in the split dose group of 250 x 4. However it was possible to explain this decrease in worm length in this latter group as there may have been earlier stage larvae still migrating into the lungs from the later administrations of *A. suum* eggs. There was an effect of dose with reduced dose size resulting in the mean length of larvae increasing. The 500 dose group had consistently larger larvae than the other two single inoculum groups but interestingly larval size was analogous to the 500 x 2 dose group.

EFFECT OF INCREASING DOSES OF EGGS ON THE GROWTH OF LARVAE IN SINGLE PULSE INFECTIONS

The mean length of larvae recovered from the lungs of mice exposed to 500, 1000 or 3000 eggs, reflecting growth over the 3 day period were examined (**fig. 4.2B.1**). There were insufficient numbers of viable larvae recovered from the 100-dose group to assess growth confidently. As expected, there was a highly significant increase in larval length during the three days that larvae were observed (Initially 3-way ANOVA, with strain and time as factors and dose as covariate, full factorial model reduced to a minimum sufficient model model R^2_{adj}= 0.33 comprising only time, dose and the 2-way interaction between time and dose, main effect of time, $F_{2,185}$=28, $P \leq 0.0001$). The minimum individual larval length was

observed on day 6 (474 μm) and the maximum length observed was on day 8 (1687 μm). The results of the *post-hoc* analysis reflected this as the day 6 larval lengths were significantly different from both the other time points ($P \leq 0.0001$), but day 7 and 8 larval lengths were not significantly different. Growth was influenced by dose of eggs administered (main effect of dose, $F_{1,185}=17.7$, $P \leq 0.0001$) reflecting density dependent constraints on growth (**fig. 4.2B.1**), with faster growth in mice exposed to the lowest inoculum, and slowest in the mice given 3000 eggs. The 500 dose group was statistically different from the other two single pulse infections both the 1000 dosage group ($P=0.002$) and the 3000 group ($P \leq 0.0001$). This was most evident on day 7 after infection (**fig. 4.2B.1**). Larval growth was less consistent in the larvae recovered from mice inoculated with 1000 eggs than the other groups, larvae failing to increase in length between days 7 and 8 (2-way interaction between time * dose, $F_{2,185}=7.9$, $P=0.001$). There was no difference between the strains in the growth of larvae (Full factorial model, main effect of strain $F_{1,179}=2.9$, P=N.S. and all interactions involving strain were not significant).

Table 4.2B.1: Mean larval length (μm) ± S.E.M. (n) on the days 6, 7, and 8 post-inoculation in the lungs of the susceptible (C57BL/6j) and resistant (CBA/Ca) strains of mice inoculated with either single inoculums of 3000, 1000 or 500 *A. suum* ova or with the split dose regimes of 500 x 2 and 250 x 4 of infective eggs of *A. suum*.

Day	Strain	n	Length (μm)	Dosage	Strain	n	Length (μm)
6	C57BL/6j	19	852 ± 27	**3000**	CBA/Ca	15	900 ± 38
7		19	895 ± 32			13	1044 ± 49
8		13	1029 ± 44			12	1022 ± 64
6	C57BL/6j	10	838 ± 27	**1000**	CBA/Ca	12	818 ± 64
7		10	1133 ± 66			7	1130 ± 92
8		12	1088 ± 71			5	939 ± 135
6	C57BL/6j	11	908 ± 36	**500**	CBA/Ca	6	818 ± 29
7		7	1230 ± 68			7	1150 ± 46
8		8	1271 ± 44			5	1286 ± 54
6	C57BL/6j	17	918 ± 64	**500 x 2**	CBA/Ca	9	1092 ± 66
7		8	1203 ± 64			8	1090 ± 97
8		9	1240 ± 51			6	1244 ±158
6	C57BL/6j	12	1093 ± 91	**250 x 4**	CBA/Ca	2	909 ± 10
7		12	1086 ± 70			6	1111 ± 143
8		3	1197 ± 267			3	872 ± 66

Table 4.2B.2: Mean larval length (μm) ± S.E.M. (n) combined from the two strains of mice on the days 6, 7, and 8 post-inoculation in the lungs of mice inoculated with either single inoculums of 3000, 1000 or 500 *A. suum* ova or with the split dose regimes of 500 x 2 and 250 x 4 of infective eggs of *A. suum*.

Dose	Day p.i.	n	length (μm)
3000	6	34	876 ± 22
	7	32	948 ± 30
	8	25	1040 ± 37
1000	6	22	827 ± 36
	7	17	1132 ± 52
	8	17	1044 ± 64
500	6	17	876 ± 27
	7	14	1190 ± 41
	8	13	1277 ± 33
500 x 2	6	26	979 ± 50
	7	16	1146 ± 58
	8	15	1242 ± 67
250 x 4	6	14	1067 ± 79
	7	18	1095 ± 64
	8	6	1035 ± 143

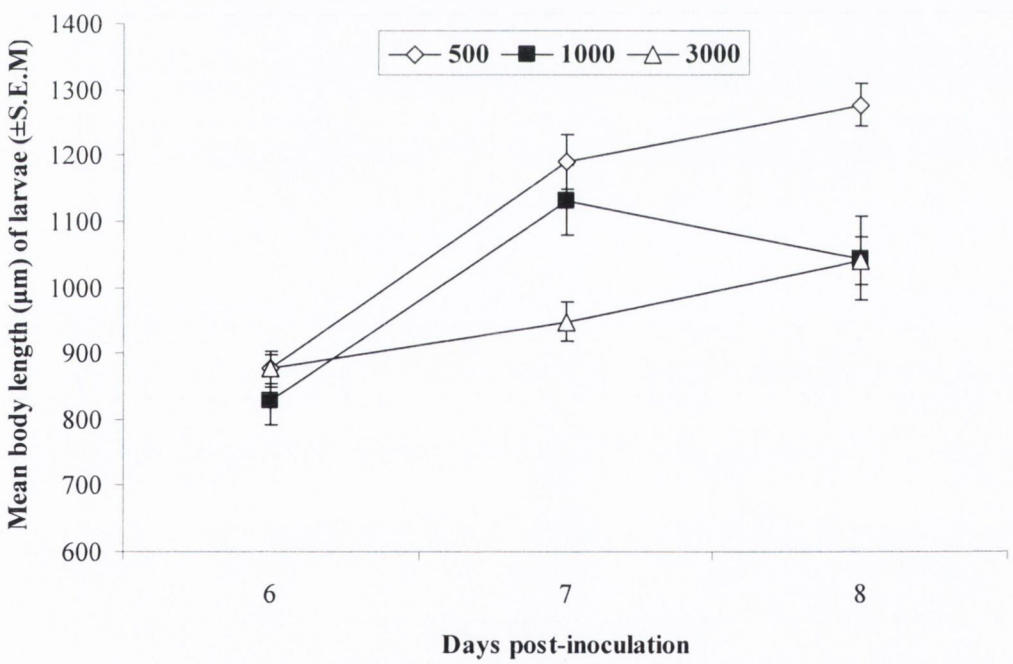


Figure 4.2B.1: Mean body length (μm) of larvae (±S.E.M.) recovered from the lungs of mice administered with a single inoculum of 3000, 1000 or 500 *A. suum* ova during the three day period (6-8) under investigation.

The second component of this experiment was the assessment of growth under single or 2 split dose regimes. In all three cases the total number of eggs administered was 1000, but one group received this dose over a period of two and another over four days. As above, there was no significant difference between the strains of mice, and no significant interactions with dose or time involving strain. Strain and all its interactions were therefore removed from the model. There was strong evidence for growth of the larvae with time after infection (**fig. 4.2B.2**; main effect of time on worm length $F_{2,146}=9.5$, $P<0.0001$), and growth was clearly affected by whether mice were inoculated in one dose or split into several doses (main effect of dose regimen $F_{2,146}=3.4$, $P=0.037$). Initially on day 6, assuming that only the larvae from the first inoculum would have arrived in the lungs, a dose dependent effect was apparent (**table 4.2B.2**), the longest larvae being from the mice infected with 4 x 250 larvae, but thereafter there was little growth in this group. Best growth was observed in mice exposed to 2 x 500 eggs. An LSD *post-hoc* analysis between the dose groups showed that the doses of 1000 and 500 x 2 were most different and that larval length differed significantly between day 6 and the two other post-inoculation days. Clearly the overall picture was not as clear-cut as in the mice exposed to single pulse infections because it was confounded by the arrival of larvae from the eggs given on days 2, 3 and 4.

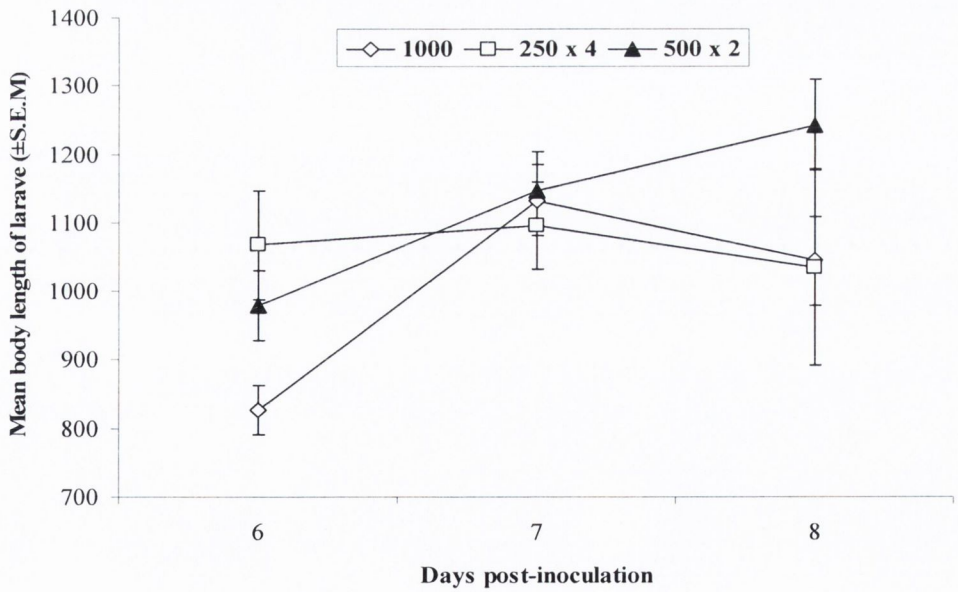


Figure 4.2B.2: Mean body length (μm) of larvae (±S.E.M.) recovered from the lungs of mice administered with a either a single inoculum of 1000 or with a split dose of 500 x 2 or 250 x 4 infective *A. suum* ova during the three day period (6-8) under investigation.

4.3B DISCUSSION

There was a correlation between dose administered and larval lengths, lower doses resulted in the increased average larval length, especially on day 7p.i.. The mean larval length was consistently higher among worms recovered from mice exposed to 500 eggs, indicating that dose exerts an important influence on the growth of worms. The observation that as dose increased, more worms were successful in completing the migration to the lungs, even though they did not grow as well as when fewer were present, suggests that there may be an active host component that underlies the difference between strains. One possibility is that increasing worm burden damage (e.g. lesions in the gut mucosa, liver, blood vessels) to host tissues, provided greater opportunities for onward migration for relatively more worms, and at the same time stimulated greater innate resistance which impeded worm development, and hence resulted in slower growth.

These results corresponded to previous investigations of larval length (Douvres & Tromba, 1971; Johnstone *et al.*, 1978). Larvae increased in length from 786 ±27μm on day 6 to 1277 ±33μm on day 8 in the group with the longest body lengths (500 eggs administered). Douvres & Tromba (1971) explored the growth of *A. suum* larvae in rabbits, guinea pigs, mice and pigs during an eleven day period to determine the comparative development of the parasite in these hosts. Each host was inoculated with 1300 eggs and mice were then euthanased 1, 2, 3, 4, 7, 9 or 11 days p.i.. They described the larval lengths in the lungs of mice and showed that they ranged from 900-1530μm (mean= 1225μm) on day 7 post-inoculation to 950-1800μm (1480μm) on days 9 and 11. These are similar to the observations outlined here with a mean larval length of 1190 ±41μm on day 7 p.i. in the 500 dose group. Recent examinations of larval development in pigs supports the larval lengths on day 7 described above, with average larval body length of 1440μm (1080-1810μm) (n=19) (Fagerholm *et al.*, 2000).

There was a high degree of variation in individual larval lengths both within the dose groups and also within the days examined and this may be the result of a number of factors. Firstly, larvae are known to bypass the liver and migrate straight to the lungs. Those individuals that circumvent this phase do not mature as quickly as their counterparts and this could explain some of the variance observed in larval lengths at this stage. Also larvae that may be trapped in the host tissues and their development arrested at an early stage would contribute to this disparity in lengths. Late stage L_3 larvae in the liver range in

size from 380-490μm (471μm) on Day 4 (Douvres & Tromba, 1971) and underdeveloped examples of larvae of this range could explain the high degree of variation in larval size. Finally, male and female adult worms are known to differ in length when fully grown (Galvin, 1968). It is proposed that this sex differentiation can be translated into the larval stages with variations in length between the sexes already apparent during these early stages. Female worms are larger than their male counterparts and a biased sex ratio may skew the observed larval lengths in favour of an upper or lower size value. Galvin (1968) noted that the sexes of *Ascaris*, in pigs and rabbits inoculated with infective ova, in most infections were unbalanced in favour of females, being 2:1 or greater in more than half of the infections in which 10 or more worms were present.

An investigation of the effects of density dependence on the growth and fecundity of *T. muris* in CBA/Ca mice found a causal link with the density dependent decline in individual worm fecundity and this appeared to be caused by the stunted growth of individual worms when occurring at high population densities (Michael & Bundy, 1989). In relation to the establishment of *T. muris* in the intestine of the murine model, it has been suggested that there is an upper limit to worm establishment that could indicate the existence of a physical carrying capacity in the mouse for this parasite (Michael & Bundy, 1989). Competition between larvae for space in the caecum results in displacement to other sites along the large intestine, in particular the colon. These observations have been observed in a number of parasitic nematode species including adult *Ascaris*. It is possible that the physiology of the worms may be affected, with adverse implications for survival and growth. Displacement or competition for protective sites of larval *A. suum* in the lungs to evade host responses may result in the unfavourable dislocation of larvae to harsh sites that impede larval growth. Increased innate resistance in the liver may also contribute to this stunted growth associated with increased worm loads.

The situation in the split dose group is not as clear as the single dose group as there are other factors influencing the observed larval growth including the issue of time as well as the possibility of an adaptive immune response. A possible indicator of these effects is the retarded growth of the four day split dose group. The mean larval length in this group is very consistent ranging from 1067 ±79 on day 6 to 1035 ±143 on day 8. The large variation on the latter day could be associated with earlier stage larvae arriving from other sites following the subsequent inoculations. Reduced rates of growth caused by an adaptive immune response have previously been demonstrated. In immunised mice, resistance

affected the stage to which *A. suum* larvae develop and also the length to which they grow (Johnstone *et al.*, 1978).

Sprent & Chen (1948) investigated variations in larval lengths in mice fed with one dose of 30-50000 *A. suum* ova associated with primary infection and mice that were reinfected with *A. suum* in both the liver and the lungs. They found that the retarding influence of the immunological process was detectable at four days after reinoculation. The length of the larvae at 8 days was significantly smaller in the reinfected mice in contrast to those initially infected. Larval size in the lungs was 1.18 ±0.04mm in primary infected mice but this was greatly reduced to 0.55 ± 0.03mm in challenged mice. Similarly, Johnstone *et al.* (1978) performed experiments examining the lengths and stages reached by larvae recovered sequentially from immunised and non-immune C57BL/6 mice on 1, 2, 5, 7, 9, and 12 days p.i.. In non-immune mice, the mean length of larvae was recorded in the livers on day 5 (559 ± 8μm) and in the lungs on day 9 (1042 ± 30μm). The maximum length reached by larvae in the livers (406 ± 13μm on day 12) and lungs (374 ± 12μm on day 7) of immune mice was significantly less than the maximum length obtained in non-immune mice. Morphologically, larvae in non-immune mice obtained a later stage (late third stage) than in immune mice (early third stage). So immunisation not only affects the number of larvae successfully migrating but also their length and rate of development.

There was clearly an effect of day on which larval length was examined but there may also be an effect associated with competition caused by increasing the number of larvae migrating through the host's tissues. This can be suggested by the reduced growth of larvae in mice with greater worm burdens. Larval length was not influenced by strain suggesting that the environment within both strains was equally suitable for the development of the worms and perhaps that the observed differences in relative susceptibility are not likely to be attributable to differences in larval fitness but rather to innate barriers to migration or differences in the early phase of the host response to the parasite. Competition between parasite strains for resources is a well documented source of retardation in growth and fecundity for the adult phase of parasitic lifecycles. The current investigation provides evidence that this competition extends to the larval stages for *A. suum* and that larvae experience stunted growth affiliated with increased larval loads.

CHAPTER 5

THE MIGRATION OF *A. SUUM* IN TWO STRAINS OF MICE (C57BL/6j AND CBA/Ca) FROM THE INTESTINE TO THE LUNGS DURING THE PERIOD FROM DAY 0 TO DAY 8 POST-INOCULATION

5.1 INTRODUCTION

The migration of *A. suum* has been described by several authors in pigs and mice as well as other experimental hosts (Galvin, 1968; Jenkins, 1968; Douvres & Tromba, 1971; Soulsby, 1982; Murrell *et al.*, 1997a; Slotved *et al.*, 1998). The infective eggs of *A. suum* readily hatch in mice of various strains (Mitchell *et al.*, 1976) and the larvae then grow and follow a similar pattern of migration through the tissues to that observed in pigs (Murrell *et al.*, 1997a). However, variations in the success of larvae migrating through the intestine, liver and lungs of mouse strains that show phenotypic heterogeneity in their susceptibility to this parasite has not previously been examined in detail. Such information on the location and timing of susceptibility/resistance to *A. suum* is vital to the understanding of the mechanism of genetic resistance.

In a previous chapter it was demonstrated that mouse genotypes differ markedly in their susceptibility to early *A. suum* infection based on the phenotypic expression of pulmonary larval burdens on days 6-8 p.i.. These observations showed that inherent differences between the mouse genotypes resulted in varying success of strains to prevent larval migration into the pulmonary tissues. The aim of the work reported in this chapter was to describe the early larval migratory pattern from day 0 to day 8 in C57BL/6j and CBA/Ca mice and to explore the location and times of worm attrition during migration.

5.2 MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The early pattern of migration in the intestine (at 6 hours), liver and lungs (days 0-8 p.i.) of the two strains of mice (C57BL/6j and CBA/Ca) inoculated with 500 *A. suum* ova was investigated. One hundred and two male inbred mice (51 C57BL/6j and 51 CBA/Ca) were purchased from Harlan, UK at 6 weeks of age and were 8 weeks old upon commencement of the initial infections. Animals were maintained as described in chapter 2.

EXPERIMENTAL PROTOCOL

Six mice inoculated with *A. suum* were euthanased from each strain daily between day 0 (at 6 hours p.i.) and 5 post-inoculation, inclusive, and a further 5 mice per strain were euthanased daily from day 6 to 8 inclusively. Six mice were used on days 0-5 because two mice from each group had their liver removed for future histological examination. At 6 hours p.i. the large intestine, the rectum, the liver and lungs were removed. The large intestine (including the colon) and the rectum were treated as separate organs. Both gastrointestinal sections were opened separately with a pair of scissors. The contents were collected by washing the relevant intestinal section with 30ml of 0.9% saline in a 50ml centrifuge tube. The capped tube containing saline and the intestinal section was shaken by hand for 10-20 seconds until all the contents were washed off, and then the clean intestinal wall was rinsed with saline and removed from the tube to which 20 ml of formalin was added. The intestinal walls were then completely submerged in 0.9% saline by suspending them from a wire in another centrifuge tube and incubated overnight at 37.5°C in a vertical position (**appendix D.1**). These methods were used for the recovery of larvae and eggs alike. The liver and lungs were examined using the modified Baermann method (see chapter 2). The recovery of larvae and eggs using centrifugation was performed on all samples including the washed contents and the incubated intestinal sections (chapter 2).

At all other post-mortem examinations, only the liver and the lungs (divided into the left and right hand side) were removed for examination of larval burdens. The spleen was weighed and processed as described in previous chapters (**appendix A.7**). The post-mortem procedure was similar to the procedure outlined in chapter 2. Post-mortem information on mouse weight etc. was noted (see list of experimental data noted in chapter 2), and recovery process were performed on all samples as described in the previous chapters. Statistical analysis was carried out as described in previous chapters.

5.3 RESULTS

LOCATION OF LARVAE IN THE INTESTINE 6 HOURS POST-INOCULATION IN C57BL/6j AND CBA/Ca MICE

Both larvae and unhatched eggs were recovered from the contents of the large intestine of both strains. Larvae were recovered from the large intestinal (LI) wall of both mouse strains at 6 hours p.i.. However, only larvae could be recovered from the contents of the rectum, there were no unhatched eggs present. Very few larvae were recovered from the wall of the rectum and these were only in C57BL/6j mice (**fig. 5.3.1**). There was a higher percentage of the total inoculum recovered from the intestine/rectum of C57BL/6j mice in contrast to CBA/Ca mice at this time point (**table 5.3.1**). The main site of larval recovery was the large intestine in both mouse strains and this was both the intestinal contents and larvae actively migrating through the large intestine wall. There was a highly significant difference in the number of eggs/larvae recovered between the two strains (**fig. 5.3.1**; 2-way ANOVA with strain and location in the intestinal tract as factors, model R^2_{adj}=0.753, main effect of strain $F_{1,50}$=8.6, P=0.005). The location within the gastrointestinal tract (**fig. 5.3.1**) from which larvae were recovered was the most significant factor in determining larval burden (main effect of location $F_{4,50}$=37.0, $P \leq 0.0001$). There was a significant interaction between strain and location of larval recovery ($F_{4,50}$=8.2, $P \leq 0.0001$).

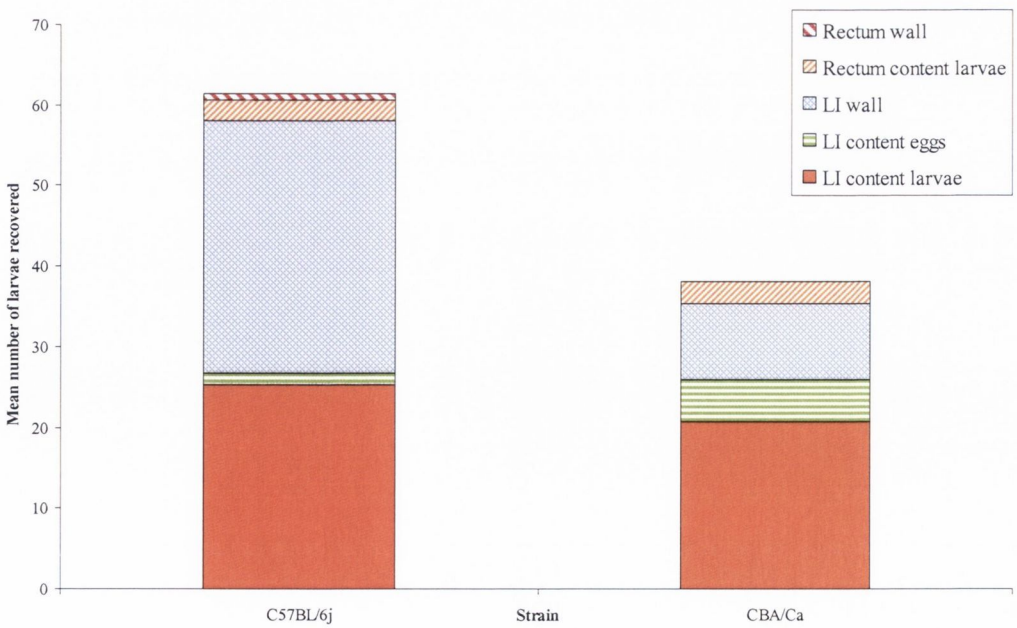


Figure 5.3.1: Mean number of larvae and unhatched eggs recovered from the large intestine (LI) and rectum in C57BL/6j and CBA/Ca mice.

Table 5.3.1: The total percentage recovered of *Ascaris suum* newly hatched larvae and eggs in each location of the intestine examined at 6 hours p.i. in both mouse strains (C57BL/6j and CBA/Ca).

	Large intestine wall (%)	Large intestine contents- larvae (%)	Large intestine contents- eggs (%)	Rectum Wall (%)	Rectum content (%)	Total (%)
C57BL/6j	6.27	5.07	0.27	0.13	0.53	**12.27**
CBA/Ca	1.87	4.13	1.07	0.00	0.53	**7.60**

A. SUUM MIGRATION IN THE LIVER OF C57BL/6j AND CBA/Ca MICE FROM DAY 0-8 P.I.

Larval burdens in the liver changed significantly with day in both strains (2-way ANOVA with day and strain as factors, model R^2_{adj}= 0.62, main effect of time, $F_{8,52}$=15.2, $P \leq 0.0001$), however, there was no significant difference in the number of larvae in this organ between the two strains during the infection period (main effect of strain $F_{1,52}$=0.9, P=N.S. and 2-way interaction strain * time $F_{8,52}$=1.2 , P=N.S.). Larvae were first observed in the liver at 6 hours p.i. There was gradual accumulation in the early days of infection (1-2 days p.i.) and building to a peak on days 3-5 p.i. Thereafter there was a steady decline in the liver coinciding with the accumulation of larvae in the lungs (**figs. 5.3.2** and **5.3.3**).

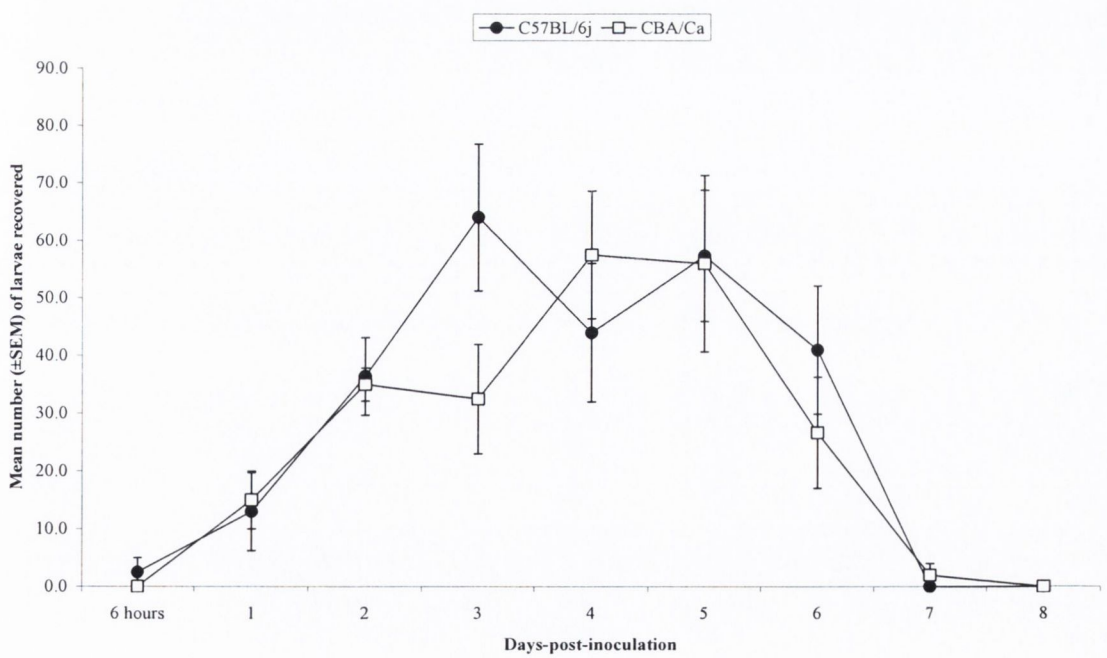


Figure 5.3.2: Larval migration in the liver of the two strains of mice (C57BL/6j and CBA/Ca) on days 0-8 inoculated with 500 *A. suum* eggs.

Larvae first appeared in the liver of C57BL/6j mice already by 6 hours p.i., however there were very few individuals recovered at this early stage (2.5 ±2.5). Larvae accumulated until day 3 at which point the number peaked. After day 5 there was steady decline to zero of the larval burden (**fig. 5.3.2**). The results of LSD *post-hoc* analysis showed that the days that differed from all others and thus were the significant days that determined the number of worms recovered in the liver, were days 2-6 p.i. as these days were significantly different from both the earlier days (0 and 1) and the latter days (7 and 8) (**table 5.3.2**).

The pattern of movement into the liver of CBA/Ca mice was very similar to C57BL/6j mice except larvae did not accumulate in this organ to its highest levels until day 4 (57.5 ±11.1). The overall movement was very similar is both strains as shown in **Figure 5.3.2**. Days 7 and 8 were significantly different from days 2-6 again reflecting the movement of larvae away from the liver (**table 5.3.2**).

Table 5.3.2: Results of the *post-hoc* LSD analysis on the differences in liver larval burdens between the days post-inoculation in both C57BL/6j and CBA/Ca mice. Only significant values are shown.

Strain	Days p.i.	Days p.i.	Probability	Days p.i.	Days p.i.	Probability
C57BL/6j	**6 hours**	2	0.0005	7	2	0.0036
		3	0.0000		3	0.0000
		4	0.0001		4	0.0010
		5	0.0000		5	0.0001
		6	0.0003		6	0.0019
	1	2	0.0349	**8**	2	0.0017
		3	0.0001		3	0.0000
		4	0.0094		4	0.0004
		5	0.0009		5	0.0000
		6	0.0178		6	0.0009
CBA/Ca	**6 hours**	2	0.0040	7	2	0.0061
		3	0.0069		3	0.0106
		4	0.0000		4	0.0000
		5	0.0000		5	0.0000
		6	0.0345		6	0.0492
	1	4	0.0007	**8**	2	0.0040
		5	0.0010		3	0.0069
	3	4	0.0325		4	0.0000
		5	0.0434		5	0.0000
	4	6	0.0159		6	0.0345
	6	5	0.0211			

The disparity in larval burdens between these two mouse strains was most evident on the latter days of infection in the lungs (**fig. 5.3.3**). As the numbers of larvae recovered from the liver declined there was an increase in the numbers of larvae recovered in the lungs on days 6-8 p.i. (**figs. 5.3.2** and **5.3.3**). There was a significant change in the worm burden of the lungs associated with time (2-way ANOVA with day and strain as factors on lung worm burdens, model R^2_{adj}= 0.56, main effect of time, $F_{8,81}$=8.7, $P \leq 0.0001$). In respect of lung worm burdens there was a marked difference between the strains compared with liver counts (**fig. 5.3.3**, main effect of strain $F_{1,81}$=42.4, $P \leq 0.0001$ and 2-way interaction strain*time, $F_{8,81}$=4.7, $P \leq 0.0001$) with larvae accumulating at different rates between strains. There was no significant variation in the predilection of larvae for the left or right hand side of the lung in either strain (**table 5.3.3**). This was unexpected as this was a consistent observation in the previous experiments (see chapters 3 and 4). The number of worms recovered in the lungs of both strains of mice was similar to previous results.

Larval counts in the lungs changed with time in C57BL/6j mice (**fig. 5.3.3**). The results of the *post-hoc* analysis (**table 5.3.4**) identified that the earlier days (0-5) are all significantly different to the latter three days (6-8) as would be expected as these are the days in which the larval burden increased dramatically in this organ (**fig. 5.3.3**). The first larvae were recovered as early as 6 hours p.i. and then there was an apparent surge of larvae on day 1 p.i. (20.7 ±12.5), larval burdens remained low thereafter until day 6 when the migration of greater numbers of juveniles into the lungs was expected. The peak worm burden observed in this group was at day 8 and not day 7 as previously demonstrated although there was no significant difference between these two days in terms of larval burden (**table 5.3.4**).

Larval migration into the lungs of CBA/Ca mice never achieved the same levels as that observed in C57BL/6j mice at any stage of infection (**fig. 5.3.3**). There was movement into the lungs of this strain at an early stage but these numbers remained low throughout the experimental procedure. Once again larvae were observed in the lungs as early as six hours post-inoculation (0.67 ±0.67), however, there was consistently lower numbers of larvae recovered during the next few days of the procedure (days 1-5). The results of the *post-hoc* analysis revealed that only day 7 differed significantly from all other days (**table 5.3.4**) when the larval burden was at its highest (11.2 ±5.4) (**fig. 5.3.3**).

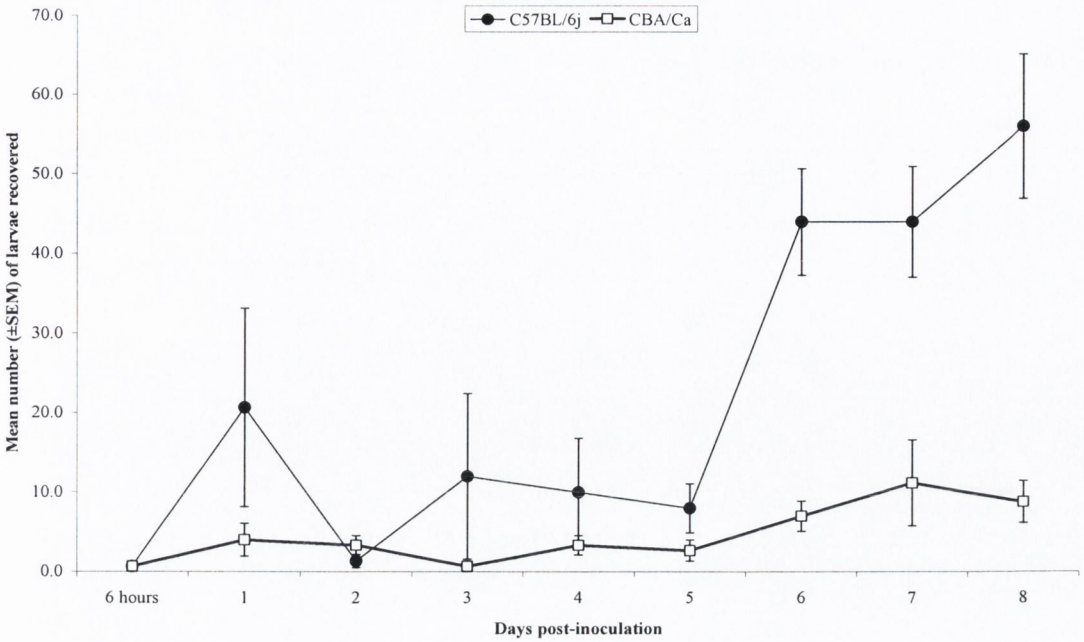


Figure 5.3.3: The mean number (±S.E.M.) of larvae recovered from the lungs of C57BL/6j and CBA/Ca mice inoculated with 500 *A. suum* ova on days 0-8.

Table 5.3.3: Mean number of larvae (±SEM) recovered from the left and right hand side of the lungs in the two strains of mice (C57BL/6j and CBA/Ca) on days 0-8 post-inoculation.

Strain	Day	n	Left lung	Right lung
C57BL/6j	6 hours	6	0.67 (±0.67)	0.00 (±0.00)
	1	6	8.67 (±5.51)	12.00 (±7.00)
	2	6	1.33 (±0.84)	0.00 (±0.00)
	3	6	6.67 (±6.67)	5.33 (±3.82)
	4	6	2.00 (±1.37)	8.00 (±5.66)
	5	5	4.80 (±2.94)	3.20 (±0.80)
	6	5	19.20 (±4.08)	24.80 (±3.20)
	7	4	26.00 (±6.22)	18.00 (±4.76)
	8	5	16.00 (±4.38)	40.00 (±7.16)
CBA/Ca	6 hours	6	0.00 (±0.00)	0.67 (±0.67)
	1	6	2.00 (±1.37)	2.00 (±1.37)
	2	6	2.67 (±1.33)	0.67 (±0.67)
	3	6	0.00 (±0.00)	0.67 (±0.67)
	4	6	1.33 (±0.84)	2.00 (±1.37)
	5	6	2.00 (±1.37)	0.67 (±0.67)
	6	4	5.00 (±1.00)	2.00 (±1.15)
	7	5	4.80 (±2.33)	6.40 (±3.25)
	8	5	2.40 (±0.98)	6.40 (±2.04)

Table 5.3.4: Results of the *post-hoc* LSD analysis on the differences in lung larval burdens between the days post-inoculation in both C57BL/6j and CBA/Ca mice. Only significant values are shown.

Strain	Day	Day	Probability	Strain	Day	Day	Probability
C57BL/6j	**6**	6 hours	0.0003	**CBA/Ca**	7	6 hours	0.0015
		1	0.0367			1	0.0251
		2	0.0003			2	0.015
		3	0.0051			3	0.0015
		4	0.0031			4	0.015
		5	0.0027			5	0.0087
	7	6 hours	0.0005		**8**	6 hours	0.0121
		1	0.0493			3	0.0121
		2	0.0006				
		3	0.0082				
		4	0.0052				
		5	0.0045				
	8	6 hours	0				
		1	0.0022				
		2	0				
		3	0.0002				
		4	0.0001				
		5	0.0001				

A. SUUM MIGRATION THROUGH THE LIVER AND LUNGS OF C57BL/6j AND CBA/Ca MICE

As expected from the global analysis of the results, there was a highly significant variation in the pattern of larval migration during the nine days that larvae were recovered from both organs (3-way ANOVA, with strain, time and organ as factors, model R^2_{adj}= 0.66, main effect of time $F_{8,106}$=7.6, $P \leq 0.0001$). There was a significant effect of strain on larval burden (main effect of strain $F_{1,106}$=15.0, P=0.0002) and the strains differed in the pattern of larval recovery between the liver and lungs (2-way interaction of strain * organ $F_{1,106}$=5.7, P=0.0190). There was greater accumulation of larvae into the lungs of C57BL/6j mice than that of CBA/Ca mice. This was more pronounced on the latter days of infection with more larvae successfully migrating into the pulmonary tissues of C57BL/6j mice on days 6, 7, and 8 (**fig. 5.3.3**). There is a significant effect of organ on the number of larvae recovered (main effect of organ $F_{1,106}$=37.9, $P \leq 0.0001$) with greater numbers of larvae accumulating in the liver initially but then in the pulmonary tissues on the latter days. Larvae migrated into the organs of the two strains at different rates, larvae accumulating faster into the liver and gradually accumulating in the lungs (main effect of

day*organ $F_{8,106}$=18.9, P≤0.0001 and the three way interaction of day * strain * organ $F_{8,106}$=2.6, P=0.013).

The number of larvae recovered from the liver of C57BL/6j mice was lower than expected when contrasted to earlier observations of migration through this organ (See chapter 2). Previously the maximum number of larvae recovered in the liver from this mouse strain was 211 (±37.4) or 42.2% of the initial inoculum on day 5. The current results show a significant reduction in the number of larvae recovered from this organ (64 ±12.8 or 12.8%) and the highest burden was on day 3. Larval counts in the liver changed significantly with time in both experimental procedures, when analysed between days 3-8 (2-way ANOVA with day and experimental procedure as factors, model R^2_{adj}=0.73, main effect of day $F_{5,40}$=15.9, P=0.0001), but the two experiments also differed significantly (main effect of experiment $F_{1,40}$=17.8, P≤0.0001 and 2-way interaction day * experimental procedure $F_{5,40}$=5.7, P=0.0005), with more larvae accumulating throughout the observation period in the livers of mice examined in chapter 2. However, and more importantly, the number of larvae accumulating in the lungs of this strain between the two experiments was consistent (2-way ANOVA with day and experimental procedure as factors, model R^2_{adj}=0.42, main effect of experiment $F_{5,40}$=0.62, P=N.S.). So while the numbers of larvae recovered from the liver of C57BL/6j mice differed between chapters 2 and 5, the number of larvae migrating into the lungs was comparable.

5.4 DISCUSSION

The observations of larval migration through the organs of the two strains of mice examined showed that the period of migration between the liver and lungs was the most decisive period determining larval success. Larval loss from the intestine following the initial inoculation not only reflects host differences but a failure of eggs to hatch and thus indicates parasite ability also. Although there were more larvae migrating through the wall of C57BL/6j mice at 6 hours p.i. this appeared to reflect difference in the speed at which larvae and eggs were passed through the intestinal tract as the larval numbers in the liver were very similar in both strains on each post-mortem day. Larval numbers were significantly reduced in CBA/Ca mice in contrast to C57BL/6j mice between the phase of migration from the liver and the lungs suggesting some hepatic/post-hepatic factor that varies between the two strains that plays a critical role in determining the success of larvae through the host tissues.

There are very few studies showing larval recovery of newly hatched larvae from the intestinal wall and it was suggested that this was due to a lack of efficient methods (Slotved, 1997). Slotved (1997) compared four procedures for recovering larvae from the intestinal wall: 1) simple incubation of the intestinal wall in 0.9% saline, 2) incubation of the intestinal wall in a Baermann apparatus, 3) incubation of mucus from the intestinal wall in a Baermann apparatus, and finally 4) incubation of mucus in agar-gel. Simple incubation of the intestinal wall proved to give significantly more larvae than the other three methods (13.6% at four hours p.i.) and therefore this method was used in the current investigation. The rate of recovery at 6 hours p.i. was approximately 9% in the investigation of larval penetration of the large intestine by Slotved *et al.* (1997a) using BALB/c mice, and a similar percentage (6%) of larvae were recovered in the large intestine wall in the strain C57BL/6j in the current work (**fig. 5.3.1.**). Thus at this early stage, large numbers of larvae had penetrated the intestinal mucosa and were beginning their onward migration. The percentage of the inoculum recovered from the intestine at 6 hours p.i. is relatively low. However, in an examination of the movement of larvae through the intestine of mice during a 12 hour period, Slotved *et al.* (1998) demonstrated that by 6 hours p.i. the number of larvae successfully migrating through the intestinal wall began to decline from 4 hours p.i.. This suggested that a large proportion of larvae capable of migrating through this organ had already done so by this period.

There were also similar numbers of free larvae in the large intestine contents at this time point (≈6% [(Slotved *et al.*, 1997a)] for BALB/c mice and 5% [**table 5.3.1**] for C57BL/6j mice). The intestinal contents of CBA/Ca mice contained comparable numbers of free larvae to C57BL/6j mice but the number of actively migrating larvae was lower (2%). However, the total number of larvae successfully migrating to the liver was equivalent in the two strains suggesting that larvae penetrated the intestine of CBA/Ca mice at a different rate in contrast to C57BL/6j. Very few larvae were recovered from the rectum and it is suggested that this is because any remaining larvae are likely to be quickly expelled from this site. Mitchell *et al.* (1976) found that resistance either to second infection in susceptible C57BL/6 mice or in relatively resistant strains of mice was not determined by the site of penetration in the intestinal wall or migration to the liver but instead during the period of residency in the liver or *en route* to the lungs, and broadly our data concur.

Larvae were observed in the liver as early as 6 hours p.i. but at very low burdens. The first substantial movement of larvae into this organ was seen on day 1 and thereafter there was continuous movement into the liver between days 2-5. The constant increase in larvae after 24 hours when all larvae capable of migrating are expected to have successfully penetrated the intestinal wall (Slotved *et al.*, 1998) indicates that larvae were arriving from other locations and perhaps had become temporarily lost or trapped in other host tissues. Overall the pattern of migration and the larval burden in the livers of the two strains was very similar on all post-mortem days and peaked on days 3-4. These observations are in concurrence to previous investigations which found the maximum larval burden in the liver of mice on day 4 (Prokopič & Figallová, 1982a). The first larvae were recovered from the lungs as early as 6 hours post-inoculation and this was consistent with the observation of Slotved *et al.* (1998). Larval burdens remained low until day 6 when the migration of greater numbers of juveniles into the lungs was expected. The peak worm burden observed in C57BL/6j mice was on day 8 and not on day 7 as previously observed, and this reflected a cohort of larvae that had undergone development in the liver and were significantly longer than those arriving freshly from the intestine (see chapter 4). However, there was no significant difference between the worm burdens of individuals at these two time points and thus the observed peak is marginal.

The period of migration from the liver into the lungs appears to be the most influential period determining the relative resistance/susceptibility of the two mouse strains. During

this period CBA/Ca mice showed a reduction in the number of larvae that successfully migrate into the lungs whereas C57BL/6j maintained a high number of larvae during this phase, showing no great reduction between the liver and the lung phases. There are three four possible mechanisms suggested for this observed variation (i) anatomical differences between the strains which impede migration to the lungs (ii) tropic difference between the strains which impede migration to the lungs (iii) differences in resistance mechanisms that either incapacitate the larvae late during the liver stage or block entry to the lungs (iv) anatomical/immunological differences in the reaction to migrating larvae in the lungs of the two strains. Some preliminary investigations examining the role of the lungs and the pulmonary inflammatory response in relation to host protection are examined in the following chapters (chapters 6 & 7).

CHAPTER 6

THE ROLE OF THE MURINE IMMUNE SYSTEM IN RESPONSE TO EARLY *ASCARIS SUUM* INFECTION DETERMINED BY THE KINETICS OF THE LEUKOCYTE POPULATION IN THE LUNGS AND THE EFFECT OF AN IMMUNOSUPPRESSANT ON LARVAL BURDENS

6.1 INTRODUCTION

When examined under natural conditions the apparent resistance or susceptibility of a host to parasites, as reflected in absence of parasites or heavy worm burdens respectively, can be the result of geographical distribution, variation in behavioural characteristics or nutritional habits, or contact with infective stages. However, there is mounting evidence that individuals are genetically predisposed to heavy/light helminth infections (Holland *et al.*, 1989; Williams-Blangero *et al.*, 1999; Williams-Blangero *et al.*, 2002) and this same predisposition is observed in pig populations infected with *A. suum* (Roepstorff *et al.*, 1997; Boes *et al.*, 1998a).

Innate resistance traditionally encompasses a physiological/morphological incompatibility between parasite and host environment that prevents invasion, establishment or survival (Wakelin, 1996) but underlying this incompatibility is the host genotype. For example parasites that enter the host orally and which require activation by specific triggers in the intestinal environment may not experience these factors or the correct combination of factors that is necessary. The eggs of *Ascaris*, for example, can hatch in a variety of mammalian hosts (Boes & Helwigh, 2000) because of the relatively low specificity of the intestinal signals required. However, this process may be host dependent and inherent variations in the genetic make-up of the host population can result in differences in susceptibility resulting from an intrinsic host factor. Even if the host provides an environment suitable for initial development, incompatibilities may well arise at subsequent stages or through incomplete stimuli for migration and reproductive maturation (Wakelin, 1996). Natural resistance to *Ascaris* is complex and appears to involve the combined effects of the humoral and cellular responses and is host-dependent (Eriksen, 1981). Resistance to *A. suum* in pigs has been suggested to involve the elimination of the parasite both during migration by white spot formation in the liver and during the early pre-patent period expulsion from the gut (Eriksen *et al.*, 1980). The role of the lungs is poorly understood in relation to its impact on the successful migration of larvae through the tissue.

This chapter consists of two separate protocols. The first experiment (hydrocortisone experiment) was the investigation of the effects of hydrocortisone on the early kinetics of migration in the lungs of the two strains of mice (C57BL/6j and CBA/Ca) inoculated with 500 *A. suum* ova. A total of 10 mg of hydrocortisone was administered over a period of

four days. Hydrocortisone administered at this intensity has previously been shown to be an immunosuppressive (Behnke & Parish, 1979; Jerzy Behnke, personal correspondence). Behnke & Parish (1979) observed that following treatment with hydrocortisone acetate formerly immune mice that were challenged with *Heligosomoides polygyrus* had significantly more worms than immune control mice, thus demonstrating the immunosuppressive effect of hyrdocortsicone. Thus, if if the adaptive immune response was involved in protection against the early stages of *A. suum*, the resistant mouse strain would be expected to demonstrate higher worm burdens in the lungs due to immunosuppression.

The second procedure (bronchoalveolar lavage experiment) was the investigation of bronchoalveolar lavage fluid (BALF) to determine the leukocyte population and larval burdens in mice inoculated with 1500 *A. suum* eggs. This experiment was designed to determine the variation in the leukocyte population between the two strains and the contrast in the differential leukocyte population on days 8, 9 and 10 p.i.. These days were chosen as previous work has suggested that the first signs of *A. suum* migration in the lungs in terms of an inflammatory reaction were observed on day 8 and by day 10 this reaction was intense (Eriksen, 1981). This experiment was designed to determine the role of the pulmonary inflammatory response in protection against primary infection with *A. suum* in relation to susceptible and resistant individuals.

6.2 MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Male mice were purchased from Harlan, UK at 6 weeks of age and were 8 weeks old upon commencement of the initial infections. Seventy inbred mice (35 C57BL/6j and 35 CBA/Ca) were purchased for the hydrocortisone experiment. In the BALF experiment, 58 inbred mice, (29 C57BL/6j and 29 of the resistant strain CBA/Ca), were purchased. Animals were maintained in conditions that were standard and constant.

EXPERIMENTAL PROTOCOL

HYDROCORTISONE EXPERIMENT

Larval migration and the administration of hydrocortisone

30 mice from each strain were inoculated with 500 fully embryonated eggs by stomach intubation as previously described on day 0. A further 5 mice from each strain were inoculated with a placebo infection of 100µl of egg medium with no infective eggs by stomach intubation, as a control group (**fig. 6.2.1**). The control mice were maintained separately at all times from infected mice and new equipment was used during the inoculation procedure to avoid accidental cross contamination.

Of the 60 mice inoculated with infective *A. suum* ova, 15 from each strain were inoculated subcutaneously, on days -1 (the day prior to infection), 1, 3 and 5 (post-inoculation), with a total of 10 mg of hydrocortisone acetate (stock solution of 12.5 mg/ml) administered each day in 0.2ml (1.25mg per 0.1 ml of hydrocortisone acetate) of the stock solution (**fig. 6.2.1**). Hydrocortisone acetate was purchased from Sigma-Aldrich® as a prepared solution. Mice were restrained and the hydrocortisone solution was administered using a 1ml sterile syringe with a hypodermic needle (25g needle, Terumo neolus 0.5 x 16mm) into the scruff of the neck ensuring that the tip penetrated the skin. The syringe was gently depressed to ensure that there was no backflow of solution from the site of inoculation. A further 15 mice of each strain were provided as controls for the hydrocortisone infection, as a measure of the effect of handling and stress on larval burden. These mice were restrained and, in an analogous procedure to that outlined above, were inoculated with physiological saline (0.9% saline). The equipment used was maintained separately from the hydrocortisone equipment to prevent accidental contamination of materials. These mice were euthanased on days 6, 7 and 8 p.i. (see below).

Five mice from each strain that were provided with a "sham" infection (0.1 N H_2SO_4 with no *Ascaris* eggs) were euthanased on day 5 post-inoculation to provide information on the spleen weights of uninfected animals and also the weight gain/loss in uninfected mice at this time point (**appendix A.8**). Five mice from each strain in the groups inoculated with both *A. suum* infective eggs and hydrocortisone acetate were euthanased on days 6, 7 and 8 (**fig. 6.2.1**). The liver (**appendix A.9**) and the two sides of the lung were removed and examined for larval migration. Similarly, the control groups, those mice inoculated with *A. suum* eggs and 0.9% saline were euthanased on days 6, 7 and 8 (five mice from each strain per day) and the post-mortem procedure was similar. The post-mortem procedure, information on mouse weight and recovery process was performed as described in chapter 2. The number of larvae migrating through the lungs was determined as previously described (chapter 2).

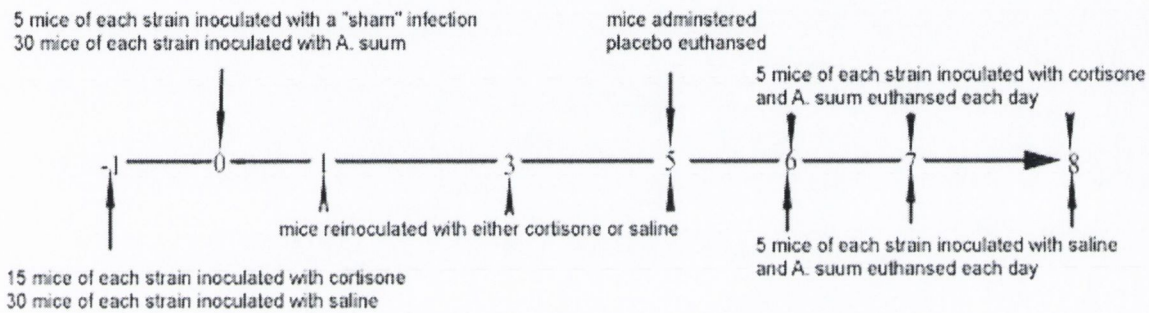


Figure 6.2.1: Inoculation and infection protocol for C57BL/6j and CBA/Ca mice in the hydrocortisone experiment and days of post-mortem are also shown.

BRONCHOALVEOLAR LAVAGE EXPERIMENTAL PROTOCOL

Bronchoalveolar lavage (BAL) of the murine lung

Forty-eight mice (24 of each strain) were inoculated with 1500 embryonated *A. suum* eggs in 100µl of solution and five mice of each strain were provided with a placebo dose consisting of 100 µl of 0.1 N H_2SO_4 and no *A. suum* eggs as controls on day 0 (**fig. 6.2.2**). Five mice from both strains were sacrificed on each day. Susceptibility was once again assessed by the number of larvae recovered from the lungs and three mice from each were euthanased daily on days 7, 8 and 9 for this purpose (**fig. 6.2.2**) and to provide comparative data between worm burdens and leukocyte populations. The mice examined for larval burdens were processed and worm numbers determined as described in chapter 2. Control mice were euthanased on day 7 to determine the base level of the leukocyte population. This experiment also provided information on the rate of recovery from an intermediate

dose not examined in chapter 4 (1500 *A. suum* eggs). The number of eggs in the inoculum was determined as described in chapter 2.

Post-mortem procedure

Each mouse was weighed at the time of infection and prior to sacrifice (**appendix A.10**). The mice utilised for larval recovery were handled as previously discussed. Post-mortems of these mice took place on days 7, 8, 9 and the groups of mice that were assessed for their bronchoalveolar lavage (BAL) fluid were euthanased on days 8, 9 and 10. Control mice were examined on day 7 (**fig. 6.2.2**).

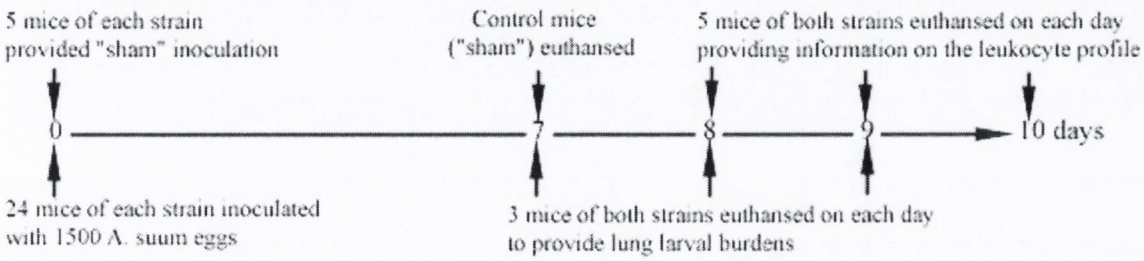


Figure 6.2.2: Infection protocol for C57BL/6j and CBA/Ca mice in the Bronchoalveolar experiment and the post-mortem procedures and days are also shown.

Depending on the procedure being carried out, the mice were killed in one of two ways. Mice that were utilised for larval counts were sacrificed by cervical dislocation. Euthanasing mice by cervical dislocation risked damage to the trachea or lungs of mice in the BAL group and thus was not appropriate. These animals were euthanased by carbon dioxide (CO_2) asphyxiation. CO_2 was used in place of chloroform to avoid damage to the lung tissue. Each mouse was placed in an appropriately sized container and the gas introduced by a tube connected to the gas cylinder via a hole in the top of the air-tight container. This procedure was performed in the fume hood as a precaution.

Mice in the BAL group were pinned, dorsal side down to a wooden work board with sterile pins. This dissection board was devised with no edges, as this allowed straight access into the trachea when performing the bronchoalveolar lavage (**fig. 6.2.3**). Using a scalpel (size 11 blade) an incision was made at the anus just underneath the epidermis. This incision was extended forward, using a round ended scissors, to the base of the lower jaw, and taking care not to incise the peritoneal membrane. This was then pinned back and an incision, using a round ended scissors, was made in the peritoneal membrane, beginning at the base of the sternum (the xiphoid cartilage) and this was extended forward to the lower

jaw, all the time ensuring that the underlying lungs and trachea were not cut. The thoracic cavity was opened by cutting the rib cage along each side. The rib cage was then removed with caution not to damage the heart or lungs. The lungs were left *in situ* and care was taken not to disturb them in any way. The trachea was exposed by extending the incision to the point revealing as much of the trachea as possible, removing tissue or fat causing an obstruction. The head of the mouse was pinned to the work board to enhance access to the trachea with the phosphate buffer solution (PBS) filled syringe (see **fig. 6.2.3**). The removal of the bronchoalveolar fluid was the next stage in this process.

Bronchoalveolar lavage (BAL)

A length of yellow cotton thread (the colour was specific so that it was easy to manipulate) was fed under the trachea using a forceps. The tip (the first 3 mm) was cut off a sterile 25g needle thus blunting the end and reducing possible accidental penetration of the trachea or lung wall. A graduated 1ml syringe was attached to the needle and 0.5ml of prepared phosphate buffer saline (PBS- a mixture of sodium phosphate and potassium phosphate, pH 7.2 commercial purchased in tablet form from Sigma-Aldrich® combined with 500 ml of water) was drawn into the syringe. The needle was then guided in through the exposed ventral surface of the wall of the trachea just above the larynx and guided delicately between the rings of cartilage ensuring that the tip did not pierce the wall. The cotton thread was tied tight around the needle and trachea (**fig. 6.2.3**) below the point of entry. The PBS was subsequently infused into the lungs slowly and the plunger withdrawn immediately to aspirate the BAL fluid. The needle was withdrawn and the thread securely tightened. The aspirated BAL fluid was deposited into a sterile Eppendorff tube labelled with mouse ID, date and contents.

Using the same needle, another 0.5ml measure of PBS were drawn into the syringe in preparation for a second BAL infusion, bringing to 1ml the amount of PBS each set of lungs was flushed with (Wilkinson *et al.*, 1990). Another length of cotton thread was passed under the trachea. The second entry point was made lower down on the trachea. The insertion of the needle was made easier by holding the two ends of the first cotton thread taut at a point anterior to the mouse head. This worked by lifting the trachea, which may had become flaccid after the first puncture. Once inserted, the needle was secured with the second length of thread. The infusion process was repeated and the fluid removed was added to the Eppendorff tube and stored on ice. This process was repeated for each

individual. The BAL fluid was examined as soon as possible (within 1 hour after removal). Subsequently, the spleen was weighed (**appendix A.10**).

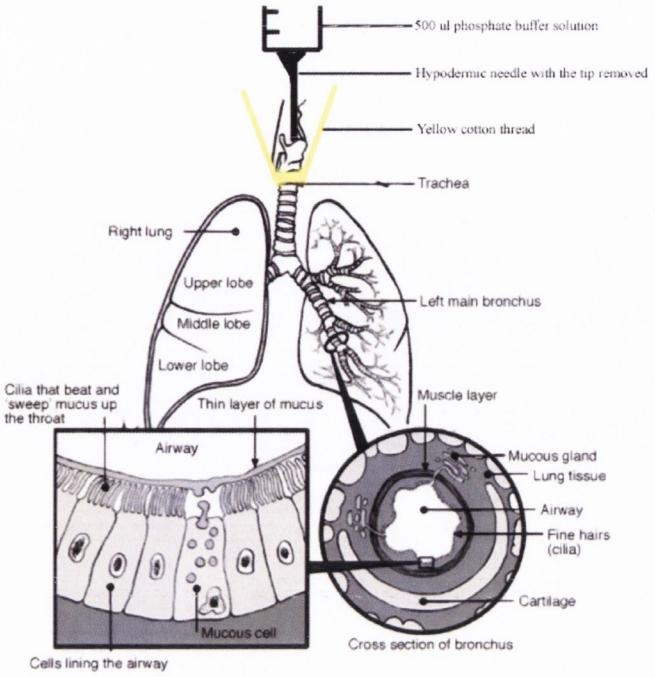


Figure 6.2.3: The procedure of aspiration by bronchoalveolar lavage of the lungs of mice with phosphate buffer saline. Also shown is the structure of the lungs (original figure).

Preparation of the BAL fluid (BALF)

The bronchoalveolar fluid removed from the mice was prepared for further analysis by means of a haemocytometer (Neubauer Counting Chamber) and slide smears as soon as possible following the BAL procedure. The cell counts were carried out within two hours of recovery; this was to prevent the lysing of leukocytes and thus inaccurate total white blood cell (leukocyte) counts (Paul Voorheis, personal correspondence). The technique used to prepare the bronchoalveolar fluid for counting and staining was that described by Kim *et al.* (2002) and is known as the undiluted erythrocyte lysing technique. 500µl of the BAL sample from an individual were added to 9.5ml of lysis solution (0.1 N HCl). The sample was centrifuged at 2100 rpm for 10 minutes at 20°C. The samples were carefully removed from the centrifuge and transferred to the fume hood with care not to disturb the pellet. Using the aspirator, the supernatant was discarded and the final volume adjusted to 500µl. The leukocyte pellet was carefully resuspended (gently shaken) and the leukocyte total cell counts were done immediately, using an improved Neubauer counting chamber (**fig.6.2.4**). The remaining sample was stored on ice to maintain the consistency of the leukocyte suspension.

Improved Neubauer counting chamber (haemocytometer)

The technique described was the most efficient and provided a constant volume of BALF from which the total number of leukocytes could be determined. The slide and cover slip were cleaned in water and dried with low lint 1-ply microscope lens cleaner tissue paper to minimise dust particles on the slide and cover slip. The cover slip was breathed upon to help adhesion to the haemocytometer and the slide was placed carefully on the coverglass mounting supports. Using a 0.1 –1.0 µl Eppendorff pipette, 0.8µl of the prepared BALF was introduced into both sides of the haemocytometer counting chamber through the sample induction point (see **fig. 6.2.4**). After the leukocytes had settled, they were counted in the squares appropriate for white blood cells (marked **W** on **fig. 6.2.4**), in all 16 squares, and this was repeated for four individual squares (**W**) and on both sides of the chamber. The volume of one grid is $0.1ml^3$ (See **fig. 6.2.4**). Thus according to the dimensions of the haemocytometer, the number of cells per cm^3 and thus per ml is calculated as follows:

$$= \frac{10^4 \text{ x number of cells in all (W) squares}}{4}$$

$$= \text{number of leukocytes/ml}$$

And the average of the two sides was then calculated. Two counts (four grids on each side of the chamber) were performed for each individual mouse and an average figure was defined as the total number of cells/ml. Note that larvae were observed migrating through the BAL fluid (**appendix B.2**).

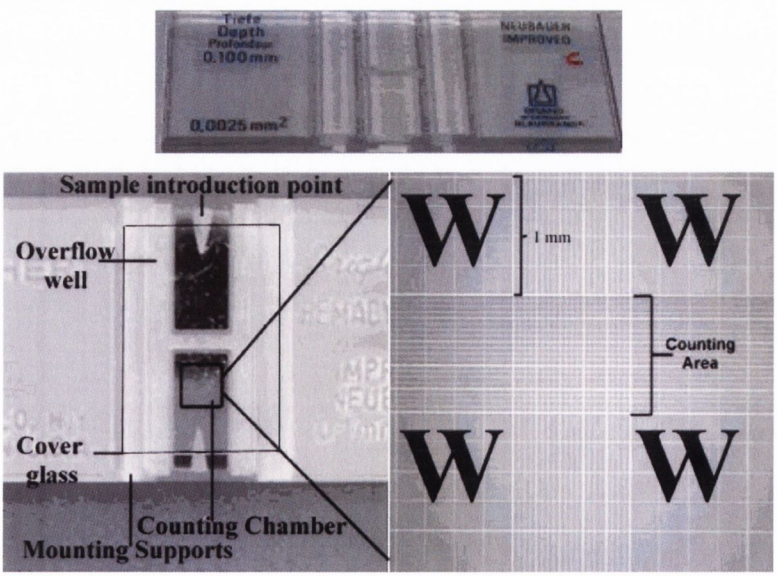


Figure 6.2.4: Haemocytometer (above), showing the raised coverslip (0.1mm) rested on the coverglass mounting supports and the sample introduction point (left). On the right is a photomicrograph of the improved Neubauer counting chamber. The boxes containing the 'W' indicate the boxes from which the white blood cells are counted.

Preparation of the BAL fluid for differential cell counts

Slides were prepared for cell smears by washing in alcohol (absolute alcohol) and air-drying in the fume hood. This provided an adherent surface for the cells. Thick films of cells were smeared, as thin films may not contain the 500 leukocytes needed for the differential counts. A 50µl aliquot of BALF from an individual was deposited twice on an alcohol washed slide and using the corner of a clean slide, using a circular motion, the drops were mixed and the BALF was spread encompassing a circular area with approximately 2 cm diameter. This process was duplicated on a second slide for each sample. The slides were left to dry in a CaptairBio dust free hood, until ready for staining. This was repeated for each BALF sample from each infected individual and also for the control mice from which BALF was taken.

Staining of the slides for differential counts

ACCUSTAIN® Wright stain (Sigma-Aldrich®) was used for the staining of the BALF film for differential staining of the cellular components of the BALF. The Wright stain was supplied ready to use. Wright stain is a modified Romanowsky stain that gives a characteristic blue or pink colouration to the nucleus and cytoplasm of the white cells. The purified dyes in the ACCUSTAIN® formulations of Wright stain eliminate inconsistency and yield reproducible batch-to-batch chromogenic staining (Sigma-Aldrich Wright stain guide). The staining procedure was modified from the literature and the Sigma-Aldrich instructions. The method applied was the horizontal staining method. The slides were placed on a slide rack in a horizontal position (level) over a well. The slide was flooded with 1-2ml of stain, covering the entire surface. It was left for three minutes, after which an equal quantity of deionised water was gently added to the slide without rinsing off the Wright stain. The deionised water was mixed with the stain by gently blowing on the surface of the fluid for 1 minute. After this time, the stain was rinsed off the slide with deionised water. The underside of the slide (which had no BALF film) was carefully wiped and the slide was suspended horizontally, face-up, and left to dry in slide boxes overnight, partially covered by a lid to protect them from dust particles in the fume hood.

Differential leukocyte counts

Once the slides were dry, the BAL film was mounted in DPX in the fume hood and a glass coverslip was placed on top. The slides were examined using an inverted phase contrast microscope (Axiovert) with x63 objective lens and a 1.4 numerical aperture. A total of 500 cells were counted per slide and the cell types were differentiated, with the assistance

of two experienced observers (Dr. Paul Voorheis and Dr. Derek Nolan, Biochemistry Department, Trinity College Dublin). This process was repeated twice for each BALF slide preparation for each individual and the average from the two counts was taken. The differentiated cells were calculated as a percentage of the 500 cells and then this was used to determine the number of specifically differentiated cells of each cell type present in the total number of leukocytes in a millilitre (ml) of BALF. The cells were differentiated as follows (Sell, 1987):

- Neutrophils: these granulocyte cells range in size from 10-12 μm and have a multi-lobed nucleus and granular staining of the cytoplasm (**Plate 6.2.1**)
- Lymphocytes: these agranulocytes can range in size from 6- 30 μm. They contain intensely staining nuclei and blue-staining cytoplasm (**Plate 6.2.2**)
- Eosinophils: these have a characteristic bi-lobed nucleus and bright pink-staining granules in the cytoplasm. They range in size from 10-14 μm (**Plate 6.2.3**)
- Macrophages: these are the largest of the leukocytes, and are characterised of an eccentrically placed nucleus (**Plate 6.2.1**)
- Basophils: these range in size from 10-12μm and are noted for their large granules in the cytoplasm, which is often obscure the nucleus (**Plate 6.2.4**)

STATISTICAL ANALYSIS

Statistitical analysis of larval recovery was similar to previous chapters. The influence of the factors on larval burden and total leukocyte and individual leukocyte counts were analysed by means of analysis of variance. *Post-hoc* analyses were used were appropriate. Statistical analysis was carried out at a confidence limit of 95% (α= 0.05).

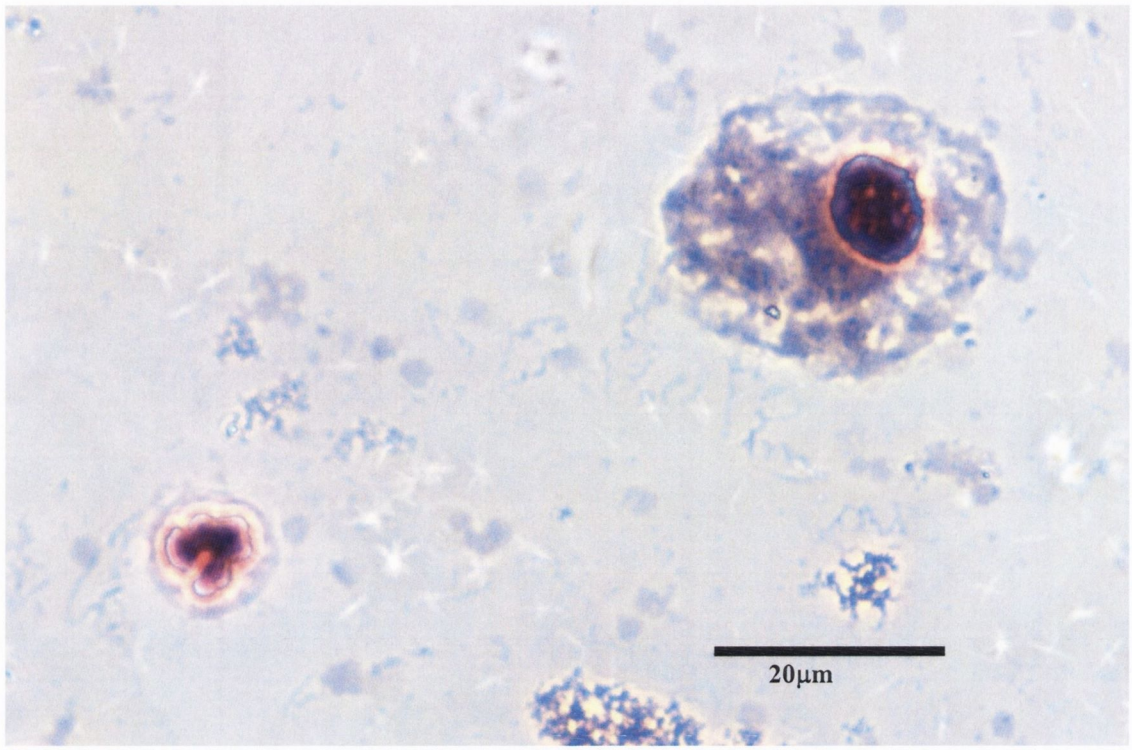


Plate 6.2.1: A neutrophil (left) and macrophage (right) in BAL fluid of an infected mouse. Note the multi-lobed nucleus in the neutrophil and the large size of the macrophage.

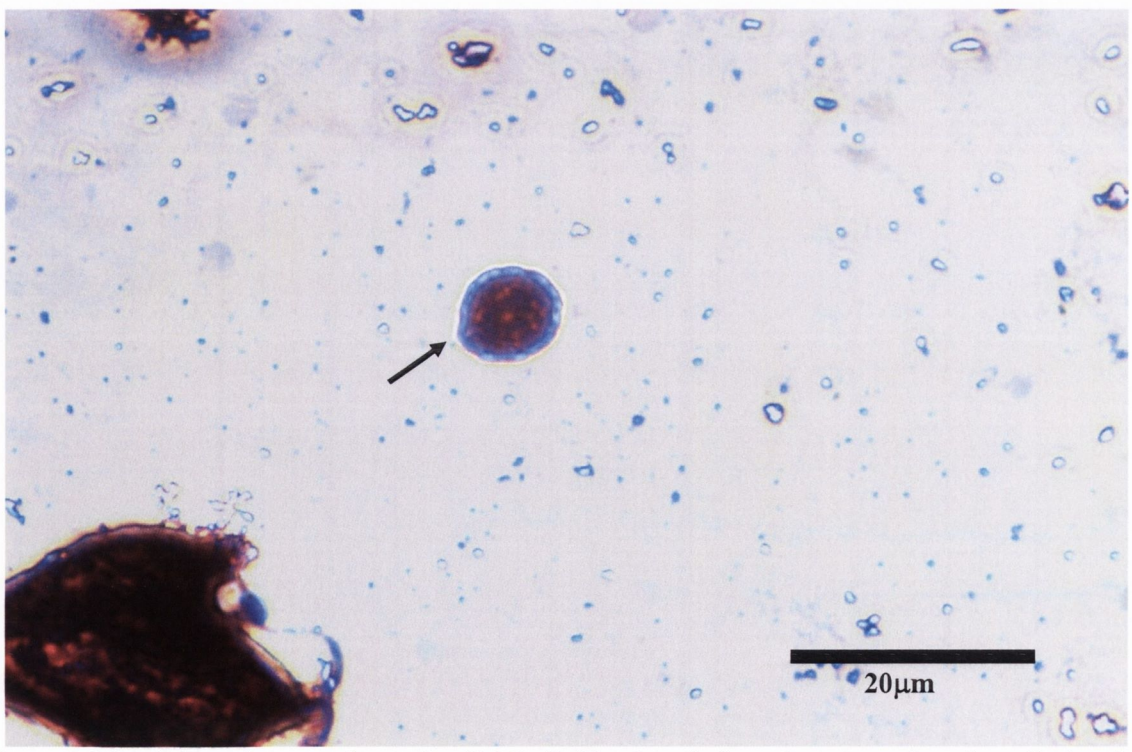


Plate 6.2.2: A lymphocyte from the BAL fluid of an infected mouse showing distinctive Wright's staining (deep blue-purple nucleus and lighter blue cytoplasm).

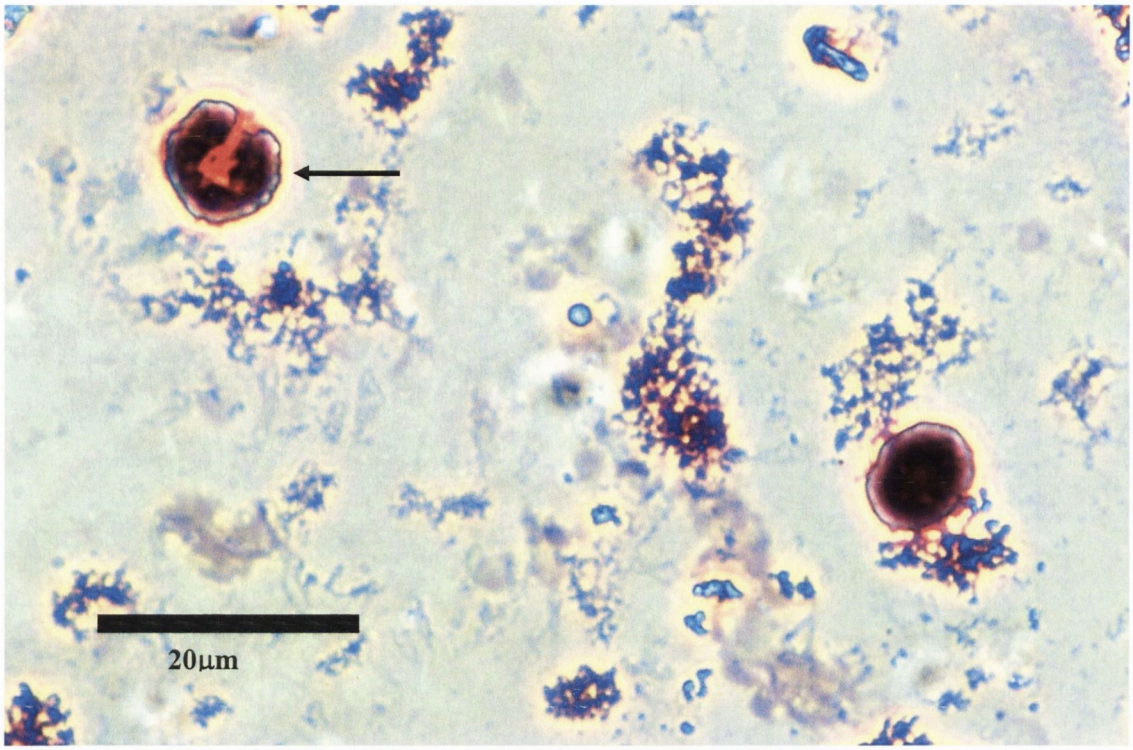


Plate 6.2.3: An eosinophil (left) and lymphocyte (right) in BAL fluid of an infected mouse. The bi-lobed nuceus and dark-pink staining of the cytoplasmic granules in the eosinophil are clearly seen.

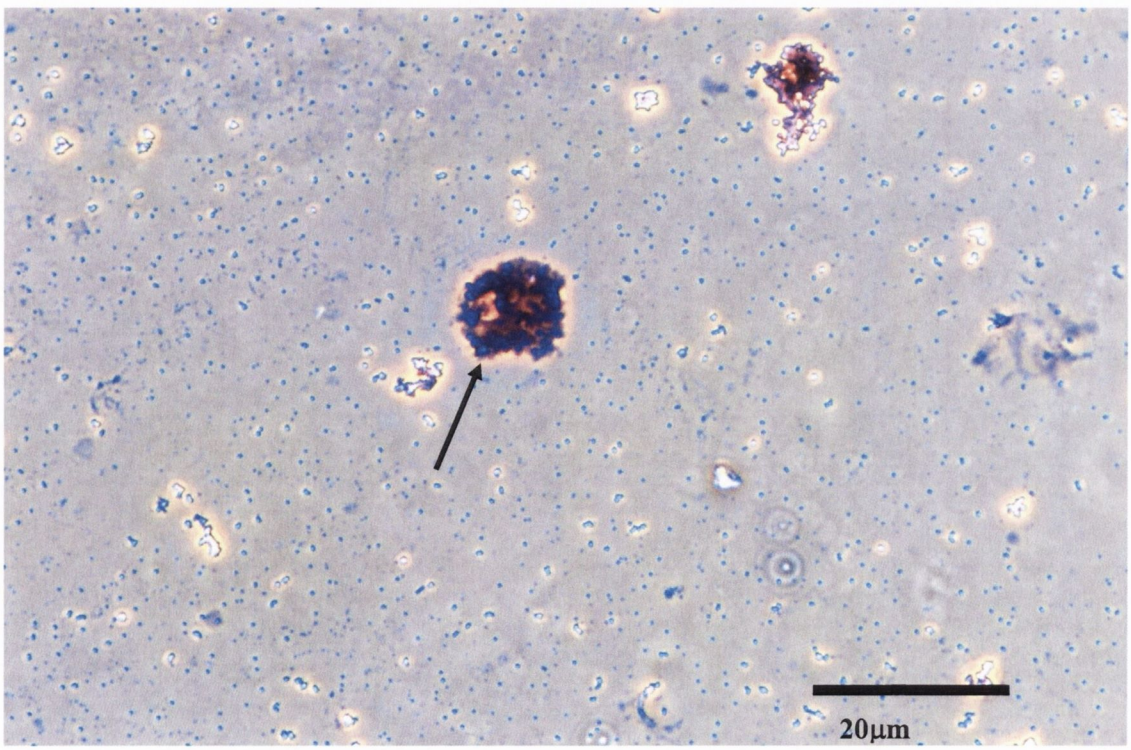


Plate 6.2.4: A basophil found in the BAL fluid of an infected mouse. Note the large cytoplasmic granules and concealed nucleus.

6.3 RESULTS

THE EFFECT OF HYDROCORTISONE ON THE NUMBER OF LARVAE RECOVERED FROM THE LUNGS OF C57BL/6j AND CBA/Ca MICE

The first stage in this analysis was the investigation of the change in spleen weight (**appendix A.8**) between the three treatment types (mice inoculated with both cortisone and *A. suum*, those infected only with *A. suum* and the uninfected control mice). There was a significant decrease in the spleen weight of both strains of mice when inoculated with both cortisone and *A. suum* on days 7-9 in contrast to the spleen weight of uninfected control mice and *A. suum* infected mice that did not receive cortisone. (**fig. 6.3.1**; initially 3-way ANOVA, with strain and treatment as factors and day as covariate, R^2_{adj}=0.684, main effect of treatment, $F_{2,63}$=72.0, $P \leq 0.0001$). Spleen weight was also influenced by the day post-inoculation (main effect of day, $F_{1,63}$=9.9, P=0.0026). There was no significant difference between the strains in spleen weight (Full factorial model, main effect of strain $F_{1,63}$=0.17, P=N.S.). An LSD *post-hoc* analysis following the inclusion of day as a factor showed that the control group were markedly different from both the infected control group (P=0.04) and also the hydrocortisone administered group ($P \leq 0.0001$). The control group had higher mean spleen weights than the cortisone group but lower than the infected group (**fig. 6.3.1**).

The movement of larvae into the lungs of fully intact infected mice followed the prescribed pattern with similar numbers of worms recovered to previous results (see chapters 3, 4 and 5). The greatest disparity was the accumulation of higher numbers of larvae into the lungs of C57BL/6j mice on day 8 rather than day 7 (**fig. 6.3.2**). The larval burden on this day was 56 ±9.1 (11.2% of the administered dose). The burden on day 7 in CBA/Ca mice was 11.2 ±5.4 (2.2%). The percentage difference between the two strains was maintained in this experiment, with a difference of 74.5% on day 7 p.i.. As expected, there were significant differences between the strains in terms of the number of worms recovered (3-way ANOVA, with strain, treatment [those inoculated with cortisone and those mice administered the saline placebo] and day as factors, full factorial model R^2_{adj}=0.53, main effect of strain, $F_{1,45}$=67.0, $P \leq 0.0001$). However, the administration of cortisone did not influence worm burdens (effect of treatment, $F_{1,45}$=0.04, P=N.S) and all interactions involving treatment were insignificant. Worm burdens in the lungs did not change significantly with time (main effect of day, $F_{2,45}$=1.08, P=N.S.). This indicated that there

was no change in larval burden or relationship between the two strains associated with the administration of cortisone (**fig. 6.3.2**).

The body weight of mice in the control groups (both C57BL/6j and CBA/Ca) increased by day 5 post-mortem in contrast to reductions of body weight of infected mice of both strains during days 6-8 (**fig. 6.3.3**). Mouse body weight was significantly reduced during the infection period (3-way ANOVA with strain and treatment as factors and day as a covariate, model R^2_{adj}=0.703, main effect of day $F_{1,60}$=26.0, $P \leq 0.0001$). There was consistently more weight loss in individuals inoculated with cortisone than those individuals that were only infected with *A. suum* in the two mouse strains (**fig. 6.3.3**; main effect of treatment $F_{2,60}$=44.1, $P \leq 0.0001$). There was an insignificant effect of strain ($F_{1,60}$=0.50, P=N.S.) but there was a marginal significant 2-way interaction (treatment*strain, $F_{2,60}$=3.4, P=0.04), so the three regimes produced different patterns of weight change during the infection period.

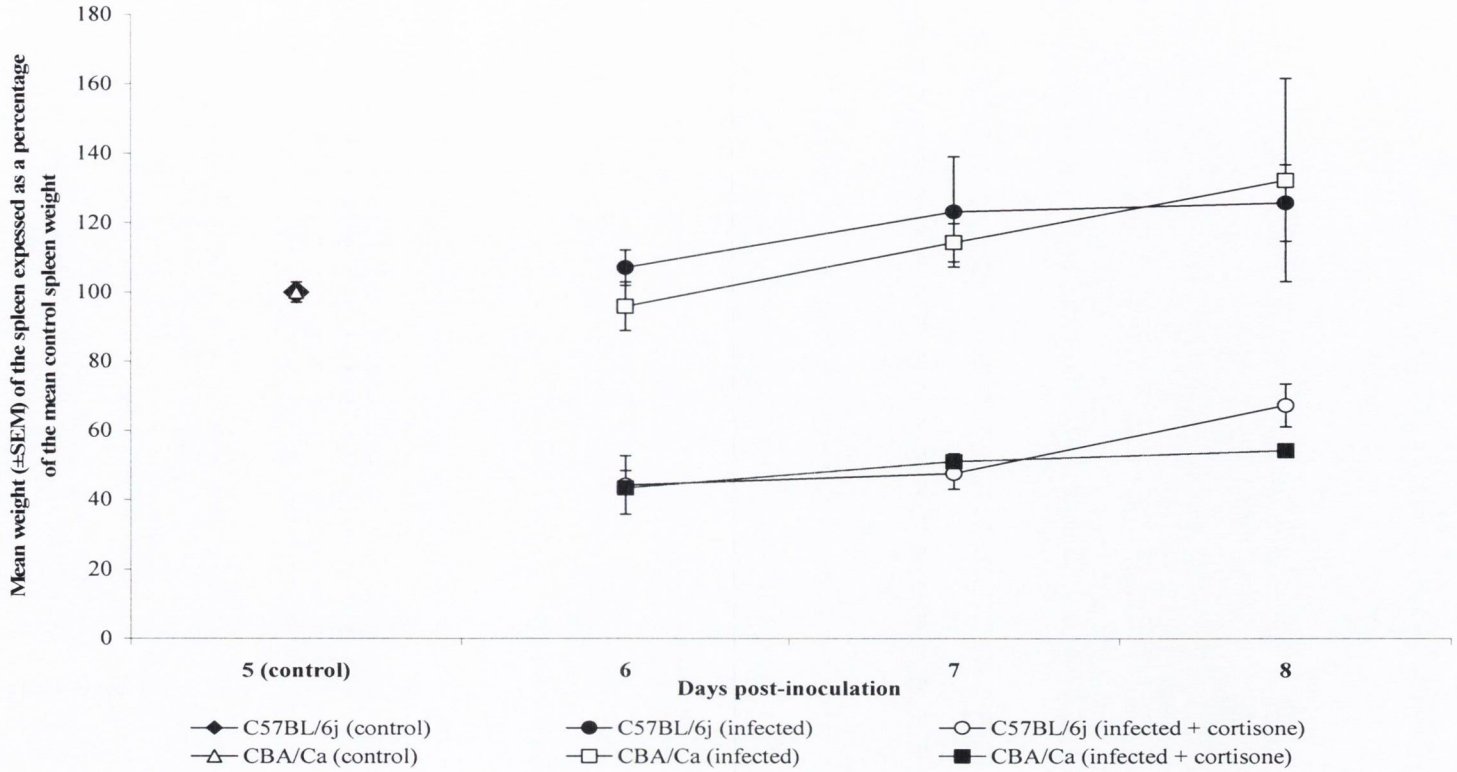


Figure 6.3.1: Mean spleen weight (±S.E.M.) expressed as a percentage of the mean weight of the control mice spleen weight on days 6, 7 and 8 and the control spleen (100%) on day 5 (C57BL/6j [♦] and CBA/Ca [Δ]). The strain C57BL/6j inoculated with *A. suum* and placebo hypodermic inoculations of water (●) and mice inoculated with *A. suum* and hypodermic inoculations of cortisone (○) are shown and CBA/Ca mice, those infected with *A. suum* (■) and those inoculated with both *A. suum* and cortisone (□).

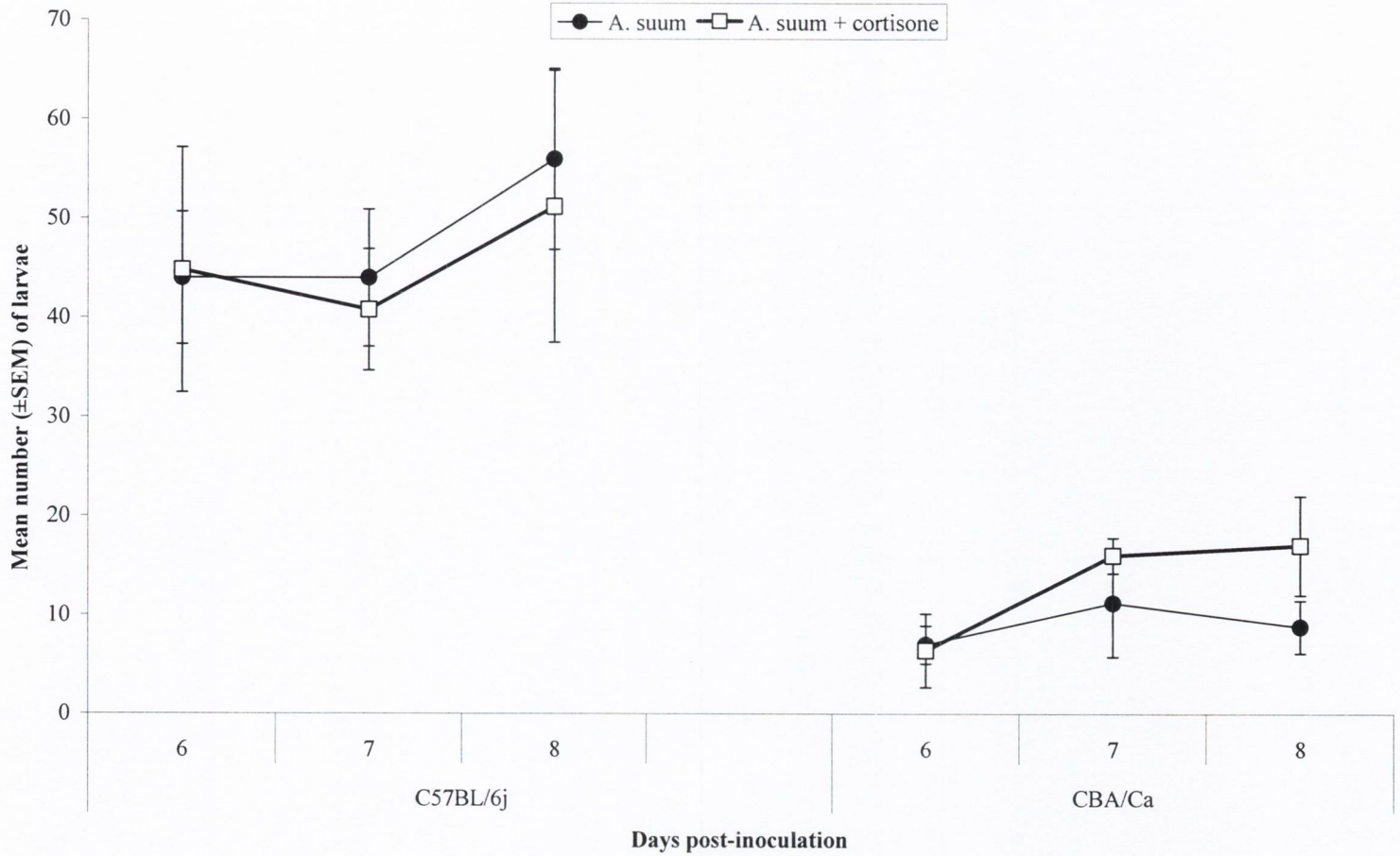


Figure 6.3.2: Mean number (±S.E.M.) of larvae recovered from the lungs of the two strains of mice on days 6, 7 and 8 post-inoculation. Shown are the larval burdens of mice inoculated with *A. suum* and a placebo (●) and mice inoculated with *A. suum* and cortisone (□).

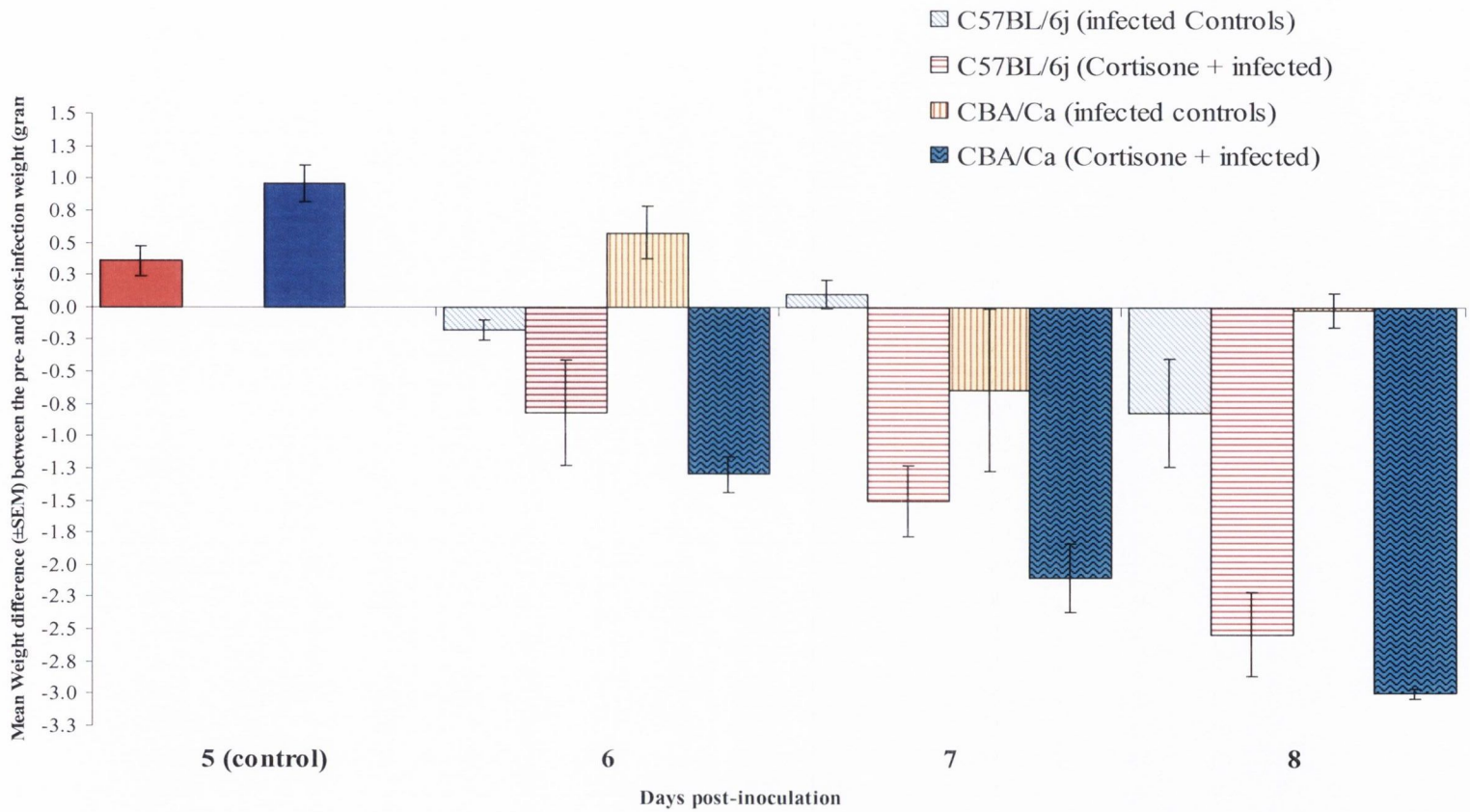


Figure 6.3.3: The mean weight difference (±S.E.M.) between the pre- and post-infection weight of the groups of mice from each strain on days 6-8 post-inoculation in those mice inoculated with 500 *A. suum* ova (C57BL/6j [\\\] and CBA/Ca [||||]) and also mice inoculated with 500 *A. suum* and cortisone (C57BL/6j[≡ and CBA/Ca [≈). The mean weight difference (±S.E.M.) in the control mice that were not infected with *A. suum* or cortisone are shown on day 5 (Solid boxes, Red box= C57BL/6j; Blue box=CBA/Ca).

Larval migration through the lungs of C57BL/6j and CBA/Ca mice on days 7-9 p.i.

As previously, there was a highly significant difference in larval burdens between the strains in the number of worms in the lungs with consistently more larvae in the tissue of C57BL/6j mice (**fig. 6.3.4;** 3-way ANOVA with strain and day and organ (left and right hand side lung) as factors, model R^2_{adj}=0.49, main effect of strain $F_{1,24}$=30.3, $P \leq 0.0001$) but no significant interaction between strain and day. Because this experiment was restricted to the later days of infection (i.e. 7-9), and given the variation in recovery within the sub groups particularly on day 8, overall worm burdens did not vary significantly over day (main effect of day $F_{2,24}$=1.5, P=N.S.). This suggests that the overall pattern of recovery did not differ between the two strains statistically, although numerically (mean worm burdens in **fig. 6.3.4**) larvae arrived later in the lungs CBA/Ca mice.

Both mouse strains had a proportional number of larvae recovered from the initial inoculum at this infection level as expected from the dose/recovery graph (**fig. 6.3.5**). Larvae accumulated in the lungs of C57BL/6j mice on day 7 (260 ±34.6) and declined thereafter (**fig. 6.3.4**). The pattern of migration in the lungs of CBA/Ca mice was consistent with previous results except that the peak of the movement into this organ was on day 8 (96.0 ±34.6) and lower larval numbers observed either side (**fig. 6.3.4**). There were consistently higher numbers of worms recovered in the right side compared to the left side of lungs (**table 6.3.1**; main effect of organ $F_{1,24}$=6.3, P=0.019) of both strains (strain*organ interaction $F_{1,24}$=0.56, P=N.S.) , particularly on day 7 p.i., however the interaction between organ and day was insignificant (organ*day interaction $F_{1,24}$=0.31, P=N.S.). The interaction of all three factors was insignificant.

Table 6.3.1: Mean number (±S.E.M.) of larvae recovered from the left and right hand side lungs in the two strains of mice on days 7, 8 and 9 post-inoculation.

Strain	n	Days p.i.	Left Lung (±S.E.M.)	Right Lung (±S.E.M.)
C57BL/6j	3	**7**	92.0 (±22.0)	168.0 (±12.9)
	3	**8**	104 (±18.5)	133.3 (±57.6)
	3	**9**	74.7 (±18.8)	93.3 (±21.5)
CBA/Ca	3	**7**	30.7 (±10.7)	45.3 (±9.3)
	3	**8**	33.3 (±17.1)	62.7 (±12.7)
	3	**9**	24.0 (±6.9)	48.0 (±8.3)

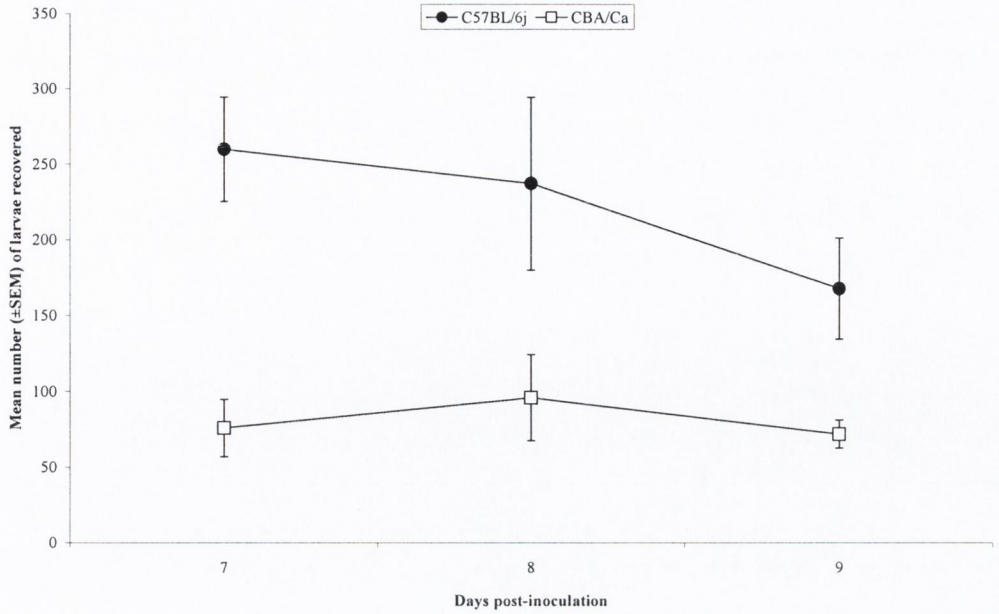


Figure 6.3.4: Mean number (±S.E.M.) of larvae recovered from the lungs of both C57BL/6j and CBA/Ca mice inoculated with 1500 infective *A. suum* eggs on days 7, 8 and 9 post-inoculation.

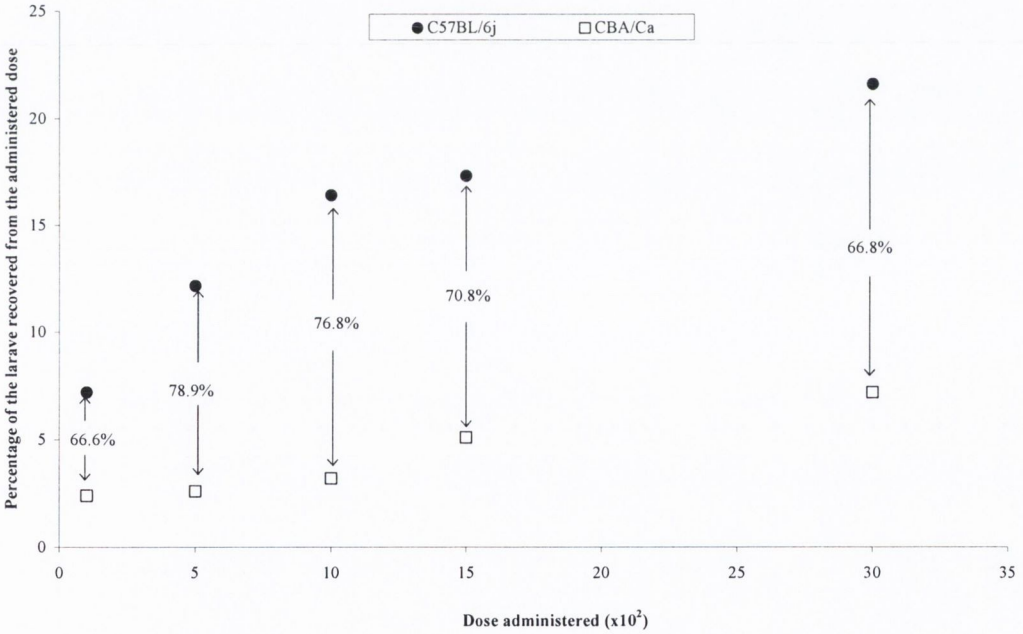


Figure 6.3.5: The percentage of larvae recovered on day 7 p.i. from the initial inoculation doses of 100, 500, 1000, 1500 and 3000 from the lungs of the mouse strain C57BL/6j (●) and the strain CBA/Ca (□) and the percentage difference between the two strains (Percentage difference calculated as: (Total number of larvae recovered from C57BL/6j-Total number of larvae recovered from CBA/Ca)/Total number of recovered from C57BL/6j).

As can be seen **(fig. 6.3.6)** the strains differed significantly in their post-infection weight (**appendix A.10**) with a constant reduction during the experimental procedure particularly in the more highly infected C57BL/6j strain. When the body weights of the infected mice were analysed against that of the control group there was a significant effect of *A. suum* infection on mouse weight (**fig. 6.3.6;** 3-way ANOVA with strain and treatment as factors and day as a covariate, model R^2_{adj}=0.84 main effect of treatment $F_{1,23}$=9.1, P=0.0061) with significant weight loss associated with the presence of *A. suum*. There was also a significant difference in the average weight loss between the two strains (main effect of strain $F_{1,23}$=7.2, P=0.0131) and the difference between strains was dependent on whether they were infected or not (naïve CBA/Ca mice generally heavier than C57BL/6j mice but the latter lost weight more rapidly after infection; the 2-way interaction strain*treatment $F_{1,23}$=12.7, $P \leq 0.0017$). In other words, the body weight of C57BL/6j mice was reduced on all days in contrast to the control (uninfected) group (**fig. 6.3.6**). The duration of infection in terms of days from inoculation with *A. suum* also played a highly significant role (main effect of day $F_{1,23}$=45.1, $P \leq 0.0001$) with groups on later post-mortem days showing greater weight reductions in both strains. The greatest weight loss of infected mice of both strains was observed on day 9. It would appear from the results that weight loss was attributable to the pulmonary larval burden.

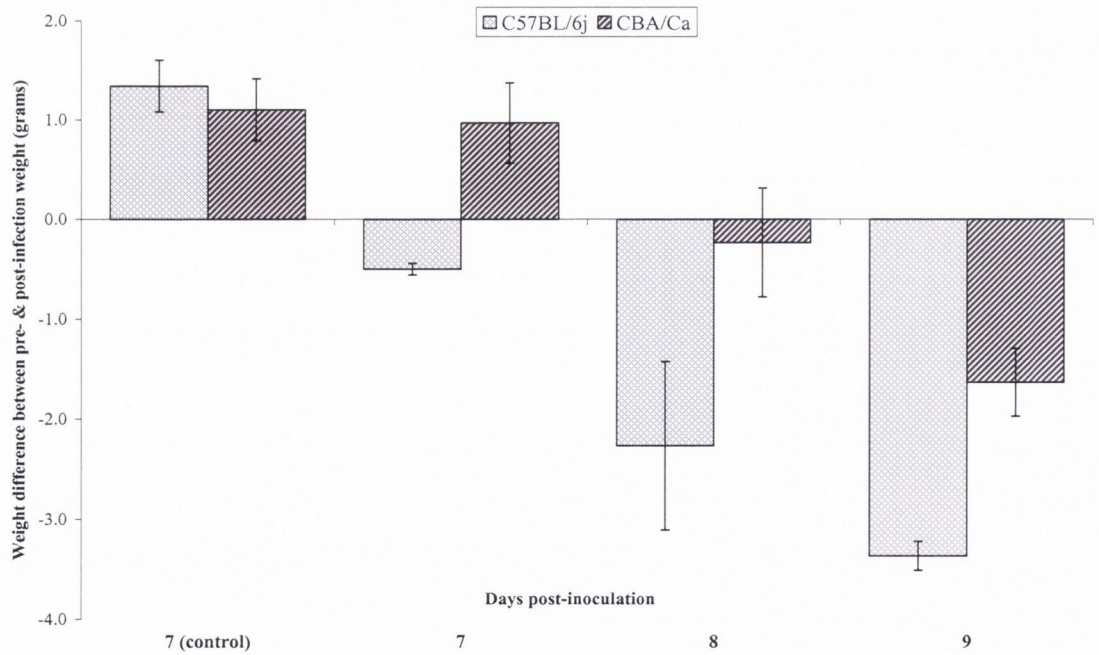


Figure 6.3.6: The mean weight difference (±S.E.M.) between the pre- and post-infection weight of the groups of mice from each strain on days 7-9 post-inoculation in the two strains of mice (C57BL/6j and CBA/Ca) inoculated with 1500 *A. suum* ova. The mean weight difference (±S.E.M.) on day 7 in the control mice that were not infected with *A. suum* is also shown.

An estimated number of larvae that would be recovered after the administration of a dose of 1500 *A. suum* eggs on day 7 can be calculated from the dose-larval recovery graph illustrated in chapter 4. The larval burden in both strains in the current investigation confirmed these expected results and the percentage difference between the two strains was within the predicted range (**fig. 6.3.5**). C57BL/6j mice had a maximum average of 17.3% of the administered dose on day 7 p.i.. The number of worms recovered from the lungs of CBA/Ca mice also lay within the predicted range of the administered dose recovered on day 7 p.i. (5.1%). Analogous to the results of chapter 4, the movement of larvae into the lungs at this higher dosage in CBA/Ca was delayed and occurred on day 8 (96 ±28.4) but there was a lot of variation in individuals on this day. There is no significant difference between this day and the days either side.

Changes in Bronchoalveolar leukocytes during infection with *A. suum*

The lungs of mice of both C57BL/6j and CBA/Ca strains inoculated with 1500 infective *A. suum* ova were assessed for changes in the classes of bronchoalveolar leukocytes present in the fluid aspirated from the lungs. Control mice provided the expected total numbers of leukocytes in uninfected individuals for comparative purposes. Differences between day 7 (control) and the other three post-mortem days (8-10), principally on the latter days of infection, highlighted the effect of *A. suum* migration on the bronchoalveolar leukocyte population. **Figure 6.3.7** represents the total number of leukocytes recovered from each group and also the total number of each class present on those days ($x10^5ml^{-1}$). The leukocyte population of the uninfected mice was also represented on this graph (day 7 control). The leukocyte count of uninfected C57BL/6j mice was approximately 1.21 (±0.390) $x10^5ml^{-1}$ and for CBA/Ca it was 1.40 (±0.684) $x10^5ml^{-1}$. The predominant cell type of uninfected control mice were macrophages, accounting for 80.5% and 75.2% for the strains C57BL/6j and CBA/Ca, respectively (**table 6.3.2**).

During the infection period there was a greater increase in the total number of leukocytes of C57BL/6j mice (2-way ANOVA with strain*day (including control) as factors, model R^2_{adj}=0.56, main effect of strain $F_{1,29}$=11.8, P=0.0018) and this appeared be correlated with worm burden. There was also a significant day effect with increasing immunological response (main effect of day $F_{3,29}$=9.8, $P \leq 0.0001$). However, the magnitude of the response in the 2 mouse strains diverged significantly with time, increasing more rapidly in respect of time in C57BL/6j mice compared with CBA/Ca mice (2-way interaction

strain*day $F_{3,29}$=3.0, P=0.046), indicating a differential response between the strains as a reaction to infection.

The results of LSD *post-hoc* analysis confirmed that the immune response was delayed in reacting to the presence of larvae. The *post-hoc* analysis identified a significant difference between the post-mortem days in terms of total white blood cells. Thus, the day 7 total bronchoalveolar leukocytes (the control group) did not differ significantly from the total number recovered in the BAL fluid of mice on day 8. However, these two days were significantly different from both day 9 (day 7; P=0.0061 and day 8; P=0.0092) and day 10 ($P \leq 0.0001$ for both days). The remainder of this analysis illustrates the changes of the individual leukocytes during the period of infection examined, from days 8-10 post-inoculation.

Table 6.3.2: Mean total number of leukocytes (±S.E.M.) recovered from the bronchoalveolar fluid (BALF) of the two mouse strains on days 7 (control mice) 8, 9 and 10 and the percentage (±S.E.M.) of each differential leukocyte in the BALF of those groups.

strain	n	Day	Total Cells Mean (±S.E.M.)	Lymphocyte (%)	Neutrophil (%)	Eosinophil (%)	Macrophage (%)	Basophil (%)
C57BL/6j	4	7	121093 ±38997	10.27 ±2.16	4.24 ±0.42	3.44 ±0.83	80.50 ±3.49	1.56 ±0.69
	5	*8*	237550 ±52851	45.80 ±1.11	44.52 ±1.87	4.04 ±0.25	5.64 ±1.59	0.00 ±0.00
	5	*9*	799980 ±187918	61.16 ±4.01	25.32 ±3.91	7.24 ±1.41	6.08 ±0.80	0.20 ±0.11
	5	*10*	887500 ±203869	48.56 ±3.10	30.80 ±2.02	14.16 ±0.95	6.36 ±0.57	0.12 ±0.08
CBA/Ca	3	*7*	140729 ±68358	13.59 ±7.13	3.24 ±0.48	2.27 ±0.67	75.20 ±9.10	5.69 ±2.60
	5	*8*	125000 ±19764	67.44 ±1.56	23.32 ±0.45	1.48 ±0.08	7.50 ±1.41	0.20 ±0.11
	5	*9*	181250 ±36175	52.28 ±6.13	29.12 ±4.37	3.16 ±0.62	15.12 ±2.03	0.32 ±0.16
	5	*10*	462500 ±72887	67.72 ±3.85	16.64 ±2.45	6.36 ±0.82	9.16 ±0.89	0.12 ±0.08

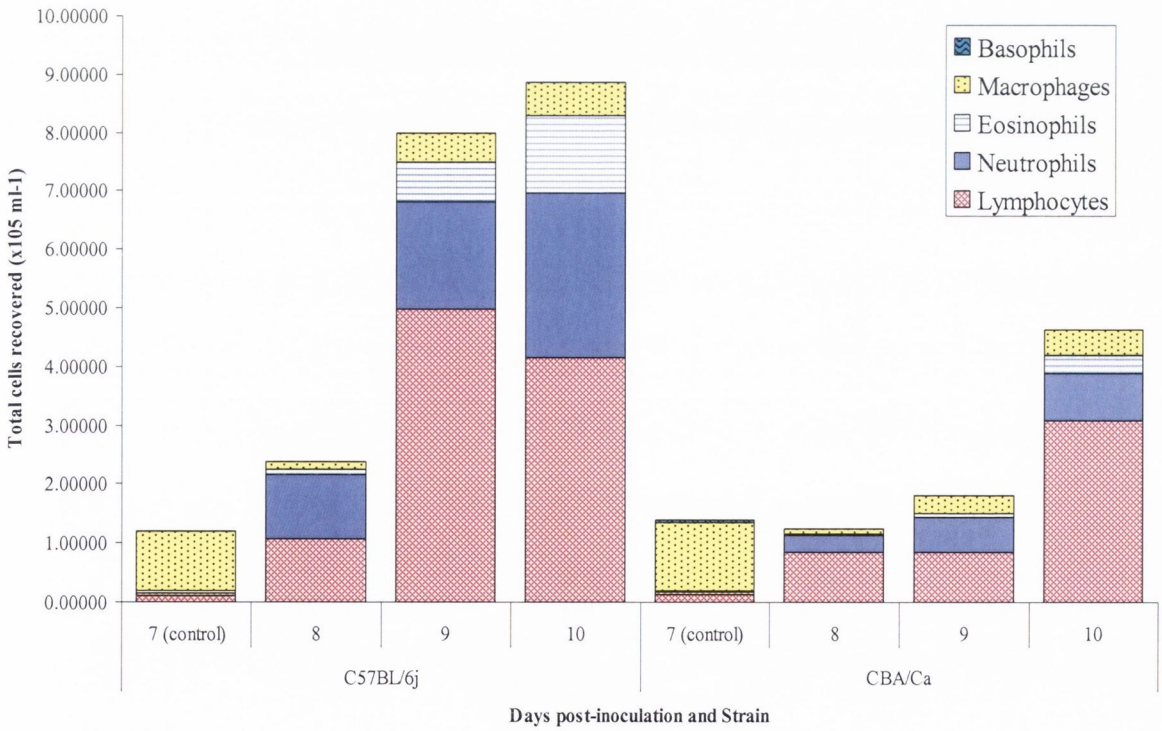


Figure 6.3.7: The number and class of bronchoalveolar leukocytes recovered following lung lavage on days 8, 9 and 10 post-infection with *A. suum* and the leukocyte population recovered from the lungs (on day 7) of control mice administered 0.1 N H_2SO_4 without *A. suum* ova.

CHANGES IN THE INDIVIDUAL LEUKOCYTE CELL TYPES ON DAYS 7-10 POST INOCULATION

Lymphocytes

Lymphocytes were the most abundant leukocyte cell type in both strains during infection period (days 8-10), comprising nearly 50% or more of the population, in contrast to control mice (day 7) in which they accounted for 10%. There was a significant increase in the numbers of lymphocytes produced associated with day (**Fig. 6.3.8;** full factorial 2-way ANOVA with strain*day (including day 7) as factors, model R^2_{adj}=0.60, main effect of day $F_{3,29}$=12.1, $P \leq 0.0001$). There was a difference in the pattern of accumulation of these cells between the two strains (2-way interaction strain*day $F_{3,29}$=4.5, P=0.01) except on day 9. Strain significantly affected the number of lymphocytes recovered ($F_{1,29}$=9.0, P=0.0054). The results of the LSD *post-hoc* analysis illustrated that both day 7 and day 8 were significantly different to day 9 (P=0.0003 and P=0.00032) and day 10 ($P \leq 0.0001$ and P=0.0002), respectively. There was vigorous production of lymphocytes in both mouse strains during the infection period (**Fig 6.3.8;** 2-way ANOVA with strain*day p.i. as factors, model R^2_{adj}=0.53, main effect of day $F_{3,24}$=8.4, P=0.002). Between day 8 and 9 C57BL/6j mice showed a five fold increase in this WBC type. Ín contrast there was a delayed increase of lymphocytes of CBA/Ca mice in which a 3-fold increase was observed on day 10 (effect 2-way interaction strain*day $F_{2,24}$=4.6, P=0.02). An examination of the lymphocytes of mice shows that strain had a significant effect on the total number of lymphocytes during the infection period ($F_{1,24}$=10.8, P=0.003).

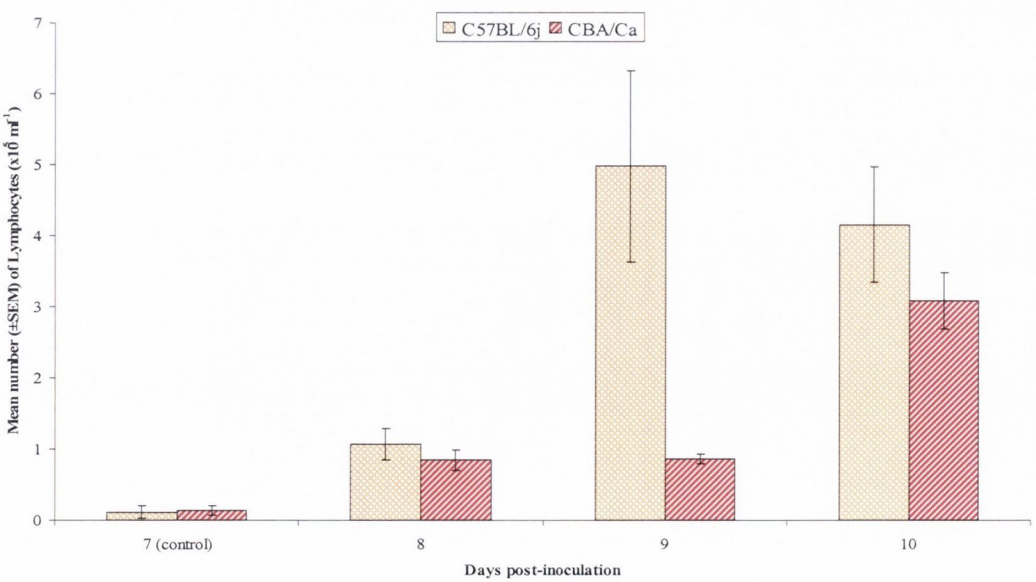


Figure 6.3.8: Mean number (±S.E.M.) of lymphocytes ($x10^5$ ml^{-1}) recovered in the BALF from the lungs of *A. suum* infected mice on days 8, 9 and 10 post-inoculation and non-infected control mice on day 7.

Neutrophils

Analysis of the neutrophil population showed that there was a significant difference in the number of these cells produced in response to infection between the two strains (**Fig. 6.3.9;** full factorial 2-way ANOVA with strain and day (including controls) as factors, model R^2_{adj}=0.38, main effect of strain $F_{1,29}$=10.4, P=0.003) and the day of examination differed (main effect of day $F_{3,29}$=2.8, P=0.05). There was a consistent disparity in the daily response between the two strains in the neutrophils produced (2-way interaction strain*day $F_{3,29}$=1.3, P=N.S.). The total number of neutrophils was lower in control mice of both strains (≤5%). An LSD *post-hoc* analysis revealed that day 7 was significantly different to both day 9 (P=0.0051) and day 10 ($P \leq 0.0001$). The day 8 response was also significantly different to day 10 (P=0.0032).The total number of neutrophils increased in the BAL fluid of C57BL/6j. There was a 2.5-fold increase in the total number of neutrophils in both mouse strains during the observation period (**fig. 6.3.9**). However, the total number of neutrophils was much lower throughout the three day period in CBA/Ca mice in contrast to C57BL/6j mice (**Fig. 6.3.9**; 2-way ANOVA with strain*day p.i. as factors, model R^2_{adj}=0.46, main effect of strain $F_{1,24}$=18.6, P=0.0002). The day post-infection had a significant effect on the production of neutrophils ($F_{2,24}$=4.3, P=0.03). There was no strain*day p.i. interaction suggesting that the pattern of neutrophil production was similar in the pulmonary tissue of the two strains i.e. an increase of 2.5x of the total.

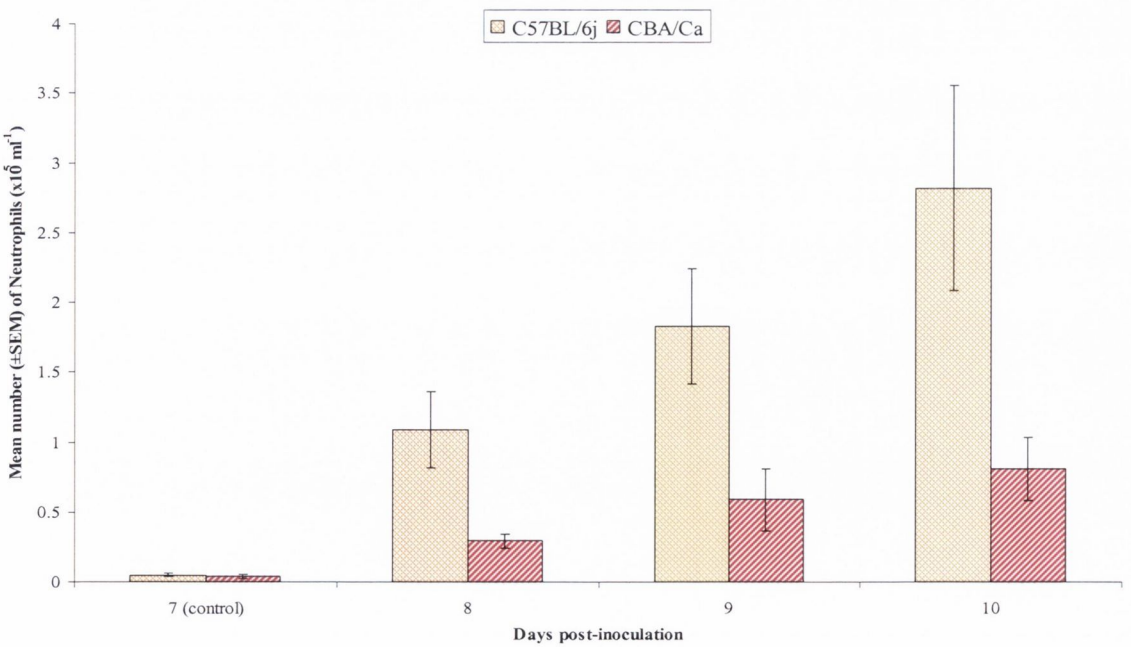


Figure 6.3.9: Mean number (±S.E.M.) of neutrophils ($x10^5$ ml^{-1}) recovered in the BALF from the lungs of *A. suum* infected mice on days 8, 9 and 10 post-inoculation and non-infected control mice on day 7.

Eosinophils

Eosinophils accounted for less than 3% of the control mice leukocyte population. They did not form an extensive part of the early stages of infection but increased appreciably on the latter days but in particular in the susceptible strain (**Fig. 6.2.10**, full factorial 2-way ANOVA with strain and day (including day 7) as factors, model R^2_{adj}=0.59, main effect of day $F_{3,29}$=9.9, $P \leq 0.0001$). There was a proportional increase in eosinophils within each strain as the total number of leukocytes increased. The eosinophilic response of C57BL/6j mice was faster than that of CBA/Ca (2-way interaction strain*day $F_{3,29}$=3.8, P=0.013). There were marked changes in the proportions of these cells by day 10 (**table 6.3.2**) and this occurred in both strains in the total number of eosinophils produced (main effect of strain $F_{1,29}$=13.6, P=0.0009). These results are revealed by the post-hoc analysis as day 10 was significantly different from all other days ($P \leq 0.0001$ days 7 and 8; P=0.0084 day 9).

There was a consistent increase in the number of eosinophils produced by C57BL/6j mice during the infection period. The total number of eosinophils increased daily and there was a 14.5-fold increase between day 8 and day 10 . The response of CBA/Ca mice was less vigorous (2-way ANOVA with strain*day p.i. as factors, model R^2_{adj}=0.57, 2-way interaction $F_{2,24}$=3.8, P=0.036). The CBa/Ca strain produced lower numbers of eosinophils when infected with *A. suum* (**fig. 6.3.10;** effect of strain $F_{1,24}$=16.5, P=0.0004). Although when considering the total number of eosinophils produced there was also a 14.5-fold increase in CBA/Ca mice during the infection period. The day post-infection significantly influenced eosinophil production with a consistent increase between days 8-10 observed in both mouse strains (effect of day $F_{2,24}$=9.6, P=0.0009).

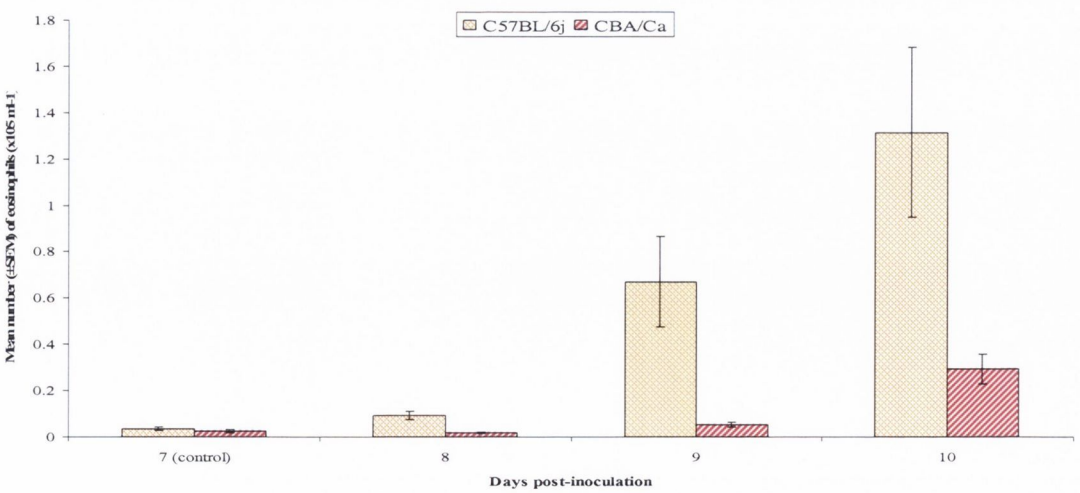


Figure 6.3.10: Mean number (±S.E.M.) of eosinophils ($x10^5$ ml^{-1}) recovered in the BALF from the lungs of *A. suum* infected mice on days 8, 9 and 10 post-inoculation and non-infected control mice on day 7.

Macrophages

The population composed of macrophages was considerably higher in uninfected individuals, although this was highly variable in all individuals on all days. The total number of macrophages was reduced in infected mice. This was demonstrated by the results of the *post-hoc* analysis as day 7 was significantly different from days 8, 9 and 10 ($P \leq 0.0001$, $P=0.0042$ and $P=0.0136$), respectively. There was no significant difference between the two strains in relation to the total number of macrophages in the BAL fluid (**Fig. 6.3.11**, full factorial 2-way ANOVA with strain and day (including day 7) as factors, model $R^2_{adj}=0.47$, main effect of strain $F_{1,29}=2.2$, P=N.S.). However, there was a significant day effect on the number of macrophages (main effect of day $F_{3,\ 29}=11.5$, $P=0.0001$). Total number of macrophages increased from day 8 to 9 but remained relatively constant thereafter in both strains. The number of macrophages present in the BALF of infected C57BL/6j increased during the period of infection. A similar trend was observed in the total number of macrophages of CBA/Ca. There was no difference in the macrophage count of the infected strains (**Fig. 6.3.11**, 2-way ANOVA with strain*day p.i. as factors, model $R^2_{adj}=0.36$, main effect of strain $F_{1,24}=2.5$, P=N.S.). An increased macrophage count was associated with the number of days post-infection (main effect of day, $F_{2,24}=8.8$, $P=0.001$) with no difference in the trend of macrophage accumulation between the two strains (main effect of strain*day p.i. $F_{2,24}=0.4$, P=N.S.).

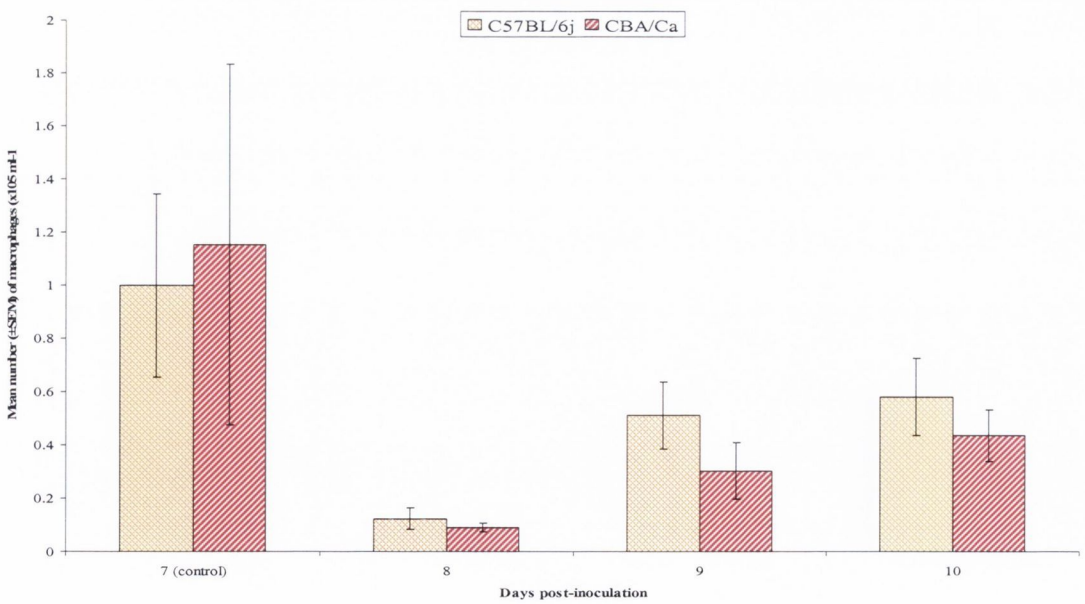


Figure 6.3.11: Mean number (±S.E.M.) of macrophages ($x10^5$ ml^{-1}) recovered in the BALF from the lungs of *A. suum* infected mice on days 8, 9 and 10 post-inoculation and non-infected control mice on day 7.

Basophils

The basophil cell type was constantly lower than other leukocytes forming a miniscule proportion of the total number of leukocytes when contrasted to the other classes and never accounted for more than 0.2% of the population C57BL/6j and 0.32% in CBA/Ca infected mice (**table 6.3.2**). The total number of basophils present in the bronchoalveolar fluid did vary between the strains due to the higher numbers of this cell type on day 7 in CBA/Ca mice (**Fig. 6.3.12;** full factorial 2-way ANOVA with strain and day as factors, model R^2_{adj}=0.69, main effect of strain $F_{1,29}$=9.1, P=0.005) and there was a day effect (main effect of day $F_{3,24}$=14.0, $P \leq 0.0001$). The pattern of basophil accumulation differed between the strains (2-way interaction strain*day $F_{3,29}$=0.29, P=0.0001) indicating the significantly higher day 7 numbers compared to the numbers during the infection period (LSD $P \leq$ 0.0001).

There was no marked change in the basophil population in the lung fluid of C57BL/6j mice associated with the day post-infection. There were no basophils recovered in the lungs of these mice on day 8 and thereafter the number was low and constant on the latter days of infection. Similarly, there was no day effect during this period in *A. suum* infected CBA/Ca mice. Proportionally the number of basophils in terms of the total leukocyte population was low in both strains (**table 6.3.2**).

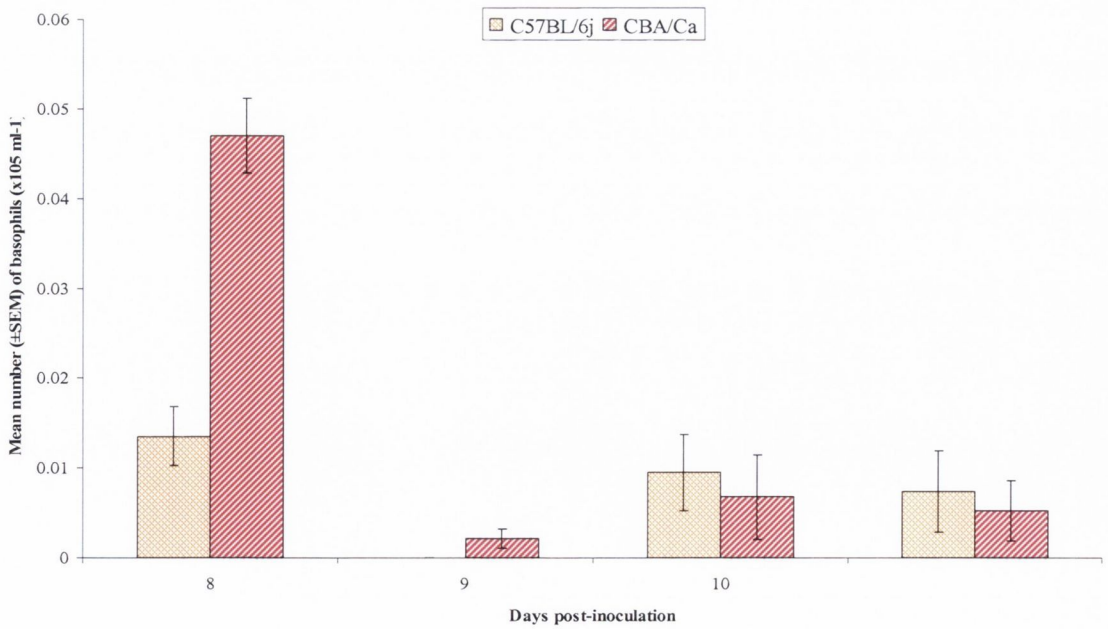


Figure 6.3.12: Mean number (±S.E.M.) of basophils (x10^5 ml^{-1}) recovered in the BALF from the lungs of *A. suum* infected mice on days 8, 9 and 10 post-inoculation and non-infected control mice on day 7.

CHANGES IN MOUSE BODY WEIGHT IN LUNG LAVAGE MICE

Both strains showed reductions in weight when infected with *A. suum* in contrast to their naïve equivalents (**Fig. 6.3.13;** 3-way ANOVA with strain and treatment as factors and day as a covariate, model R_{adj}^2=0.57 main effect of treatment $F_{1,35}$=32.1, $P \leq 0.0001$). **Figure 6.3.13** describes the differences between the pre- and post-infection body weight and illustrates that there was a constant reduction during the experimental procedure (**appendix A.10**) particularly in the more highly infected C57BL/6j strain (2-way ANOVA with day and strain as factors (only infected individuals contrasted) main effect of strain $F_{1,24}$=5.1, P=0.03). The pattern of changes in weight with time differed significantly between strains (2-way interaction strain*day $F_{2,24}$=4.1, P=0.029). C57BL/6j lost significantly more weight on day 8 after which the two strains had comparatively identical weight loss patterns. There was no influence of day on the changes of weight in infected individuals (main effect of day $F_{2,24}$=1.8, P=N.S.).

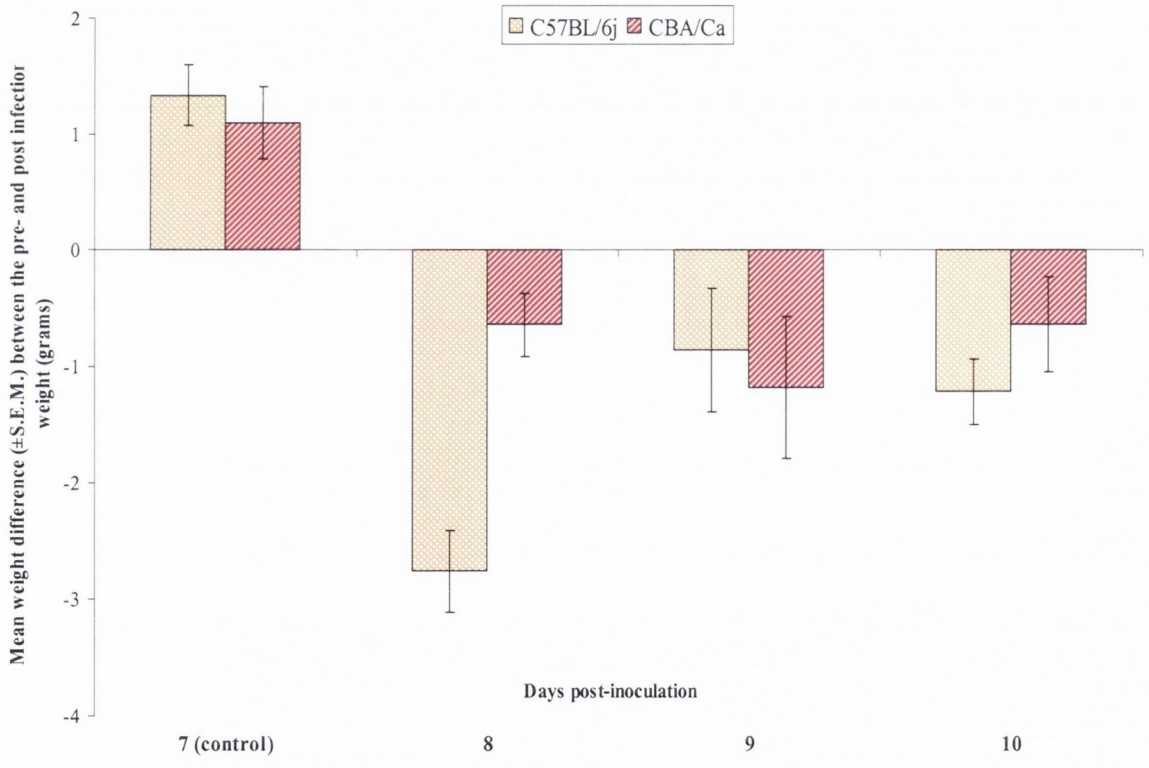


Figure 6.3.13: The mean weight difference (±S.E.M.) between the pre- and post-infection weight of the groups of mice from each strain on days 8-10 post-inoculation in the two strains of mice (C57BL/6j and CBA/Ca) inoculated with 1500 *A. suum* ova and examined for leukocytes by lung lavage. Also shown is the mean weight difference (±S.E.M.) on day 7 in the control mice that were not infected with *A. suum*.

6.4 DISCUSSION

The combined results of the inoculation of mice with corticosteroids and the examination of the change in profile and number of leukocytes present in bronchoalveolar lavage fluid suggested that the inflammatory immune response was not prominently involved in the protection of mice to *A. suum* infection in the latter days of infection in the lungs and that the eradication of the parasite from host tissues occurred at an earlier stage. C57BL/6j mice produced a BAL response almost twice as intense as that of CBA/Ca mice with stronger neutrophil, lymphocyte and eosinophil but not macrophage components. Overall, the peak of the response occurred after the maximum accumulation of larvae in the lungs during the period they were expected to migrate through the alveolar wall and return to the intestine. Therefore mice with heavier burdens responded more intensely, but this was a response to damage rather than a protective response. The number of worms invading the pulmonary tissues appears to be determined during the period of residence in the liver and the migration to the lungs. This was evident by the significant loss in larval numbers in CBA/Ca between the liver and lungs in contrast to C57BL/6j (see chapter 5). If the difference between the two strains was primarily an inflammatory reaction in the lungs then the administration of cortisone should have reduced the level of resistance of CBA/Ca mice. This was not the case. Finally, there was a significant effect on weight determined by larval burden. Infected animals lost weight in comparison with the uninfected controls, an effect of infection previously observed in *T. canis*-infected mice (Epe *et al.*, 1994).

Corticosteroids act as anti-inflammatory agents and they prevent the accumulation of inflammatory cells at the site of infection (Behnke & Parish, 1979). The cells active in the inflammatory process are polymorphonuclear neutrophils, lymphocytes and macrophages (Sell, 1987). Eosinophils have been associated with the inflammatory response to parasitic infections (Wakelin, 1996; Meeusen & Balic, 2000; Klion & Nutman, 2004) but their function as protective cells in GI nematode infections is controversial (Klion & Nutman, 2004). *A. suum* larval tissue migration in pigs is known to stimulate an inflammatory response, involving tissue infiltration by neutrophils, mononuclear cells, eosinophils, and mast cells at the site of infection (Eriksen *et al.*, 1980; Jungersen, 2002). Corticosteroids interfere with signals from T cells, such as cytokines and they also result in the depopulation of T-cell dependent areas of lymphoid organs, thus reducing T-cell counts in the blood stream and tissues following treatment (Behnke & Parish, 1979). Committed B-cell lymphocytes, plasma cells and related responses such as the secretion of antibodies are

thought to be relatively resistant to the effect of steroids. It is known that committed cells are relatively cortisone resistant and hence cortisone would not be expected to markedly reduce pre-existing levels of serum antibody (Behnke & Parish, 1979). Steroid treatment in mice almost completely abolishes mast cell responses and the changes in villus structure that occur during the expulsion of parasites such as *Trichinella* (Behnke, personal correspondence) and the bronchoalveolar response should be analogous.

The reduction in the spleen weight of cortisone-inoculated mice was consistent with steroid treatment, thus suggesting that cortisone provided the desired effect as an anti-inflammatory agent. The failure of the corticosteroid to increase worm burdens in CBA/Ca mice provides evidence that the resistant nature of this strain is either a structural/morphological barrier or the result of a rapid steroid-resistant response and this possibly operates between the liver and the lung stages. Furthermore, the consistency in worm burdens between C57BL/6j mice infected with *A. suum* and those that were also administered cortisone indicates that the observed larval burdens in the lungs are the maximum possible establishment and that the numbers of successfully migrating larvae cannot be enhanced in the lungs for this strain by immunosuppression. Thus the lung burden represents the maximum possible number of juveniles that can migrate through the host's tissues supporting the hypothesis that the lung burden is a good indicator of the resistant/susceptible phenotypes. The lung may represent a site of further entrapment of larvae prior to migration into the intestine.

Eriksen (1981) indicated that the elimination of *A. suum* larvae from the lungs of mice during primary and secondary infection was influenced by the thymus-dependent immune responses. The response to primary inoculation in thymus-deficient (nu/nu) nude mice was different from that of thymus-bearing (+/+) mice, with the former harbouring worm burdens of approximately fifteen larvae in the lungs and the later with worm burdens of eleven larvae on day 8 p.i. from primary inoculation doses of 1000 eggs. Although there is a difference in the larval burden, the larval recoveries were extremely low in this organ and there may be speculation as to possible error experienced with such a low recovery rate. There was no marked eosinophilia in the peritoneal cavity and the peripheral eosinophil response was negligible and accompanied by no marked serum antibody response in nu/nu mice. Although the author implicated eosinophils in protection, it could also suggest that eosinophils do not play a major role as the difference in larval burdens between the nu/nu and wild-type mice appeared to be insignificant at this stage. The numbers of larvae

migrating in the liver during primary infection were not influenced by T-cell deficiency. Mitchell *et al.* (1976) also found that T-cell deficient BALB/c mice were not more susceptible to increased liver larval burdens during primary infection with *A. suum*.

The liver has been cited as an important site in the destruction of primary migrating larvae (Taffs, 1968; Mitchell *et al.*, 1976; Johnstone *et al.*, 1978) rather than the previous suggestions of the GI tract (Bindseil, 1970a) and appears, at least in part, to play a defensive role. Migrating *A. suum* larvae cause an inflammatory reaction in the liver in experimental groups of pigs that was not observed in uninfected animals (Frontera *et al.*, 2003). The importance of this stage of infection between the liver and the lungs was highlighted by the significant reduction in the number of larvae between these two stages in CBA/Ca mice. Although the numbers of larvae recovered from the livers of the two strains were similar there was a reduction in the number of worms that successfully migrated from the liver to the lungs in both strains, but this was greater in CBA/Ca mice further providing evidence of a differential response between the two strains during this time period (see chapter 5). Although the loss of some individual larvae may also be attributed to the failure to locate the organ (Crompton & Pawlowski, 1985), there was an obvious consistent disparity in the number of larvae successfully arriving in the lungs between the two strains throughout previous experiments and that suggested some intrinsic difference between them.

Bronchoalveolar lavage (BAL) is a useful tool in the sampling of the lower respiratory tract. One important parameter for the analysis of BAL is the quantification of the number and types of cells recovered. A comparison between cytocentrifugation and slide smear preparation of BALF cells has shown that the microscope slide smear technique yielded well-preserved cell morphology (Thompson *et al.*, 1996). The comparative analysis of microscope slide smears for samples with predetermined numbers of cells yielded lymphocyte and neutrophil percentages which did not differ from the calculated differentials. Thus, microscope slide smear preparation was proposed as a simple and accurate method for the quantification of bronchoalveolar lavage fluid cytology (Thompson *et al.*, 1996). The inoculation of mice in the present study with an increased dose size (1500 infective *A. suum* ova) was to ensure sufficient antigenic exposure and thus elicit a sufficient immune response, as certain antigenic doses can be required to stimulate the immune response as shown with *Trichuris muris* in the mouse model (Bancroft *et al.*,

2001). This degree of antigenic exposure could be dose dependent and also strain dependent (Bancroft *et al.*, 2001).

In this study the control group tended to present lower leukocyte counts than infected mice on all days examined p.i.. The ideal experimental design for this experiment would be to examine white blood cell counts on all days p.i. in both *A. suum* infected and uninfected mice. However, due to financial restrictions and time constraints this was unfeasible. The day 7 control mice were the same age as the infected group and the leukocyte count of uninfected individuals is not expected to change over this short period of time as the immune system is at rest. Therefore control mice of approximately the same age would not vary in their total or individual leukocyte counts during the period of investigation i.e. from day 7 to day 10. A similar experimental design was employed by Wilkinson *et al.* (1990) in the examination of BAL of mice inoculated with *Necator americanus.*

The total number of bronchoalveolar leukocytes differed not only in the composition of the population but also in the total numbers of leukocytes that were recovered. In both strains the inflammatory response was slow to appear, and the leukocyte numbers were not significantly different between day 7 controls and day 8 infected mice. The number of neutrophils, lymphocytes and eosinophils did not differ between day 7 and 8, but these showed a dramatic increase on the latter days. However, there was a marked inflammatory response after this day. These observations correspond to those of Eriksen (1981) who described the first signs of pulmonary inflammation in histological sections on day 8 but this became more intense on day 10. The level of response to parasitic invasion or tissue damage caused by the migrating larvae appeared to be strain specific. Whitehead *et al.* (2003) examined the total number of leukocytes, and the eosinophil and neutrophil profile of whole lung lavage fluid after ovalbumin (OVA) sensitisation and aerosol challenge. They found that strains of inbred mice showed divergent temporal responses of airway hyper-reactivity (AHR) and inflammation in the lung airway. The severity of the eosinophilic inflammation of the mouse strains ranged from relatively unresponsive to responsive and this did not always correlate with the development of AHR. The strains BALB/cJ, C3H/HeJ, and DBA/2J displayed significantly increased airway responsiveness after OVA treatment, whereas the strains A/J and C57BL/6J did not. The results of the present study suggest that although C57BL/6j mice are responding with increased leukocyte responses including eosinophils this was not a protective function as the larval burden in the lungs was consistently greater in this strain. Thus this merely represented a

response to the presence of larvae and was not responsible in determining the number of larvae that could successfully migrate from the liver to the lungs.

There was a significant contrast in the BAL fluid of CBA/Ca mice and C57BL/6j mice inoculated with *A. suum*. Lymphocytes were the majority cell type on day 9 composing 52.3% and 61.2% of the population, respectively. Neutrophils were the next most prominent cell type. There were increases in the total number of neutrophils found from day 8 to 10 in both strains. However, the percentage of the total leukocytes that they accounted for decreased. C57BL/6j mice showed a reduction from 44.5% on day 8 to 30.8% on day 10. CBA/Ca showed a different pattern with an initial increase on day 9 (from 23.1-29.1%) but then a reduction on day 10 to 16.6%. Recent studies have suggested that human neutrophils respond rapidly to nematode body fluid constituents (Wood *et al.*, 1998; Falcone *et al.*, 2001). Neutrophils are rapid response cells that are not initially T cell driven. They form part of the acute early response to damage and infection, and precede influx by eosinophils and lymphocytes to sites of infection. This corresponds to the kinetic pattern of leukocyte infiltration in the current investigation. Human neutrophils may express a receptor that interacts with both IL-8 receptors and that could enable the early immune system to detect, target, and ultimately destroy tissue-migrating larvae (Falcone *et al.*, 2001). The significant numbers of neutrophils present on the days around the peak of accumulation in the organs suggest that neutrophils are reacting to the presence of the larvae in the lung but the protective function of the response is unknown.

Wilkinson *et al.* (1990) noted that the passage of the larval stages of *N. americanus* through the lungs of BALB/c mice was associated with marked changes in the leukocyte population of the organ. However, during primary infection this response occurred after the majority of the larvae had left the lungs and therefore was unlikely to have a significant protective role. The BAL fluid of control mice generally yielded about 4.03×10^5 cell ml^{-1}, comprising 98.7% alveolar macrophages. During primary infection there was a steady increase in the total number of leukocytes recovered, and this was marked by changes in the proportions of the differential cell types. Macrophage cell counts depleted from 94.4% on day 2 p.i. to 46.1% by day 9. There was a small rise in the number of lymphocytes (2-8.5%) and no change in the number of neutrophils observed (3.2%). The greatest increase related to the eosinophils, which had dramatic increase from 0.5% on day 5 to 42.1% of the total cell count by day 9. It must be noted that *N. americanus* has a longer period of development in the lungs (5 days to one week) and there is no liver phase. Secondary

infection was accompanied by an analogous pattern but was distinguished by more marked changes that were initiated earlier in relation to the day of exposure (Wilkinson *et al.*, 1990). The role of leukocytes in this case appears to be associated predominantly with secondary infection and a faster response in resistant individuals results in the effective trapping of more larvae in these individuals.

Frontera *et al.* (2004) showed that the bronchoalveolar fluid of control pigs contained 93.3 ±1.1% alveolar macrophages. There was a significant reduction in the percentage of macrophages in *A. suum* infected pigs 14 days p.i. (43.1 ±8.5%) compared to uninfected pigs. There were significant increases in the percentages of granulocytes at 14 days (50.6 ±9.5%) in contrast to the percentage in control animals (0%) and the majority of these were eosinophils. Lymphocytes and neutrophils were present at levels of <5% (Frontera *et al.*, 2004). The current investigation found that the proportion of eosinophils consistently rose during the examination period and were at their highest on day 10 in both strains and this appears to coincide with the observations in pigs. The percentage of the population of leukocytes accounted for by eosinophils rose from 4.0-14.2% in C57BL/6j (a 25-fold increase in the total leukocytes accounted for by these WBCs) and from 1.5-6.4% in CBA/Ca (a 9-fold increase) during the three days examined in the current investigations. Although this was a much lower percentage in mice, this could be expected to continue increasing up to day 14 as observed in the pig model (Dawson *et al.*, 2005).

Macrophages formed a small part of the population on day 9, 6.36% (C57BL/6j) and 15.12% (CBA/Ca) and there was no significant difference between the strains in relation to the total number of macrophages. Islam *et al.* (2005a) found an absence of eosinophils and macrophages in the lung tissues of recombinant *A. suum* 24 kilodalton antigen immunised BALB/c mice suggesting that these cells were not involved in larval killing at least in relation to that particular antigen. Thus the overall conclusion from previous studies and the current observations would be to suggest that the inflammatory response observed in the lungs is not an effective mechanism in the entrapment of larvae and that larval burdens in the lungs were determined at some stage prior to the pulmonary phase. Migration of L_3 larvae of *Nippostrongylus brasiliensis* through the lungs of the rat, during primary infection revealed evidence of damage to the lung epithelial cells and microvasculature but BAL fluid showed very little evidence of inflammatory cell recruitment. The leukocytes that dominated were macrophages on all days and the rest were lymphocytes, eosinophils and neutrophils, respectively (McNeil *et al.*, 2002).

Loeffler (1956) first remarked on the presence of rapidly occurring pulmonary infiltrates associated with eosinophils in the lungs of *Ascaris*-infected individuals. The characteristic eosinophilia was of short duration, appearing and diminishing quickly. Andersen *et al.* (1973) observed that the numbers of liver granulomas or "milk spots" was very low in piglets irrespective of dose suggestive of a moderate response to migrating larvae in this organ. In the lung, there was a corresponding increase in eosinophil response but it was suggested that, although reflecting immune reactions, the eosinophil granulocyte was not involved in the defence mechanisms against *A. suum* larvae. This was suggested by the conclusion that piglets inoculated with 1,000 and 5,000 *A. suum* eggs during the early neonatal period (0-3 days of age) inhibited the infection as effectively as older pigs although they did not respond with peripheral eosinophilia. If the eosinophilic response of the mice investigated was protective the expectation would be for the resistant strain to respond more vigorously but the level of eosinophils in CBA/Ca mice infected with *A. suum* was consistently lower than that of C57BL/6j both in total numbers and also as a proportion of the total cell count during the examination period.

The role of eosinophils in protection against parasitic infections is incompletely understood and controversial (Behm & Ovington, 2000; Meeusen & Balic, 2000; Klion & Nutman, 2004) but they have been associated with the inflammatory response to parasitic infections for many years (Wakelin, 1996). The accumulation of eosinophils, for example, in *T. canis*-infected animals was shown to result in significant thickening of the mucosal smooth muscle layers of the airways and was implicated in airway and vascular damage but may not have a protective role in immunity (Yoshikawa *et al.*, 1996). Perhaps there is a genetic deficiency associated with the eosinophilic response. C57BL/6j mice elicited high levels of eosinophils; however, this may not necessarily enable this strain to control infection, whereas the lower response of CBA/Ca may constitute a balanced reaction allowing efficient control of larval migration.

Eosinophils normally comprise only a small fraction (<1-5%) of circulating leukocytes and are predominately found in the tissues at the surface of the body that interact with the external environment (Behm & Ovington, 2000). The early stages of their differentiation are controlled by the cytokines granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-3 which also control other granulocytes such as neutrophils, basophils and mast cells. The latter stages are controlled by IL-5 which in turn is produced by activated T-cells and mast cells. Helminths with rapid transit through the tissues and intestines do

not normally encounter large numbers of activated eosinophils as it takes the host seven days or more post-infection, to mount an eosinophilopoietic response (Behm & Ovington, 2000). However, *Nippostrongylus brasiliensis* and *Angiostrongylus cantonensis* larvae that experienced large amount of eosinophils early in infection in transgenic mice that over expressed the gene coding IL-5 were possibly ill-equipped against a rapid eosinophilic response and this dramatically decreased worm burdens in contrast to normal mice (Behm & Ovington, 2000). Heterogeneity in the capacity of mice to mount eosinophilia has been described. BALB/c and C3H are eosinophil high responder strains and CBA and A/J are eosinophil low responder strains, judged by the response of blood eosinophils to *A. suum* antigen (Vadas, 1982). However, these strains showed disparate larval burdens when examined in chapter 3. BALB/c had intermediate larval burden, whereas the latter three strains harboured low larval burdens. Thus these observations of pulmonary larval burdens did not correlate to protection by the production of increased eosinophils as described by Vadas (1982). The general conclusion drawn from these observations and the results described in this chapter suggest that eosinophils are present and they react to larval migration but they do not correspond to a protective function.

The relative importance of cytokines in protective immunity against larval *Ascaris* infection is not clear. There was a related increase in the Th2-like pattern of gene expression during the period of expulsion of *A. suum* L_4 larvae from the small intestine of pigs, coupled with low Th1-like and anti-inflammatory cytokine gene-expression (Mahida, 2003; Dawson *et al.*, 2005). Jackson *et al.* (2004) found that increased Th2 cytokine production correlated with the age-dependent reduction of *A. suum* in a hyperendemic community. Protective immune responses operate at different stages of the life cycle and the role the Th1-like responses appear to be more influential in determining the success of the tissue stages. Overall a balanced immune response coupling the Th1/Th2 response appears to successfully protect the host against larval migrans.

A combination of Th1/Th2 response appears to be an important element in the elimination of migrating larvae at the different stages. Islam *et al.* (2005a) proposed that vaccination of mice with recombinant *A. suum* 24-kilodalton antigen induces a Th1/Th2-mixed type response and that this mixed response was responsible for protection against the parasite lung-stage infection in BALB/c mice following a challenge infection with *A. suum* eggs. In the present study, both strains increased the number of neutrophils and eosinophils during the days of infection in the lungs but there are significantly more present in the BAL fluid

of C57BL/6j mice than CBA/Ca mice. In contrast, the lower levels of these cell types in CBA/Ca may prevent a prevalent Th2 response, which would normally down-regulate the Th1-type responses, and thus infection could be controlled by the alliance of the Th1 and Th2 responses. Thus a delicate balance between these T-helper type responses rather than the predominance of one may be mutually effective in combating larval infection. In relation to this hypothesis, the resistance to *Litomosoides sigmodontis* was characterised by a strong Th1/Th2 immune response mounted against the worm in C57BL/6j. It was suggested that the Th2 bias was less pronounced in the resistant strain, in relation to *L. sigmodontis*, than in the susceptible BALB/c mice allowing a stronger mixed response in the former (Babayan *et al.*, 2003). In terms of parasite evasion there appears to be a suppressive effect of *Ascaris* components that can inhibit both the humoral and cell-mediated immune response therefore having effects upon the Th1- and Th2- dependent immune responses (Macedo & Barbuto, 1988; Faquim-Mauro & Macedo, 1998; Lynch *et al.*, 1998; Souza *et al.*, 2002; Oshiro *et al.*, 2005). This adds a further dimension as hosts may also vary in their susceptibility to these evasive techniques. A host with a genotype capable of generating a strong response to the presence of the parasite may nevertheless be unable to do so if it is susceptible to immunomodulation (Behnke *et al.*, 2000).

At this stage, we cannot be certain about the mechanisms underlying the greater susceptibility of C57BL/6j mice to infection with *Ascaris* but it is likely to be genetic and to consist of innate or molecular/structural/physiological differences between the strains with one set of alleles enabling *Ascaris* to migrate in large numbers and the other impeding this migration. The lack of variation in the larval burdens observed in the liver between C57BL/6j and CBA/Ca and loss of larvae between this organ and the lungs suggest that this is the area and time that is most decisive in determining the success of worms. It is conceivable that more larvae are entrapped in the livers of the resistant strain, or that their onward migration from the liver is less successful than in the susceptible strain. The latter is likely to be dependent on host cues that are exploited by the parasite to guide their homing to the pulmonary tissues, and it may be that the relevant signals vary between mouse strains. Finally it is possible that on arriving in the lungs more larvae fail to establish in resistant mice, or are expelled possibly through the bronchial airways by coughing or increased inflammatory responses, although this latter explanation can be discounted due to the failure of cortisone to reduce the resistance of CBA/Ca mice in the current investigation.

CHAPTER 7

A COMPARISON OF THE HISTOPATHOLOGICAL LESIONS IN THE LUNGS OF C57BL/6j AND CBA/Ca MICE INDUCED BY MIGRATING *A. SUUM*

7.1 INTRODUCTION

A number of possible locations have been cited at which larval migration may be impeded by host defences, both as a result of natural and also aquired immunity. Migrating *Ascaris* larvae are known to cause an inflammatory reaction in the liver, the lungs and the small intestine of infected animals, as well as human hosts (Vogel & Minning, 1942; Beaver & Danaraj, 1958; Ronéus, 1966; Spillmann, 1975; Eriksen *et al.*, 1980; Eriksen, 1981; Urban *et al.*, 1988; McSharry *et al.*, 1999; Cooper *et al.*, 2000; Pérez *et al.*, 2001; Frontera *et al.*, 2003; Frontera *et al.*, 2004; Miquel *et al.*, 2005). The intestine has been cited as a barrier to larval migration in pigs that have been continually exposed to *A. suum* larvae. Urban *et al.* (1988) suggested that continual exposure of the intestinal mucosa to larvae eventually elicits the appropriate effector components necessary to prevent larval migration from the intestines.

The inflammatory reaction of the liver has been suggested as a barrier to successful migration. Although the incidence of this reaction in the human liver has been disputed, experimentally infected pigs produced an inflammatory reaction in the liver caused by migrating *A. suum* in contrast to the lack of response in uninfected animals (Frontera *et al.*, 2003). Focal parasitic granulomata with eosinophils and lymphocytes were the main histopathogical lesions in the liver of reinfected pigs, while more marked cellular infiltrates and abundant fibrous/scar tissue were seen in the livers of immunised animals (Frontera *et al.*, 2003). This inflammation was characterised by an intensive infiltration with eosinophils and mononuclear cells in accordance with earlier reports (Ronéus, 1966; Eriksen, 1981). The protective nature of these responses was reflected in the reduction of larvae migrating through the visceral organs of reinfected/immunised animals in contrast with challenged control pigs.

A similar response occurs in the lungs of *A. suum* infected pigs (Frontera *et al.*, 2004) and was associated with a protective immune response in immunised individuals (Ronéus, 1966; Eriksen, 1981; Frontera *et al.*, 2004). Histopathological examination of pulmonary inflammation in pigs was characterised by intensive infiltrations of eosinophils and mononuclear cells in the lungs of both reinfected and antigen-immunised pigs (Frontera *et al.*, 2004). There were abundant granulomatous formations in the lungs of pigs receiving repeated *Ascaris* infections and these may have been caused by trapped and decomposing larvae, similar to those described in the liver lesions (Frontera *et al.*, 2003). Eriksen (1981)

suggested that the intensity of the pathological lesions caused by *A. suum* was related to the degree of resistance of the host. However, there has been no evidence that the pulmonary inflammatory response has a protective function in primary infected animals or the degree of variation in this response between naturally susceptible/resistant individuals.

The passage of *A. lumbricoides* and *A. suum* through the pulmonary tissue of their natural hosts is known to be associated with severe respiratory distress (Matsuyama *et al.*, 1998). Additional symptoms include substernal pain, fever, and skin rash. Peripheral eosinophilia has also been reported. Loeffler (1932) described a respiratory illness, later termed "Loeffler's syndrome", and this was caused by the migrating larvae of *A. lumbricoides* in human populations (Vogel & Minning, 1942; Loeffler, 1956). These symptoms of respiratory distress are also seen in pigs infected with larval *A. suum* (Slotved, 1997). Bronchovascular damage caused by the parasite may result in secondary infection by opportunistic bacteria that may proliferate in the inflammed pulmonary tissues and invade the host vasculature (Keller *et al.*, 1932; Liljegren *et al.*, 2003; Tjørnehøj *et al.*, 1992).

The innate inflammatory response has been implicated in resistance to adult *A. lumbricoides* in the intestine (McSharry *et al.*, 1999). The aim of the present research was to provide a comparison of the histopathological lesions induced by migrating *A. suum* in susceptible and resistant mice. These results may help to clarify what role, if any, the inflammatory process plays in host protection to larval ascariasis.

7.2 MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Twenty eight inbred male mice (14 each of; C57BL/6j, and CBA/Ca) were purchased from Harlan, UK at 7 weeks of age and were 8 weeks old upon commencement of the initial inoculations. Animals were maintained as described in chapter 2.

INOCULATION OF MICE AND POST-MORTEM PROCEDURE

Eighteen mice (9 of each strain) were inoculated with 1500 embryonated *A. suum* eggs in 100µl of solution and five mice of each strain were provided with a placebo dose consisting of 100µl of 0.1 N H_2SO_4 and no *A. suum* eggs as controls on day 0 as described in chapter 6. The number of eggs administered in the inoculum was determined as described in chapter 2. This experiment was designed to determine the heterogeneity in the inflammatory response in the lungs by histological examination between the two strains on days 8, 9 and 10 p.i.. The inflammatory response is believed to be most prominent on these days following inoculation with *A. suum* (Eriksen, 1981). Three mice from both strains were sacrificed on each day post-inoculation. Mice were analysed on days 8, 9 and 10 and control mice were examined on day 6 as detailed in chapter 6. All mice were infected at the same time whether they were used for larval burden quantificantion or histology. Uninfected animals were only analysed on day 6, however, since no inflammation response is expected this is not believed to be a limitation.

HISTOLOGY

A length of yellow cotton thread was passed under the trachea using a fine forceps. The tip was removed from a sterile 25g needle to prevent penetration of the trachea or pulmonary tissue during fixation. The hypodermic needle was inserted into the anterior end of the trachea and the thread was tied tightly around it to prevent fixative from flowing out of the tissue. 0.5ml of Carnoy's fixative (60ml absolute ethanol, 30ml chloroform and 10ml concentrated acetic acid) was drawn into a graduate syringe and attached to the needle and was subsequently infused into the lungs. The hypodermic needle was withdrawn and the thread securely tightened around the trachea. The lungs were left *in situ* for 5 minutes and then excised as a whole and immersed in Carnoy's fixative for 4 hours. After this period the lungs were removed from the fixative and divided into the left and right sides and transferred to separate containers with 70% ethanol for histological examination. All

containers were marked with the mouse ID (strain and ID), day of post-mortem, and the organ (left or right lung).

Lungs were embedded in paraffin wax prior to sectioning (**appendix C**). Five μm sections were stained with haematoxylin and eosin (H&E) (**appendix C**). Three μm sections were stained with Alcian blue, Periodic Acid Schiff (PAS), Discombe's eosin and Haematoxylin (**appendix C**). Longituindal lung sections were cut from each tissue sample. A total of 25 sections were cut from each lung. A preliminary examination was performed on lung sections of both mouse strains stained with H&E which stained the nuclear chromatin and cytoplasm of cells. The cytoplasm was uniformly stained pink by eosin. The cell nuclei were stained a contrasting blue to purple by haematoxylin. Cells containing an abundance of ribosomes had a purple to blue tinge in the cytoplasm due to the presence of cytoplasmic RNA. The secondary histological process was a combination of Alcian Blue and Periodic Acid Schiff's (PAS) reagent for mucins and Discombes eosin for eosinophils and Harris's haematoxylin was used as a counterstain. Using these procedures acid mucins stained blue, and eosinophil granulues brown to pink. The pulmonary inflammatory response observed was scored semi-quantitatively ranging from + (very mild inflammation) to ++++ (severe inflammation) by an investigator (Dr. J. Cassidy, Dept. of Veterinary Pathology, UCD) without prior knowledge of the relative susceptibility or immune responses of C57BL/6j and CBA/Ca mice to migrating *A. suum*.

HISTOPATHOLOGICAL EXAMINATION OF LUNG SECTIONS

General observations

Qualitatively similar pulmonary inflammation consisting of multifocal infiltrates of neutrophils, eosinophils, macrophages and lymphocytes, particularly centred around bronchioles and blood vessels and within the walls of adjacent alveoli was observed in both C57BL/6j and CBA/Ca mice. However, quantitative differences were noted with a more severe degree and extent of inflammation detected in C57BL/6j animals (**plate 7.3.1**). In both mouse strains, cross-sections of helminth larvae were frequently noted in association with this inflammatory response within alveolar and bronchiolar lumens at all three time-points (days 8, 9 and 10) (**plate 7.3.2**). In addition to the leucocytic infiltrates, areas of inflammation exhibited intra-alveolar haemorrhage (**plate 7.3.3**), fibrin deposition and oedema and haemosiderophages (macrophages that have phagocytosed and broken down erythrocytes) were detected (indicates previous and recent injury to blood vessels walls by the migrating larvae). From day 9 p.i., larvae were noted within the lung parenchyma surrounded by dense aggregates of neutrophils, eosinophils, macrophages, (**plate 7.3.4**) multinucleate macrophage giant cells (**plate 7.3.4e**) and lymphocytes (**plate 7.3.4**). This represented early granuloma formation (**plate 7.3.2**). However, the presence of larvae within these granulomatous regions could not always be detected. Semi-quantitative grading of the inflammatory response (**table 7.3.1 and plate 7.3.1**) indicated that in both mouse types the inflammation became more severe between day 8 and 10 p.i.. No significant changes were observed in the lungs of the control mice of both strains. The structure of the pulmonary tissue of the control mice of both strains was similar.

Table 7.3.1: The table illustrates the severity of pulmonary inflammation in C57BL/6j and CBA/Ca mice ranging from + (very mild inflammation) to ++++ (severe inflammation) on days 8-10 p.i.. The assignment of this semi-quantitative inflammation score is based on both the distribution and on the amount of inflammatory infiltrate.

Mouse type	Day 8	Day 9	Day 10
C57BL/6j	++	+++	+++
CBA/Ca	+	++	++

HISTOPATHOLOGICAL EXAMINATION OF LUNG SECTIONS

Day 8 post-inoculation

Severe intra-alveolar haemorrhage was observed in the pulmonary tissue of C57BL/6j mice on day 8 post-infection. Both clusters of erythrocytes and proteinaceous material were observed within adjacent alveolar lumens. The presence of larvae in the bronchioles was accompanied by inflamed bronchiolar walls. Greater mucus production was demonstrated in the bronchioles of C57BL/6j mice particularly associated with cross-sections of larvae (**plate 7.3.3a**). The leukocyte response was mixed consisting of neutrophils, lymphocytes, eosinophils and macrophages. Neutrophils were the most prominent white blood cell type present with smaller numbers of admixed eosinophils. The presence of haemosiderophages at this stage suggested that vascular damage had occurred prior to this day. There was a less prominent response on this day in the lungs of CBA/Ca mice, with less inflammation and haemorrhage occurring in the pulmonary tissue of these animals in contrast to C57BL/6j (**figure 7.3.1** and **plate 7.3.1**). The cell types involved in the inflammatory response was similar to that in C57BL/6j mice.

Day 9 post-inoculation

Overall there was a quantitative difference in the inflammatory response in both mouse strains between days 8 and 9 p.i.. The formation of granulomas was first noted within the lung parenchyma surrounded by dense aggregates of neutrophils, eosinophils, macrophages, multinucleate macrophage giant cells (macrophages that have fused during chronic inflammation) and lymphocytes at day 9 p.i. (**plate 7.3.4e**). During this period of observation there was a plateau in the inflammatory response observed in the pulmonary tissue in both strains between day 9 and 10 p.i.. Although both strains show a plateau, the response of C57BL/6j was more severe (score +++) in contrast with CBA/Ca mice (score ++). This plateau coincided with larval migration away from the lungs (**figure 7.3.1**).

Day 10 post-inoculation

C57BL/6j mice exhibited pulmonary inflammation on this day but there was no change in the score between day 9 and 10. There was persistent infiltration of white blood cells and these had continued to accumulate between the two days. Granuloma formation was again observed, containing multi-nucleated macrophage giant cells, with the presence of fibrin as exudates in surrounding alveoli. Larvae were observed associated with these regions in some cases. An inflammatory response was sustained in the lungs of CBA/Ca mice but there was only a subtle difference between day 9 and 10. Furthermore the bands of white

blood cells were less dense at this stage suggesting that the response had begun to plateau in this mouse strain (**figure 7.3.1**).

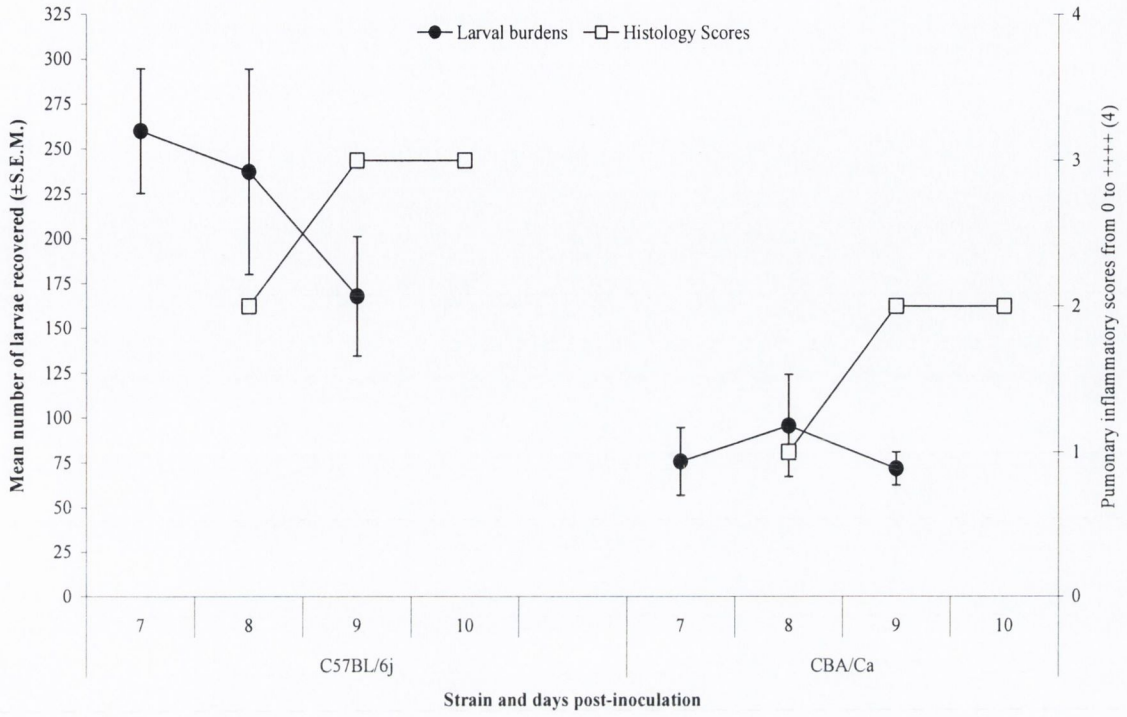


Figure 7.3.1: The mean lung larval burden (±S.E.M.) of C57BL/6j and CBA/Ca mice administered 1500 *A. suum* ova and the associated change in the pulmonary inflammatory response (scoring ranged from 0 {uninfected}- ++++ {4}) [results of larval burdens are taken from chapter 6].

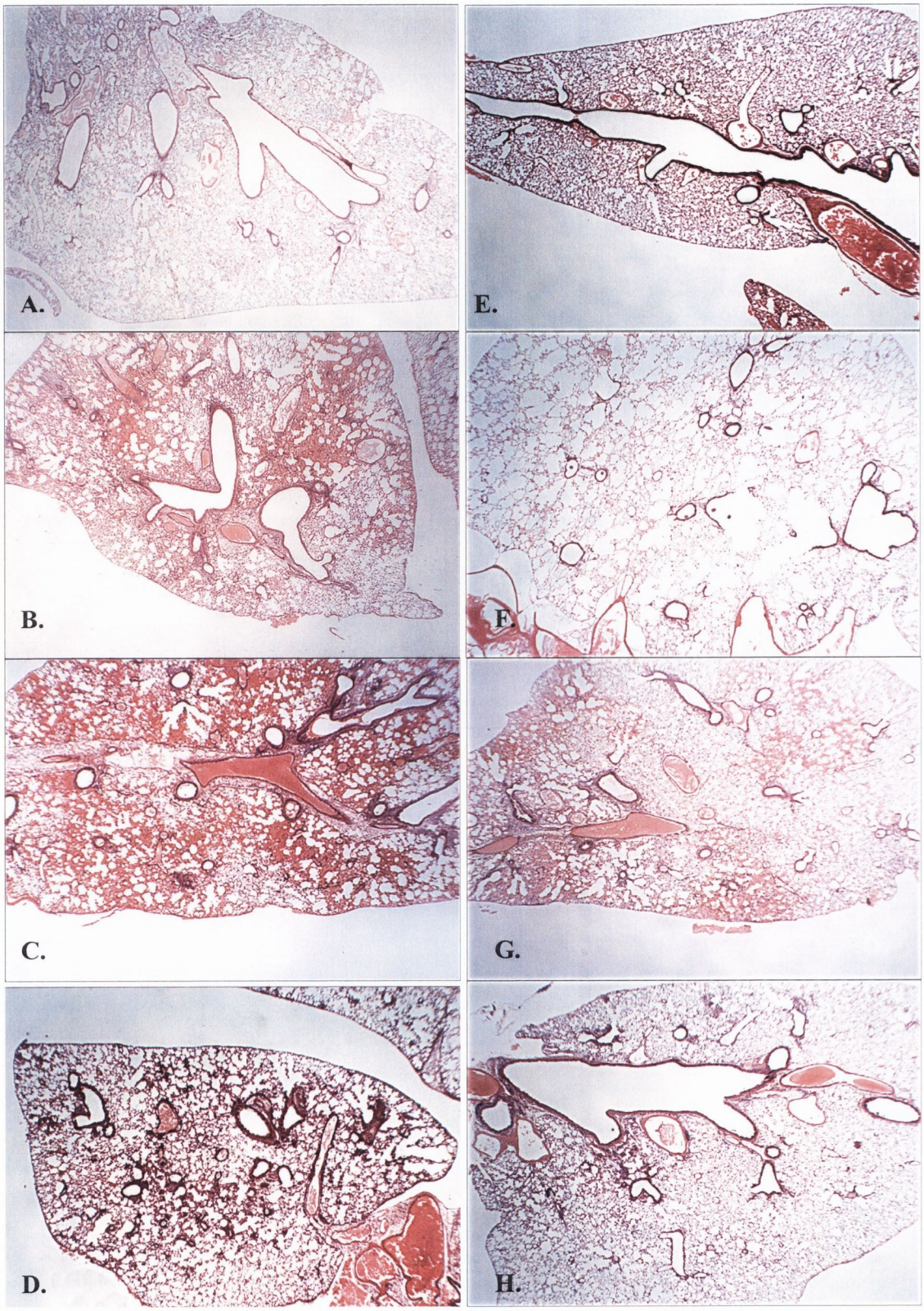


Plate 7.3.1: Composite low power (x2) photomicrographs illustrating; **(A)** uninfected lung tissue and the extent of intra-alveolar haemorrhage and of pulmonary inflammation **(B)** at day 8 p.i. (score ++) **(C)** at day 9 p.i. (score +++) and; **(D)** at day 10 p.i. (score +++); from C57BL/6j mice (A-D) and **(E)** uninfected lung tissue and the extent of intra-alveolar haemorrhage and of pulmonary inflammation **(F)** at day 8 p.i. (score +) **(G)** at day 9 p.i. (score ++) and **(H)** at day 10 p.i. (score ++); from CBA/Ca mice (E-H). Haematoxylin and Eosin (HE) stain.

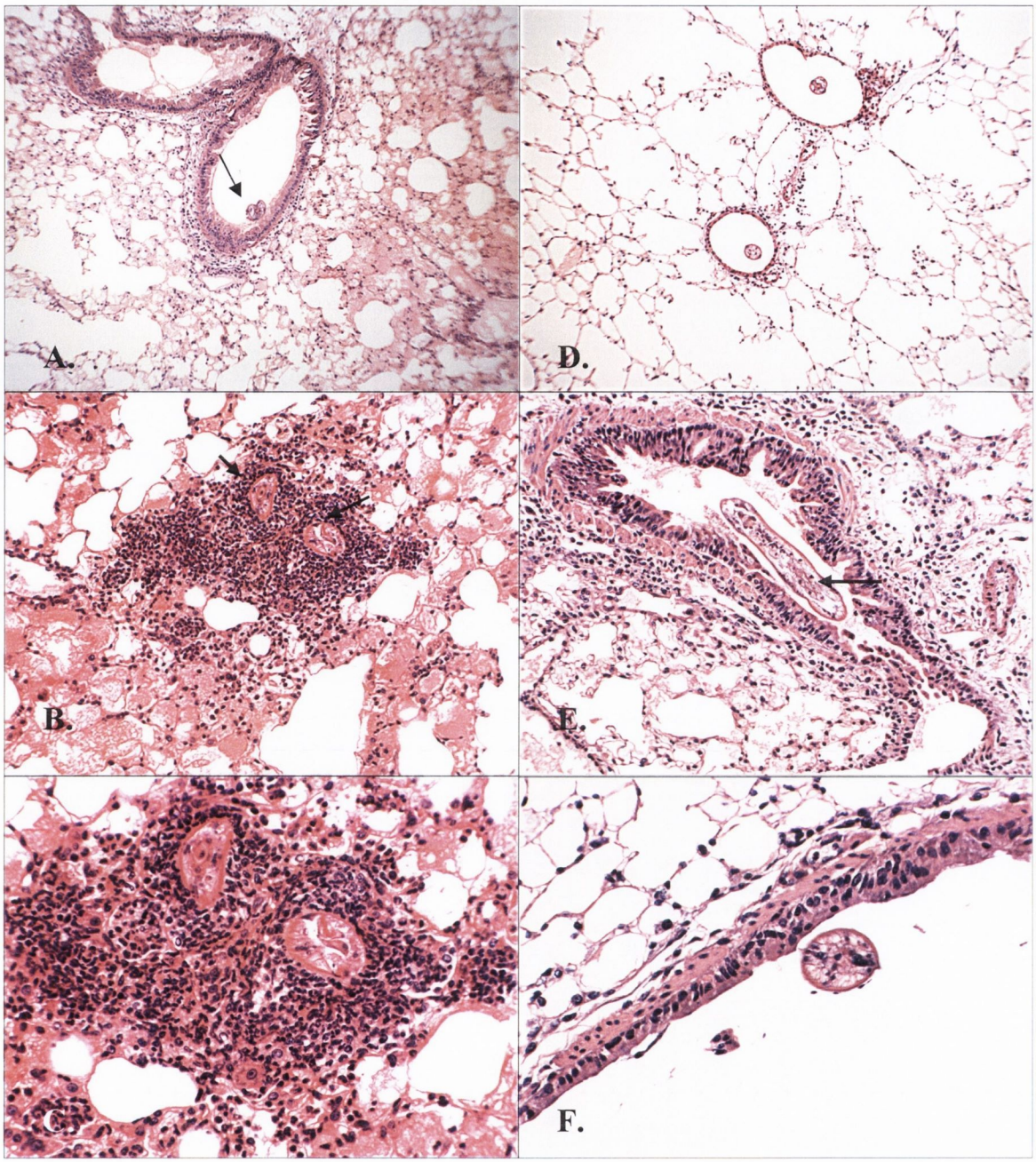


Plate 7.3.2: Photomicrograph illustrating transverse cross-section of *A. suum* (→); **(A)** larva adjacent (x10) to the surface of the bronchiolar epithelium with an accompanying peri-airway inflammation (++) at day 8 p.i. Light eosinophilic mucus is noted in an adjacent bronchiole and a proportion of the surrounding alveoli are filled with erythrocytes and proteinaceous exudates **(B)** two larvae (x20) surrounded by a dense infiltrate of inflammatory cells (+++) and with a proportion of the surrounding alveoli filled with erythrocytes and proteinaceous exudates at day 9 p.i. **(C)** high magnification (x40) of previous slide, two *A. suum* larvae surrounded by dense aggregates of admixed neutrophils, eosinophils and macrophages at day 9 p.i.; in C57BL/6j mice (A-C) and; **(D)** larvae (x10) within the lumens of adjacent bronchioles with accompanying light (+) peri-airway inflammation **(E)** saggital cross-section through an *A. suum* larva (x20) within a bronchiole at day 9 p.i.. There is accompanying peri-airway inflammation (++) **(F)** larva (x40) within a bronchiole with mild accompanying inflammation; in CBA/Ca mice (D-F) at day 10 p.i. HE stain.

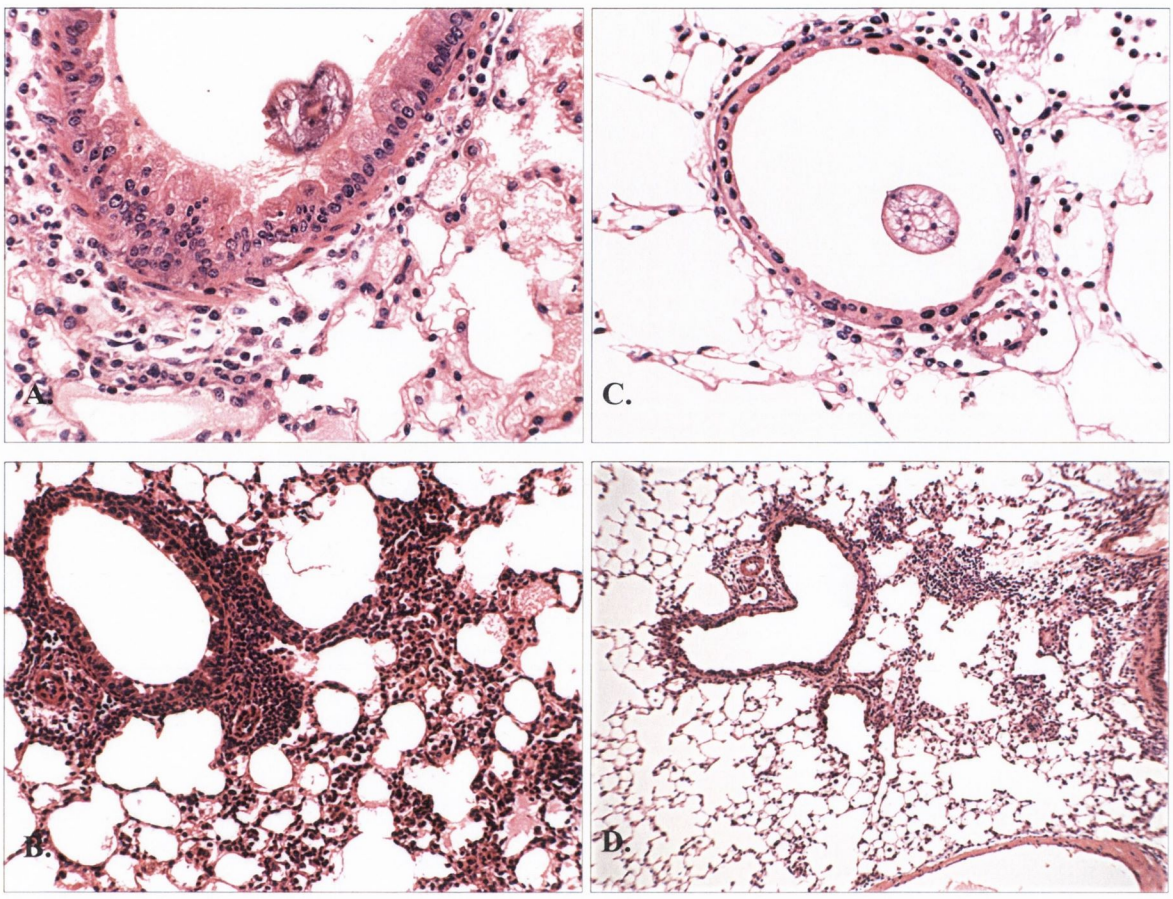


Plate 7.3.3: Photomicrograph illustrating; **(A)** high power magnification (x 40) transverse cross-section of *A. suum* larva adjacent to the surface of the bronchiolar epithelium at day 8 p.i.. There is a layer of pale eosinophilic mucus between the parasite and the epithelium. A light infiltrate of eosinophils, neutrophils and lymphocytes is noted surrounding the airway **(B)** dense infiltrates of inflammatory cells (+++) around a bronchiole and blood vessels and extending into the surrounding alveolar spaces at day 10 p.i. (X 20) in C57BL/6j mice and; **(C)** transverse cross-section (x40) of an *A. suum* larva within the lumen of a bronchioles with accompanying very mild peri-airway inflammation at day 8 p.i. **(D)** inflammation (++) extending from around bronchioles and blood vessels into the adjacent lung parenchyma at day 10 p.i. in CBA/Ca mice. HE stain.

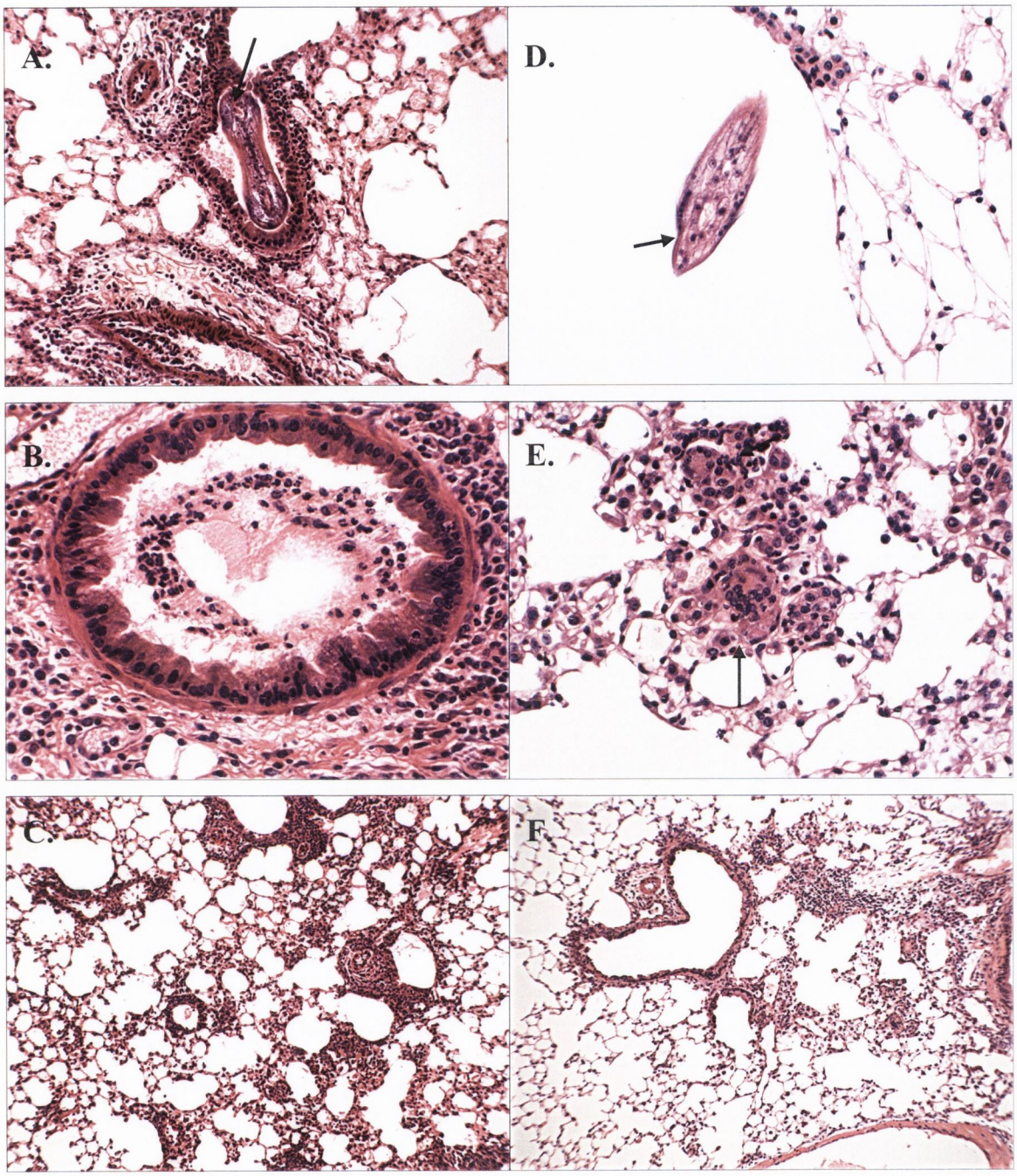


Plate 7.3.4: Photomicrograph illustrating; **(A)** a saggital cross-section through an *A. suum* larva (→) (x20) within the lumen of a bronchiole at day 8 p.i.. An infiltrate of inflammatory cells surround the airway **(B)** admixed neutrophils, eosinophils, macrophages and mucus within the lumen of a bronchiole at day 8 p.i. (x40) **(C)** peri-airway and peri-vascular inflammation (+++) at day 10 p.i.;in C57BL/6j mice (A-C) and **(D)** saggital cross-section through an *A. suum* larva (x40) within the pulmonary parenchyma at day 8 p.i. **(E)** a number of multinucleate macrophage giant cells (→) within the pulmonary parenchyma at day 9 p.i. (x 20) **(F)** inflammation (++) extending from around bronchioles and blood vessels into the adjacent lung parenchyma at day 10 p.i. in CBA/Ca mice (D-F). (HE)

7.4 DISCUSSION

The inflammation in the lungs of C57BL/6j and CBA/Ca mice infected with *A. suum* were compared to determine the potential role of this response in killing the parasite. The investigation provided strong evidence that these distinct mouse genotypes have divergent responses to the presence of migrating larvae in their tissues. The passage of the larval stages of *A. suum* through the mouse lungs was clearly associated with a marked inflammatory response in both strains when contrasted to their uninfected counterparts. C57BL/6j mice exhibited increased inflammation relative to CBA/Ca mice. However this did not appear to be related to protection against the parasite, as larvae persisted in greater numbers in C57BL/6j (chapter 6). This confirmed the observation of the BAL study in which total numbers of bronchoalveolar leukocytes was greater in C57BL/6j mice. Larval migration induced haemorrhage with oedema and on the latter days there was distinct entrapment of larvae within some granulomas. However, the more intense reaction in the lungs of C57BL/6j mice, but higher larval burdens, would suggest that the entrapment of larvae in the lungs of these mice may only retard but not prevent their ultimate migration. Although eosinophils were present, the eosinophil-rich inflammatory response was not a particular feature associated with the presence of larvae in the lungs on these days. The overall conclusion from these results was that the pulmonary inflammatory response was not a major factor contributing to the differences between the two strains of mouse in relation to protection against primary migration in the lung tissues.

Massive intra-alveolar haemorrhage was noted in both mouse strains but was more severe in the susceptible C57BL/6j mice. This suggested that there was more damage caused by migrating larvae penetrating blood vessels and migrating into the alveolar lumen in this strain. The more severe damage observed in the pulmonary tissue of C57BL/6j mice would support the presence of more larvae in the lungs of these mice that were capable of onward migration to the intestine. The reduction of haemorrhage on the latter day was consistent with the completion of movement of larvae from the lungs, observed in earlier chapters, already by day 8 and with the majority of larvae having been expelled from the lungs by day 9. The occurrence of haemosiderophages on day 8 suggested that there had been previous haemorrhage caused by larval migration prior to this day. The presence of these haemosiderin-loaded macrophages was previously demonstrated by Eriksen (1981) in the lungs of *A. suum* infected mice. The continued accumulation of erythrocytes into lungs of C57BL/6j mice, in contrast with CBA/Ca, showed the maintenance of greater worm

burdens on latter days in this strain. The accompanied haemorrhage related to larval migration from the blood vessels in the alveolar walls into the alveolar spaces and this has implications in relation to secondary opportunistic bacterial infections. Tjørnehøj *et al.* (1992) demonstrated that on days 8-12, following inoculation with *A. suum*, mice were more susceptible to pneumonia and septicaemia resulting from aerosol exposure to *Pasteurella multocida*. The lesions and severe haemorrhaging caused by *A. suum*, which were shown to be very extensive in the present study, provide a protein-rich fluid in the alveolar spaces that favours proliferation and invasion by bacteria.

These present observations correspond to the results of Sprent & Chen (1948) and Bindseil (1969b) who observed haemorrhage in the alveolar spaces until days 7-9 after which the cellular reaction was minimal, and limited to small concentrations of macrophages and lymphocytes associated with the bronchi and blood vessels. Prior to this time extensive haemorrhage and cellular infiltrations were observed in the alveolar lumen. They observed that the cellular population was composed of lymphocytes, neutrophils and eosinophils accumulating in the alveolar spaces particularly around bronchi and blood vessels. Bindseil (1969b) found that the majority of larvae were lying without association to inflammatory reactions. The present study observed intense inflammatory reactions and these appeared to be associated with the migratory tracks of larvae emerging from the blood vessels in the alveolar walls rather than larval entrapment and this was exhibited by increased inflammatory response of the bronchi particularly where larvae were observed in close proximity. The migratory tracks of the larvae were marked by the infiltraion of white blood cells along the migratory route and the damage caused to the blood vessels and the surrounding tissue. The bronchi were thickened in C57BL/6j in contrast to CBA/Ca mice, reflecting the more severe inflammatory response of the former. This was further evidence that the inflammatory response was not primarily involved in larval entrapment or killing but merely defined the route of migration through the pulmonary tissues. These observations are supported by the results of bronchoalveolar lavage in chapter 6, which showed an increase in the total BAL leukocyte population of both mouse strains on days 8-10 p.i.. Furthermore the BAL white blood cell count of C57BL/6j mice was consistently higher than that of CBA/Ca mice supporting the observation that this was an association between the inflammatory response and the presence of larvae rather than a protective response.

Previously, necrotic foci were described forming at day 8 in the lungs of mice but were not associated with larvae in the majority of cases (Sprent & Chen, 1948) however, these results must be interpreted with caution as multiple sections are required to rule out possible entrapped larvae within necrotic loci or granulomas. Pulmonary granulomas were observed but the presence of entrapped larvae was not always observed. Sprent & Chen (1948) detected increased infiltrations of lymphocytes, neutrophils and eosinophils in the peri-bronchial and peri-portal connective tissue but the larvae were found free of these infiltrations. Analogous to the current observations this group found that eosinophils were not the prominent leukocyte type in primary infected mice. Eriksen (1981) noted that larvae were trapped in inflammatory tissue reactions consisting of mononuclear cells, neutrophils and eosinophils on day 8 p.i. in primary infected +/+ mice from a closed mouse colony. This reaction intensified on days 10 and 14 in the region of *A. suum* larvae and in the peri-bronchiolar areas. In contrast nu/nu mice had decreased cellular reactions in the lungs and larvae were not recognised until day 14 p.i. while maintaining higher larval burdens. However, qualitatively the response was analogous between +/+ and nu/nu mice. This corresponds to the observations of the present study but in contrast the innately resistant strain exhibited less inflammation but also exhibited fewer larvae in this pulmonary tissue. In pigs there appears to be a dose-associated granulomatous inflammation in the lungs caused by trapped and decomposing larvae suggesting that in challenged animals the lung plays a role in protection (Frontera *et al.*, 2004).

The association of increased eosinophilic responses in the presence of migrating larvae in the tissues has been reported for many years (Wakelin, 1996). Previous studies have reported peripheral eosinophilia associated with migrating larvae but its protective function is debated (Behm & Ovington, 2000; Meeusen & Balic, 2000; Klion & Nutman, 2004). In the examination of BAL fluid there was a three fold increase in the percentage eosinophil count (the total leukocyte count increased 25-fold in C57BL/6j mice and 9-fold in the BAL fluid of CBA/Ca mice) between the control day (day 7) and day 10 of infection. However, the percentage of these cells in relation to the total number of leukocytes remained low because of the marked increase in the total number of lymphocytes and neutrophils (see chapter 6). The presence of eosinophils may be more directly linked to their function as phagocytes of immune complexes, and Andersen *et al.* (1973) illustrated that the time of maximum peripheral eosinophilia (day 10 p.i.) coincided with the appearance of circulating antibodies against *A. suum* reported by Taffs (1968). Andersen *et al.* (1973) observed a corresponding increased peripheral eosinophil response but it was suggested

that, although reflecting inflammation, however, eosinophils were not involved in the primary defence mechanisms against *A. suum* larvae. There are contradictory reports associated with the recruitment of eosinophils in other helminth infections. A positive role for eosinophils was demonstrated during *Strongyloides stercoralis* infection (Dombrowicz & Capron, 2001). In this case, eosinophils (and IL-5) are involved in larval killing upon reinfection. In *S. ratti* infection, a correlation was found between peripheral eosinophilia and protection during primary infection. The accumulation of eosinophils was correlated with significant thickening of the airways and vascular damage in T. canis-infected animals but again there was no protective function associated with this response (Yoshikawa *et al.*, 1996).

Larvae are continually found in large numbers in the liver up to day 5 after which they migrate to the lungs and the number of larvae successfully penetrating the pulmonary tissue is considerably reduced. Thus, as previously suggested it would appear that *A. suum* larval migration and the relative susceptibilities of hosts were determined in the liver or the post-hepatic period in primary inoculated individuals, in accordance with earlier studies (Taffs, 1968; Eriksen *et al.*, 1980; Eriksen, 1981; Frontera *et al.*, 2003; Jungersen *et al.*, 1999b). The migration through the liver may stimulate or contribute to the development of an anti-parasite response as well as successfully trapping larvae in the liver of resistant individuals. Thus this period between the residence of larvae in the liver and migration to the lungs appears to determine the success of migrating larvae into the lungs in the two strains of mice. This study provided the first description of histopathological changes that accompanied the development of *A. suum* in the lungs of mice that show divergent susceptibility to this parasite. The observations were compatible with those of the previous chapter and demonstrated that the pulmonary inflammatory response was not directly involved in host protective immunity to migrating *A. suum*.

CHAPTER 8

GENERAL DISCUSSION

The overall aim of this study was to provide a model for the study of larval ascariasis in order to investigate inherent host factors that contribute to phenotypic differences in the number of larvae that successfully migrate into the lungs between individual hosts. The investigation initially concentrated on the influence of extrinsic factors on larval *A. suum* worm burdens in divergent mouse strains and then examined intrinsic differences between mouse genotypes that may influence the observed phenotypes of susceptibility and resistance in relation to pulmonary larval burdens. There had been little conclusive evidence that mouse strains vary in their susceptibility to this parasite due to experimental variation, resulting from the protocols employed, on worm larval burdens in the lung. This thesis provides strong evidence that different mouse genotypes show consistent differences in their susceptibility to *A. suum* and that this is the result of variation in the response of inherent host factors to the presence of migrating larvae, influenced by the genetic heterogeneity of the strains.

Previously, Jenkins (1968) and other authors added infective *A. suum* to the food of mice to explore resistance and larval migration. However the administration of eggs in this way meant that exact information on the dose administered or the timing of migration was not easily determined and these factors influenced the observed larval burdens. The standard oral inoculation procedure utilised in this thesis, adapted from Slotved *et al.* (1997a) provided mice with exact egg doses and precise timing of administration. This gave comparative worm burdens because the dose administered could be related directly to the number of larvae recovered and the precise time larvae where migrating through the tissues was clearly evident. The present study also provided a refined Baermann method for the recovery of larval *A. suum* from visceral organs. This technique resulted in increased worm recoveries in comparison to previous methods. The application of the refined protocol developed in this study also produced consistent larval recoveries between experimental groups and removed extrinsic factors as a source of heterogeneity in the observed worm burdens.

The pattern of migration and accumulation in the liver and lungs was initially determined in two reference strains C57BL/6j (susceptible) and BALB/c (resistant) and provided evidence that mouse susceptibility to larval ascariasis could be distinguished by their day 7 pulmonary larval burdens. This confirmed previous observations of the pattern of

migration, with the majority of larvae capable of migrating recorded in the liver on day 4/5 p.i. and there was a shift in the organ of highest recovery to the lungs on day 7 p.i. (Sprent & Chen, 1948; Sprent, 1962; Douvres & Tromba, 1971; Eriksen, 1981; Prokopič & Figallová, 1982; Wade *et al.*, 1987). This provided the template on which subsequent experiments would be based.

Inbred mouse strains are known to show pronounced heterogeneity in their capacity to reject GI nematodes and this variability has a genetic basis. Heterogeneity in larval burden associated with strain was the next aspect of *A. suum* migration that was investigated. There was strong evidence that mouse genotypes differ markedly in their susceptibility to early *A. suum* infection based on the phenotypic expression of pulmonary larval burdens on days 6-8 p.i.. The observations showed that there were distinct mouse genotypes that responded with varying degrees of success to the presence of larvae in their lungs from an equivalent administered dose. The mouse phenotypes based on pulmonary larval burdens on day 7 were; susceptible (C57BL/6j), intermediate resistant (NIH, SJL and BALB/c) and finally the resistant strains (A/J, C3H/HeN, CBA/Ca, DBA/2, and SWR). It was established that C57BL/6j mice were uniquely susceptible to infection with *A. suum*. Worm burdens recovered from lungs of this strain markedly exceeded those recovered from other similarly infected strains of which the most resistant was the CBA/Ca mouse. The variation in susceptibility of inbred mouse strains corresponded to the observations of Mitchell *et al.* (1976) who demonstrated a direct link between mouse genotype and pulmonary larval burden. These two strains therefore represent opposite response phenotypes, mimicking the extremes of predisposition detected in humans (Holland *et al.*, 1989; Guyatt *et al.*, 1990; Hallam & Anwar, 1992; Chan *et al.*, 1994; Palmer *et al.*, 1995; Peng *et al.*, 1996; William-Blangero *et al.*,, 1999; Wong *et al.*, 1999; Williams-Blangero *et al.*, 2002) and pigs (Boes *et al.*, 1998a).

Previous investigations have provided conflicting results as to the effect of altering the initial inoculum size (Galvin, 1968; Andersen *et al.*, 1973; Mitchell *et al.*, 1976; Johnstone *et al.*, 1978; Slotved *et al.*, 1996) and the effects that increased exposure had on the relative susceptibility of a host strain. Having defined two distinct phenotypes and demonstrated that the experimental protocol did not alter larval recoveries the next stage was to investigate the influence of dose. Dose did not influence the relative susceptibility of the two strains. Therefore it can be assumed that there are underlying intrinsic host factors resulting in the observed phenotypes, as the difference in the migrating larval population

between the two strains remained constant irrespective of the initial dose size. There was a positive density dependent relationship between dose and the percentage of the initial inoculum that was recovered, as percentage larval recovery increased with increasing inoculum size. This pattern was observed in both the susceptible and resistant strains of mice, and is consistent with observations by Johnstone *et al.* (1978) on *A. suum* in the lungs of C57BL/6 mice, but in other host-parasite systems it is more usual to observe a reduction in percentage establishment with increasing parasite density (Norozian-Amiri & Behnke, 1993)

The two contrasting host environments were equally suitable for the development of the worms as larval length was not influenced by strain. This suggested that the observed differences in relative susceptibility are unlikely to be attributable to differences in larval fitness but rather to innate barriers to migration or differences in the early phase of the host response to the parasite. There was a correlation between dose administered and larval lengths, lower doses resulting in an increase in the average larval length, especially on day 7p.i.. The mean larval length was consistently higher among worms recovered from mice exposed to 500 eggs, indicating that dose exerts an important influence on the growth of worms. The observation that as dose increased, more worms were successful in completing the migration to the lungs, even though they did not grow as well as when fewer were present, suggests that there may be an active host component that underlies the difference between strains.

Mitchell *et al.* (1976) suggested that resistance by immunising doses or in relatively resistant strains of mice did not appear to be affected at the level of entry of larvae through the intestinal wall or migration to the liver but rather during the period in residence in the liver or *en route* to the lungs. Time course studies have shown that the period of residence in the liver or migration to the lungs were the stages in the lifecycle in which larvae were targeted by the host's immune system (Sprent, 1962; Douvres & Tromba, 1971; Eriksen, 1981). The present study determined that the period of residence in the liver or the stage of migration prior to larval accumulation in the lungs was one of the critical phases in the life cycle of *A. suum*. The number of worms capable of successfully migrating to the lungs was determined prior to the pulmonary stage. Following the examination of larval migration from day 0 to day 8 in the two mouse genotypes, there was evidence that similar numbers of larvae were capable of reaching the liver of both strains, although there was a difference in the number of larvae penetrating the large intestine at 6 hours post-infection. This early

difference might be attributed to a variation in the physiology of the intestine and the speed at which eggs and larvae pass through the large intestine. Slotved *et al.* (1998) suggested that individual mice of the same strain show variation in the rate at which larvae were passed through the large intestine. However, the number of larvae that migrated into the pulmonary tissues was substantially reduced in CBA/Ca in contrast to C57BL/6j, and this was a consistent observation throughtout the experimental procedures. This preliminary evidence suggests that the major difference in larval burden between the strains was first evident upon arrival in the lungs not the liver stage following primary inoculation.

The migration of *A. suum* in the pulmonary tissue of pigs is known to stimulate an inflammatory response, involving tissue infiltration by neutrophils, mononuclear cells, eosinophils, and mast cells at the site of infection (Eriksen *et al.*, 1980; Jungersen, 2002). It was vital to determine whether it was pulmonary involvement that contributed to the successfully entrapment and killing of larvae in CBA/Ca mice in contrast to C57BL/6j which resulted in resistance to this parasite. The combined results of the inoculation of mice with corticosteroids, the examination of the change in profile and number of leukocytes present in bronchoalveolar lavage fluid and the results of the histopathological examination suggested that the inflammatory immune response was not involved in the eradication of larvae in the lungs although there was a prominent inflammatory response and this supports the earlier observations of a pre-pulmonary factor. C57BL/6j mice produced a BAL response almost twice as intense as that of CBA/Ca mice and this was reflected in the histopathological changes of the pulmonary tissues observed during this period. There was a positive correlation between the presence of worms in the pulmonary tissues and the inflammatory response but larvae were not impeded by the greater response initiated by C57BL/6j mice. If the difference between the two strains was primarily attributed to an inflammatory reaction in the lungs then the administration of hydrocortisone should have reduced the level of resistance of CBA/Ca mice and there was no increase in susceptibility observed in this strain following hydrocortisone administration.

Wilkinson *et al.* (1990) demonstrated that the passage of the larval stages of *Necator americanus* through the lungs of BALB/c mice was associated with an increased inflammatory reaction, especially eosinophils. However, during primary infection this response occurred after the majority of the larvae had left the lungs and therefore was unlikely to have a significant protective role. Eosinophils have been associated with the

inflammatory response to parasitic infections (Wakelin, 1996; Meeusen & Balic, 2000; Klion & Nutman, 2004) including in the bronchoalveolar fluid during pulmonary larval ascariasis (Dawson *et al.*, 2005) but their function as protective cells is controversial (Klion & Nutman, 2004). Andersen *et al.* (1973) observed an increase in eosinophil response associated with larval *A. suum* migration in the lungs but suggested that, although reflecting immune reactions, the eosinophil granulocyte was not involved in the defence mechanisms against *A. suum* larvae. This was concluded from the evidence that piglets inoculated with *A. suum* eggs during the early neonatal period (0-3 days of age) inhibited the infection as effectively as older pigs although they did not respond with peripheral eosinophilia. Islam *et al.* (2005a) found an absence of eosinophils and macrophages in the lung tissues of recombinant *A. suum* 24 kilodalton antigen immunised BALB/c mice suggesting that these cells were not involved in larval killing at least in relation to that particular antigen. The present investigation found that the proportion of eosinophils consistently rose during the examination period and were at their highest on day 10 in both strains and this appears to coincide with the observations in pigs (Dawson *et al.*, 2005). However, if the eosinophilic response of the mice investigated was protective the expectation would be for the resistant strain to respond more vigorously but the level of eosinophils in CBA/Ca mice infected with *A. suum* was consistently lower than that of C57BL/6j both in total numbers and also as a proportion of the total cell count during the examination period.

Thus the overall conclusion from previous studies and the current observations would suggest that the inflammatory response observed in the lungs is not an effective mechanism in the entrapment of larvae in a primary infection. This study provided evidence that the liver or post-hepatic environment was more destructive to larvae in the resistant CBA/Ca mice than that of C57BL/6j resulting in reduced pulmonary larval burdens in the former. At least three possible mechanisms may be operating at this stage resulting in the maintenance of greater worm burden in C57BL/6j in contrast to CBA/Ca which include (i) tropic differences between the strains impeding larval homing to the lungs (ii) anatomical differences between strains impeding migration to the lungs (iii) differences in resistance mechanisms that either incapacitate the larvae late during the liver stage or block entry to the lungs. The liver has previously been cited as an important site in the destruction of migrating larvae (Taffs, 1968; Mitchell *et al.*, 1976; Johnstone *et al.*, 1978). Migrating *A. suum* larvae cause an inflammatory reaction in the liver in experimental groups of pigs that was not observed in uninfected animals (Frontera *et al.*,

2003). The results of the current investigation showed a positive correlation between administered dose and the pulmonary larval burden. This has implications for the incidence of severe morbidity with this stage in the host population as both resistant and susceptible individuals would harbour increased numbers of worms in their lungs as the density of infective stages increased in the environment. Furthermore the results of the histopathological analysis reveal that susceptible individuals, in addition to the increased leucocytic infiltrates exhibited intra-alveolar haemorrhage, fibrin deposition and oedema and these could be associated with secondary complications such as invasion by opportunistic microorgansisms.

This study has provided a more definitive mouse model for the investigation of the mechanisms that determine host susceptibility to early *A. suum* migration. Thus future work can proceed in the knowledge that the model is consistent and that observed differences in susceptibility are the result of innate host factors which are operating at the hepatic stage of migration. The benefits of such an assessment relate to the identification of the mechanism underlying the greater susceptibility of individuals to infection with *Ascaris* which is likely to be genetic and to consist of innate or molecular/structural/physiological differences between the individuals with one set of alleles enabling *Ascaris* to migrate in large numbers and the other impeding this migration. Once the innate host features determining larval success have been identified work can concentrate on those genes that result in predisposition to heavy/light larval burdens. This could contribute to the successful management of this disease, through targeted control strategies, and the reduction of morbidity and mortality in the host population by the successful identification of individuals that are heavily parasitized.

FURTHER WORK

It is important to clarify precisely the locations and times of worm attrition during migration of *Ascaris* larvae in the visceral organs of mice. This could be addressed using conventional approaches and modern radiolabelling techniques. At post-mortem, live larvae can be isolated by the refined Baermann technique, histological sections of all tissues will provide information on the encapsulation of larvae by the immune response of the host and radiolabelled larvae can be tracked. Radiolabelling will detect both living and dead larvae whereas the Baermann technique is only appropriate for living worms. This approach will enable sites and times of worm accumulation and attrition to be identified,

worm attrition to be quantified and will clarify the mechanism involved (blockage of migration, larval entrapment, encapsulation etc.). In conjunction with this it will be of interest to assess cellular responses in liver, peritoneal fluid and intestine. The interpretation of histopathological changes, both qualitatively and quantitatively, in these tissues will provide data on the cellular responses and correlate the inflammatory response with migrating larvae similar to the BAL/histopathological study in the lung of the two strains examined earlier.

The role of innate immunity must be assessed to determine its role in the observed host variation in response to larval ascariasis. A difference between strains in pulmonary larval burdens is observed as early as day 5 p.i. although the peak difference occurs on day 7. T cell-deprived resistant strains retain resistance and the effect of cortisone shows equivocal effects. Therefore innate mechanisms of resistance may impede worm migration. A number of approaches may be employed (i) Peritoneal macrophages harvested from uninfected and infected mice of both strains exposed to larval ES antigen (ii) RNA isolated and used to detect differential gene expression in microarrays, covering appropriate immunological genes, associated with innate resistance and the early stages of adaptive immunity. (iii) It will be of interest to assess the susceptibility of mouse strains lacking specific genes for components of innate immunity.

A productive area of research would be to introduce *A. lumbricoides* to the developed mouse system as the study of human ascariasis is hampered by logistical issues. Intitially it is proposed to build on the ideas developed by Peng *et al.* (1998) and determine a distinct human *Ascaris* genotype and pig *Ascaris* genotype from a number of sources worldwide. Eggs from human and pig hosts in both endemic and non-endemic areas will be included in this study and this encompasses areas where only *A. lumbricoides* would be expected to be endemic and similarly areas in which *A. suum* is the prevalent species. This would enable the evaluation of the early migration of *A. suum* and *A. lumbricoides* in the mouse model and the possibilities that *A. lumbricoides* migration is very similar to that of *A. suum*, and this can only be determined conclusively with the distinction of *Ascaris* genotypes such as those identified by Peng *et al.* (2003). These experiments have the potential to distinguish *Ascaris* as two distinct species or finally provide conclusive evidence as to the existence of one species with a number of genotypes.

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APPENDIX A: TABLES

Table A.1: Results for measuring a standard oral inoculation dose of *A. suum* eggs. Egg counts in 10 x 10 µl from individual doses of 500 eggs and the estimated number of eggs per inoculation dose and the mean (± S.E.M.) number of larvae in six randomly examined samples.

Sample number	Infective egg count											Estimated number of eggs in inoculation dose
	1	2	3	4	5	6	7	8	9	10	Mean	
A	95	47	47	47	35	32	46	32	30	86	49.7	497
B	93	47	49	46	45	31	31	31	37	70	48	480
C	34	131	31	46	30	62	40	59	28	34	49.5	495
D	30	58	64	43	58	40	43	46	26	99	50.7	507
E	50	58	45	50	47	82	35	46	42	40	49.5	495
F	44	55	56	63	51	38	48	44	43	45	48.7	487
Mean (± S.E.M.) number of larvae in the 6 samples												**493.5 (± 3.8)**

Table A.2: Mean weight (±S.E.M.) of mice infected with *Ascaris suum* pre-infection and on the day of post-mortem (Weight difference=post – pre infection) in the two strains of mice (C57BL/6j and BALB/c) in chapter 2.

Strain	Days p.i.	n	Pre-infection weight (grams)	post-mortem weight (grams)	Weight Difference
C57BL/6j	3	5	23.14 ±0.98	23.00 ±0.99	-0.14
	4	5	23.30 ±0.68	22.80 ±0.56	-0.50
	5	5	22.60 ±0.50	22.06 ±0.40	-0.54
	6	5	23.10 ±0.54	22.52 ±0.51	-0.58
	7	5	22.94 ±0.62	21.92 ±0.32	-1.02
	8	5	22.24 ±0.89	21.56 ±0.81	-0.68
	9	4	23.20 ±0.09	21.20 ±0.72	-2.00
	Mean	**34**	**22.93 ±0.25**		
BALB/c	3	5	20.96 ±1.12	20.32 ±1.02	-0.64
	4	5	19.34 ±2.11	18.40 ±1.78	-0.94
	5	4	20.38 ±1.13	20.93 ±1.69	0.55
	6	5	17.86 ±0.10	17.50 ±0.84	-0.36
	7	5	20.78 ±0.93	21.10 ±0.98	0.32
	8	5	22.44 ±0.46	21.82 ±0.54	-0.62
	9	5	20.46 (±1.30)	19.86 ±1.05	-0.60
	Mean	**34**	**20.32 ±0.47**		

Table A.3: Mean weight (±S.E.M.) of the spleen and the mean weight of the spleen expressed as a percentage of body weight (±S.E.M.) and also the mesenteric lymph nodes and the mean weight of the lymph nodes expressed as a percentage of body weight (±S.E.M.) in the two strains of mice (C57BL/6j and CBA/Ca) on each day of post-mortem from chapter 2.

Strain	Day	n	Spleen Weight (grams)	Spleen weight (percentage of body weight)	Mesenteric lymph node weight (grams)	Mesenteric lymph node (percentage of body weight)
C57BL/6	3	5	0.0639 ±0.0045	0.2768 ±0.009	0.0257 ±0.0099	0.1125 ±0.043
	4	5	0.0565 ±0.0045	0.2470 ±0.016	0.0299 ±0.0051	0.1299 ±0.019
	5	5	0.0598 ±0.0036	0.2716 ±0.018	0.0381 ±0.0047	0.1733 ±0.022
	6	5	0.0623 ±0.0053	0.2773 ±0.026	0.0298 ±0.0021	0.1325 ±0.010
	7	5	0.0677 ±0.0026	0.3088 ±0.010	0.0251 ±0.0038	0.1152 ±0.018
	8	5	0.0648 ±0.0056	0.3032 ±0.030	0.0205 ±0.0020	0.0963 ±0.011
	9	4	0.0670 ±0.0039	0.3160 ±0.015	0.0239 ±0.0055	0.1138 ±0.027
BALB/c	3	5	0.0819 ±0.0118	0.4398 ±0.062	0.0234 ±0.0087	0.1206 ±0.045
	4	5	0.0835 ±0.0097	0.4629 ±0.048	0.0321 ±0.0073	0.1671 ±0.037
	5	4	0.1015 ±0.0148	0.4767 ±0.036	0.0178 ±0.0043	0.0892 ±0.023
	6	5	0.0726 ±0.0094	0.4107 ±0.057	0.0288 ±0.0014	0.1655 ±0.010
	7	5	0.0918 ±0.0141	0.4263 ±0.049	0.0223 ±0.0044	0.1039 ±0.019
	8	5	0.1179 ±0.0100	0.5392 ±0.041	0.0301 ±0.0048	0.1386 ±0.022
	9	5	0.0980 ±0.0117	0.4874 ±0.036	0.0256 ±0.0035	0.1298 ±0.017

Table A.4: Mean body weight (grams) of mice (±S.E.M) of individual strains pre-infection (time= 0) and the mean body weight (±S.E.M.) on the day of post-mortem and the weight difference (Pre-infection – post-infection weight= weight difference) from chapter 3.

Strain	n	Day	Pre-infection weight	Post-mortem weight	Weight difference
A/J	5	6	21.16 ±0.00	21.68 ±0.17	0.52
	5	7	21.96 ±0.01	21.86 ±0.17	-0.09
	5	8	20.95 ±0.00	21.07 ±0.12	0.13
	5	9	21.36 ±0.01	21.46 ±0.05	0.10
SJL	5	6	22.57 ±0.18	23.35 ±0.28	0.78
	4	7	22.45 ±0.71	23.55 ±0.69	1.10
	5	8	20.20 ±0.16	21.65 ±0.30	1.45
	5	9	21.66 ±0.52	22.33 ±0.44	0.67
DBA/2	4	6	19.57 ±0.90	21.08 ±0.77	1.51
	5	7	21.80 ±1.09	21.20 ±0.91	-0.60
	5	8	18.40 ±1.39	19.25 ±1.14	0.86
	4	9	21.04 ±1.50	22.27 ±1.33	1.23
SWR	5	6	20.76 ±0.58	20.99 ±0.57	0.23
	4	7	19.10 ±0.68	19.36 ±1.11	0.26
	5	8	18.36 ±0.84	19.44 ±1.03	1.09
	5	9	19.01 ±0.69	18.84 ±0.93	-0.17
C3H/HeN	5	6	22.90 ±0.28	23.11 ±0.28	0.22
	5	7	22.50 ±0.58	23.40 ±0.66	0.90
	5	8	20.91 ±1.13	21.94 ±1.21	1.03
	5	9	22.44 ±0.25	22.77 ±0.33	0.33
C57BL/6j	5	6	24.36 ±0.49	24.86 ±0.81	0.50
	5	7	23.04 ±0.37	23.27 ±0.37	0.24
	5	8	23.77 ±0.58	22.98 ±0.38	-0.79
	5	9	24.41 ±0.46	22.74 ±0.43	-1.67
CBA/Ca	5	6	19.99 ±0.33	19.62 ±0.46	-0.37
	5	7	21.04 ±0.49	21.01 ±0.49	-0.03
	5	8	20.53 ±0.56	21.24 ±0.73	0.71
	5	9	19.50 ±0.27	19.97 ±0.33	0.46
NIH	5	6	25.80 ±0.54	26.94 ±0.58	1.14
	5	7	24.60 ±0.52	25.58 ±0.63	0.98
	5	8	26.26 ±0.65	27.51 ±0.82	1.25
	5	9	24.86 ±0.60	25.61 ±0.63	0.75
BALB/c	5	6	19.60 ±1.00	21.45 ±0.50	1.84
	5	7	20.56 ±0.42	21.19 ±0.41	0.63
	5	8	20.83 ±0.57	20.81 ±0.85	-0.02
	5	9	21.20 ±1.14	21.66 ±1.09	0.46

Table A.5: Mean weight (±S.E.M.) of the liver (grams) and the mean weight of the liver expressed as a percentage of body weight (±S.E.M.) and the mean weight (±S.E.M.) of the spleen (grams) and the mean weight of the spleen expressed as a percentage of body weight (±S.E.M.) in the nine strains of mice on each post-mortem day from chapter 3.

Strain	n	Day	Liver weight (grams)	Liver weight (percentage of body weight)	Spleen weight (grams)	Spleen weight (percentage of body weight)
C57BL/6j	5	**6**	1.317 ±0.129	5.29 ±0.40	0.0908 ±0.0071	0.3638 ±0.0204
	5	**7**	1.230 ±0.045	5.28 ±0.11	0.0834 ±0.0048	0.3573 ±0.0152
	5	**8**	1.133 ±0.057	4.92 ±0.17	0.0838 ±0.0084	0.3644 ±0.0358
	5	**9**	1.045 ±0.059	4.58 ±0.19	0.0980 ±0.0036	0.4324 ±0.0211
NIH	5	**6**	1.416 ±0.048	5.25 ±0.11	0.1358 ±0.0041	0.5042 ±0.0120
	5	**7**	1.257 ±0.054	4.90 ±0.09	0.1235 ±0.0075	0.4814 ±0.0205
	5	**8**	1.379 ±0.063	5.00 ±0.11	0.1444 ±0.0083	0.5242 ±0.0233
	5	**9**	1.178 ±0.024	4.60 ±0.07	0.1222 ±0.0086	0.4762 ±0.0288
SJL	5	**6**	1.183 ±0.033	5.06 ±0.11	0.1252 ±0.0041	0.5361 ±0.0173
	4	**7**	1.245 ±0.027	5.31 ±0.22	0.1395 ±0.0140	0.5924 ±0.0559
	5	**8**	1.165 ±0.009	5.39 ±0.11	0.1331 ±0.0057	0.6154 ±0.0296
	5	**9**	1.148 ±0.021	5.14 ±0.02	0.1426 ±0.0075	0.6405 ±0.0395
BALB/c	5	**6**	1.205 ±0.074	5.66 ±0.48	0.1487 ±0.0065	0.6941 ±0.0297
	5	**7**	1.074 ±0.029	5.07 ±0.16	0.0992 ±0.0017	0.4685 ±0.0077
	5	**8**	1.077 ±0.026	5.22 ±0.32	0.1124 ±0.0187	0.5370 ±0.0784
	5	**9**	1.079 ±0.057	4.99 ±0.12	0.1011 ±0.0037	0.4695 ±0.0191
SWR	5	**6**	1.188 ±0.050	5.67 ±0.23	0.0857 ±0.0039	0.4080 ±0.0131
	4	**7**	0.993 ±0.082	5.11 ±0.22	0.0733 ±0.0065	0.3783 ±0.0239
	5	**8**	0.997 ±0.066	5.13 ±0.24	0.0841 ±0.0090	0.4316 ±0.0343
	5	**9**	0.944 ±0.050	5.01 ±0.08	0.0688 ±0.0082	0.3643 ±0.0359
A/J	5	**6**	1.090 ±0.035	4.88 ±0.14	0.0682 ±0.0018	0.3148 ±0.0082
	5	**7**	0.976 ±0.040	4.47 ±0.85	0.0695 ±0.0076	0.3228 ±0.0414
	5	**8**	0.946 ±0.026	4.49 ±0.23	0.0682 ±0.0018	0.3672 ±0.0168
	5	**9**	1.021 ±0.050	4.76 ±1.00	0.0662 ±0.0057	0.3096 ±0.0241
C3H/HeN	5	**6**	1.342 ±0.045	5.80 ±0.16	0.0809 ±0.0018	0.3506 ±0.0113
	5	**7**	1.200 ±0.042	5.13 ±0.13	0.0867 ±0.0039	0.3708 ±0.0144
	5	**8**	1.183 ±0.078	5.38 ±0.09	0.0711 ±0.0052	0.3258 ±0.0238
	5	**9**	1.138 ±0.031	4.99 ±0.07	0.0816 ±0.0049	0.3852 ±0.0198
CBA/Ca	5	**6**	1.096 ±0.040	5.58 ±0.12	0.0521 ±0.0017	0.2651 ±0.0033
	5	**7**	1.228 ±0.051	5.87 ±0.35	0.0542 ±0.0020	0.2578 ±0.0077
	5	**8**	1.145 ±0.053	5.38 ±0.11	0.0630 ±0.0029	0.2352 ±0.0591
	5	**9**	1.084 ±0.026	5.43 ±0.06	0.0532 ±0.0036	0.2657 ±0.0143
DBA/2	4	**6**	1.194 ±0.033	5.69 ±0.24	0.0962 ±0.0084	0.4538 ±0.0252
	5	**7**	0.963 ±0.030	4.58 ±0.26	0.0843 ±0.0059	0.3985 ±0.0237
	5	**8**	1.021 ±0.070	5.31 ±0.17	0.0784 ±0.0081	0.4044 ±0.0226
	4	**9**	1.179 ±0.065	5.30 ±0.08	0.0909 ±0.0084	0.4093 ±0.0334

Table A.6: Mean pre- and post-infection body weight of each inoculation group (±S.E.M.) of both mouse strains (C57BL/6j and CBA/Ca) strain and the weight difference between the pre- and post-infection weight (±S.E.M.) from chapter 4.

Strain	day	n	Dose	pre-infection body weight	post-infection body weight	Weight difference
	6	5	**3000**	15.84 (±0.64)	15.30 (±0.57)	-0.54(±0.32)
C57BL/6j	7	5		16.34 (±0.46)	14.40 (±0.19)	-1.94(±0.47)
	8	5		15.42 (±0.62	12.68 (±0.61)	-2.74(±0.18)
	6	5	**3000**	20.54 (±0.90)	20.94 (±0.77)	0.40 (±0.23)
CBA/Ca	7	5		21.10 (±0.62)	20.02 (±0.56)	-1.08 (±0.13)
	8	5		20.80 (±0.51)	18.04 (±0.45)	-2.76 (±0.16)
	6	5	**1000**	16.46 ±0.26	17.20 ±0.08	0.74 (±0.20)
C57BL/6j	7	5		15.92 ±0.52	15.28 ±0.24	-0.64(±0.31)
	8	5		16.02 ±0.51	14.34 ±0.07	-1.68(±0.57)
	6	5	**1000**	20.32 ±0.28	20.84 ±0.32	0.52 (±0.15)
CBA/Ca	7	5		20.16 ±0.34	21.04 ±0.28	0.88 (±0.18)
	8	5		20.22 ±0.29	21.00 ±0.35	0.78 (±0.22)
	6	5	**500**	16.72 (±0.23)	17.64 (±0.27)	0.92 (±0.14)
C57BL/6j	7	5		17.06 (±0.51)	17.54 (±0.39)	0.48 (±0.26)
	8	5		16.40 (±0.37)	16.38 (±0.33)	-0.02(±0.21)
	6	5	**500**	22.42 (±0.79)	23.24 (±0.83)	0.82 (±0.14)
CBA/Ca	7	5		22.96 (±0.51)	23.52 (±0.49)	0.56 (±0.06)
	8	5		21.82 (±1.02)	22.22 (±0.92)	0.40 (±0.39)
	6	5	**100**	16.06 ±0.54	16.78 ±0.61	0.72 (±0.12)
C57BL/6j	7	5		16.10 ±0.62	16.52 ±0.61	0.42 (±0.31)
	8	5		16.54 ±0.36	17.06 ±0.28	0.52 (±0.23)
	6	4	**100**	23.58 ±0.70	24.00 ±0.87	0.43 (±0.36)
CBA/Ca	7	5		21.54 ±1.15	21.80 ±0.97	0.26 (±0.27)
	8	5		22.72 ±0.38	22.86 ±0.34	0.14 (±0.66)
	6	5	**250 x 4**	17.10 ±0.29	15.98 ±0.26	-1.12 (±0.09)
C57BL/6j	7	5		16.10 ±0.36	14.96 ±0.25	-1.14 (±0.41)
	8	4		16.65 ±0.61	16.03 ±1.11	-0.63 (±0.65)
	6	5	**250 x 4**	23.04 ±0.32	23.50 ±0.44	0.46 (±0.26)
CBA/Ca	7	5		21.66 ±0.18	21.28 ±0.24	-0.38 (±0.11)
	8	4		22.20 ±0.87	21.48 ±1.18	-0.73 (±1.81)
	6	5	**500 x 2**	16.52 ±0.38	16.20 ±0.42	-0.32 (±0.19)
C57BL/6j	7	5		15.66 ±0.57	15.74 ±0.55	0.08 (±0.38)
	8	5		16.76 ±0.16	15.98 ±0.43	-0.78 (±0.55)
	6	5	**500 x 2**	21.88 ±0.69	22.88 ±0.51	1.00 (±0.20)
CBA/Ca	7	5		20.52 ±0.44	21.10 ±0.32	0.58 (±0.23)
	8	5		22.04 ±0.65	22.22 ±0.70	0.18 (±0.10)

Table A.7: Mean pre- and post-infection body weight of each inoculation group (±S.E.M.) of both mouse strains (C57BL/6j and CBA/Ca) strain and the weight difference between the pre- and post-infection weight. Also shown, the mean weight (±S.E.M.) of the spleen (grams) and the mean weight of the spleen expressed as a percentage of body weight (±S.E.M.) in the nine strains of mice on each post-mortem day from chapter 5.

Strain	Day	n	Pre-infection weight	post-infection weight	Weight difference	Spleen Weight (grams)
C57BL/6j	6 hours	6	23.07 ±0.76	21.62 ±0.74	-1.45 (±0.14)	0.0678 ±0.0042
	1	6	20.23 ±0.84	19.63 ±0.70	-0.60 (±0.16)	0.0546 ±0.0040
	2	6	21.23 ±0.50	21.15 ±0.45	-0.07 (±0.14)	0.0581 ±0.0029
	3	6	22.00 ±1.04	21.83 ±1.15	-0.15 (±0.21)	0.1069 ±0.0143
	4	6	20.24 ±0.66	20.15 ±0.61	-0.09 (±0.17)	0.0602 ±0.0049
	5	5	21.08 ±0.37	20.58 ±0.19	-0.54 (±0.21)	0.0490 ±0.0023
	6	5	21.31 ±1.08	21.12 ±1.05	-0.18 (±0.07)	0.0656 ±0.0031
	7	4	22.61 ±0.95	22.73 ±1.02	0.10 (±0.11)	0.0755 ±0.0098
	8	5	22.27 ±0.57	21.44 ±0.51	-0.82 (±0.42)	0.0771 ±0.0068
CBA/Ca	6 hours	6	21.09 ±0.33	19.47 ±0.26	-1.63 (±0.16)	0.0404 ±0.0019
	1	6	21.16 ±0.44	20.30 ±0.38	-0.87 (±0.11)	0.0424 ±0.0007
	2	6	21.11 ±0.54	20.93 ±0.52	-0.20 (±0.28)	0.0438 ±0.0008
	3	6	21.23 ±0.35	20.37 ±0.47	-0.87 (±0.22)	0.0490 ±0.0019
	4	6	21.12 ±0.62	20.95 ±0.47	-0.17 (±0.17)	0.0468 ±0.0018
	5	6	21.03 ±0.37	21.77 ±0.31	0.75 (±0.13)	0.0467 ±0.0015
	6	4	21.01 ±0.67	21.60 ±0.65	0.58 (±0.20)	0.0418 ±0.0031
	7	5	21.98 ±0.43	21.34 ±0.63	-0.64 (±0.63)	0.0499 ±0.0024
	8	5	20.32 ±0.51	20.30 ±0.46	-0.02 (±0.14)	0.0577 ±0.0127

Table A.8: Chapter 6 mean (±S.E.) pre- and post-infection body weight of mice and the mean spleen weight (±S.E.) of mice inoculated with *A. suum* and placebo hypodermic inoculations of water and mice inoculated with *A. suum* and hypodermic inoculations of cortisone and also shown are the day 5 control mice.

Strain	Day	Pre-infection weight (grams)	Post-infection weight (grams)	Weight Difference	Spleen weight (grams)
C57BL/6j					
Control	*5*	*21.37 ±0.57*	*21.72 ±0.58*	*0.36 ±0.12*	*0.0613 ±0.0031*
Placebo	6	21.31 ±1.08	21.12 ±1.05	-0.18 ±0.07	0.0656 ±0.0031
	7	22.61 ±0.95	22.73 ±1.02	0.10 ±0.11	0.0755 ±0.0098
	8	22.27 ±0.57	21.44 ±0.51	-0.82 ±0.42	0.0771 ±0.0068
cortisone	6	21.16 ±0.98	20.34 ±1.34	-0.82 ±0.40	0.0271 ±0.0052
	7	20.47 ±0.79	18.98 ±0.62	-1.50 ±0.28	0.0292 ±0.0028
	8	21.99 ±0.80	19.46 ±0.81	-2.54 ±0.32	0.0413 ±0.0038
CBA/Ca					
Control	*5*	*20.78 ±0.19*	*21.72 ±0.24*	*0.96 ±0.14*	*0.0436 ±0.0013*
Placebo	6	21.01 ±0.67	21.60 ±0.65	0.58 ±0.20	0.0418 ±0.0031
	7	21.98 ±0.43	21.34 ±0.63	-0.64 ± 0.63	0.0499 ±0.0024
	8	20.32 ±0.43	20.30 ±0.46	-0.02 ±0.14	0.0577 ±0.0127
cortisone	6	20.20 ±0.48	18.90 ±0.45	-1.30 ±0.14	0.0189 ±0.0022
	7	19.89 ±0.54	17.78 ±0.56	-2.10 ±0.26	0.0223 ±0.0010
	8	21.63 ±0.72	18.65 ±0.77	-3.00 ±0.04	0.0236 ±0.0009

Table A.9: The mean number of larvae (±S.E.M.) recovered from the liver of both C57BL/6j and CBA/Ca mice inoculated with either *A. suum* and cortisone or *A. suum* only on days 6-8 post-inoculation.

Strain	Procedure	Days p.i.	n	Liver
C57BL/6j	cortisone + *A. suum*	6	5	24.5 ±6.1
	cortisone + *A. suum*	7	5	0.0 ±0.0
	cortisone + *A. suum*	8	5	4.0 ±2.3
	A. suum only	6	5	41.0 ±11.1
	A. suum only	7	4	0.0 ±0.0
	A. suum only	8	5	0.0 ±0.0
CBA/Ca	cortisone + *A. suum*	6	5	35.0 ±10.8
	cortisone + *A. suum*	7	5	6.0 ±3.8
	cortisone + *A. suum*	8	4	2.7 ±2.7
	A. suum only	6	4	26.7 ±9.6
	A. suum only	7	5	2.00 ±2.0
	A. suum only	8	5	0.0 ±0 .0

Table A.10: Chapter 6 mean (±S.E.M.) pre- and post-infection body weight of mice and the mean spleen weight (±S.E.M) of mice inoculated with 1500 *A. suum* in the BAL experiment and also the spleen weight expressed as a percentage of the control spleen weight (±S.E.M.) and also shown are the day 7 control mice.

Strain	Procedure	n	Days	Pre-infection weight (grams)	Post-infection weight (grams)	Weight difference	Spleen Weight (grams)	Spleen weight as % of control mean
C57BL/6j	Control	5	**7**	19.14 (0.67)	20.48 (0.51)	1.34 (0.26)	0.0658 (0.0049)	100 (7.47)
	BAL	5	**8**	20.58 (0.86)	17.82 (0.61)	-2.76 (0.35)	0.0599 (0.0053)	91.0 (8.16)
		5	**9**	19.0 (0.89)	18.14 (0.93)	-0.86 (0.53)	0.0715 (0.0078)	108.7 (11.88)
		5	**10**	19.7 (1.45)	18.48 (0.1.22)	-1.22 (0.29)	0.0738 (0.0047)	112.1 (7.14)
CBA/Ca	Control	5	**7**	20.4 (0.45)	21.5 (0.43)	1.1 (0.31)	0.0453 (0.0019)	100 (4.09)
	BAL	5	**8**	19.72 (0.85)	19.08 (0.78)	-0.64 (0.27)	0.0524 (0.0029)	115.6 (6.39)
		5	**9**	19.84 (0.65)	18.66 (1.04)	-1.18 (0.61)	0.0567 (0.0051)	125.1 (11.35)
		5	**10**	20.46 (0.73)	19.82 (0.99)	-0.64 (0.41)	0.0627 (0.0051)	138.4 (11.31)

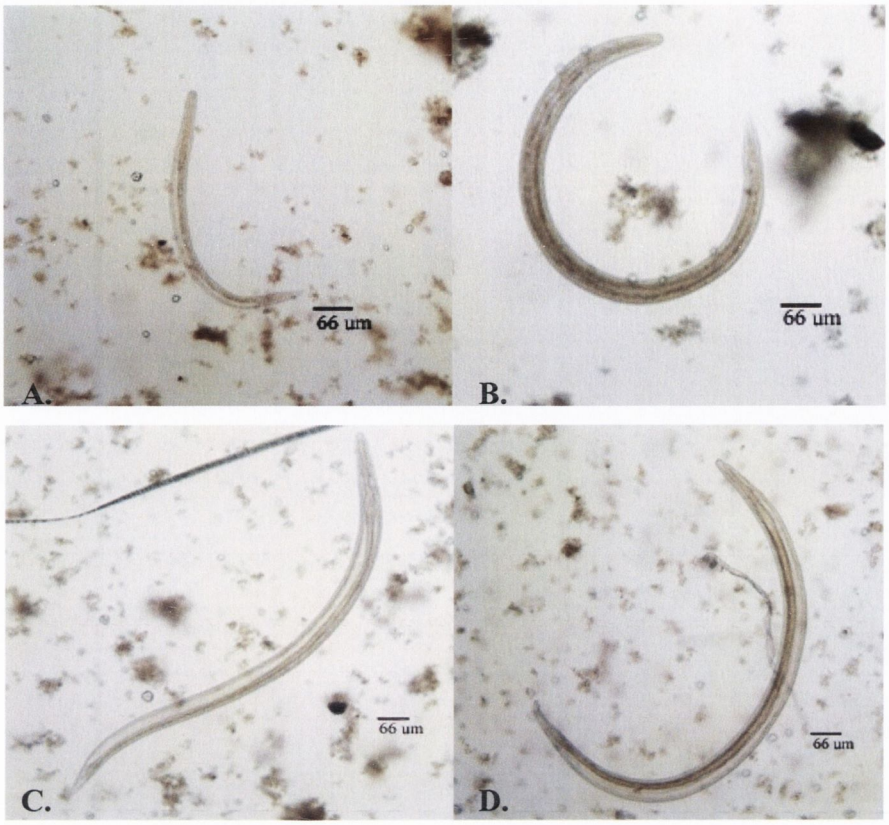


Plate B.1: Photomicrographs of larvae of variable length representing different phases of growth and different post-mortem days (day 6 (a), 7 (b) and 8 (c and d) respectively) recovered from the lungs of mice (Scale= μm) from chapter 4.



Plate B.2: Video capture still of an *A. suum* larvae moving through the bronchoalveolar fluid of a C57BL/6j mouse under x20 magnification (the surrounding cells are red and white blood cells) from chapter 6.

APPENDIX C: LABORATORY PROCEDURES

EMBEDDING AND STAINING OF FIXED TISSUE FROM CHAPTER 7

Embedding process

1. The lung was placed in a glass vial. To this a label was added with organ, strain, day of p.m. and date. 70% alcohol was added and left for 1 hour.
2. The 70% alcohol was removed and to the vial 90% alcohol was added and left for 1 hour.
3. The 90% alcohol was discarded and replaced with absolute alcohol I for 1 hour
4. After one hour, the Absolute alcohol I was replaced with Absolute II for 1 hour.
5. Absolute II was extracted and the samples were placed in Histoclear™ for 1 hour.
6. After this period, the Histoclear™ was siphoned off and the samples immersed in a wax: Histoclear™ (50:50) mixture and placed in the incubator for 1 hour.
7. The wax: Histoclear™ mix was removed and molten paraffin wax I (100% wax) replaced it for 1 hour.
8. After one hour the wax I was removed and replaced with wax II (100% paraffin wax) and placed in the incubator overnight.
9. After overnight incubation the individual sections were blocked, with the tissue lying on its dorsal surface, in paraffin wax and allowed to solidify on a cold-plate.
10. After this process the individual blocks were sectioned transversely through the middle part of the tissue on a microtome (Leica® RM2135) and each section floated in albumin and placed on a slide and allowed to dry overnight.

Haematoxylin and Eosin Staining procedure

(a) Dewax slides in histoclear for 1-3 minutes

(b) Hydrate the tissue as follows:

-Absolute Alcohol for 1-2 minutes

-90% alcohol for 2 minutes

-70% Alcohol for 2 minutes

(c) Wash in water

(d) Stain in Haematoxylin using progressive staining for between 2-10 minutes

(e) Wash briefly in water

(f) Differentiate the tissue in Scott's tap water for 1-4 minutes examining continuously to obtain the right degree of blueing

(g) Wash in water

(h) Counterstain with aqueous Eosin (0.35% in water)

(i) Dehydrate the tissue as follows:

-70% Alcohol for 2 minutes

-90% Alcohol for 2 minutes

-Absolute Alcohol for 1-2 minutes

-Histoclear™ for 2-5 minutes

(j) Mount with DPX

Discombes Eosin staining procedure

(a) Sections were placed in an incubator for 20-30 minutes at 57°C. They were then dewaxed by placing them in histoclear and then rehydrated by passing them through decreasing concentrations of alcohol from absolute ethanol down to 70% ethanol and finally in water. This process was performed in using an automated system (Varistain, Gemini).

(b) The slides were treated with 1% Alcian blue at pH 2.5 prepared from 1 gram Alcian blue and 3% Acetic acid for 5 minutes

(c) They were then washed gently in water

(d) Slides were the treated with 1% periodic acid for 7 minutes

(e) Again slides were washed with water

(f) The slides were then placed in Schiff solution for 10 minutes

(g) The slides were the placed under gently running warm water for 10 minutes

(h) Slides were removed from the warm water and placed in Discombes eosin (0.5% aqueous eosin and 5% acetone in distilled water) for 10 minutes

(i) The slides were removed from this satin and washed with water

(j) They were then placed in Harris's haematoxylin for 1 minute

(k) The stained slides were then washed in warm water for 1 minute

(l) Finally the slides were dehydrated by immersing the tissue in 50%, 70%, 90% and 2-3 changes of Absolute alcohol and finally xylene. This process was performed in using an automated system (Varistain, Gemini). The prepared slides were then mounted in DPX.

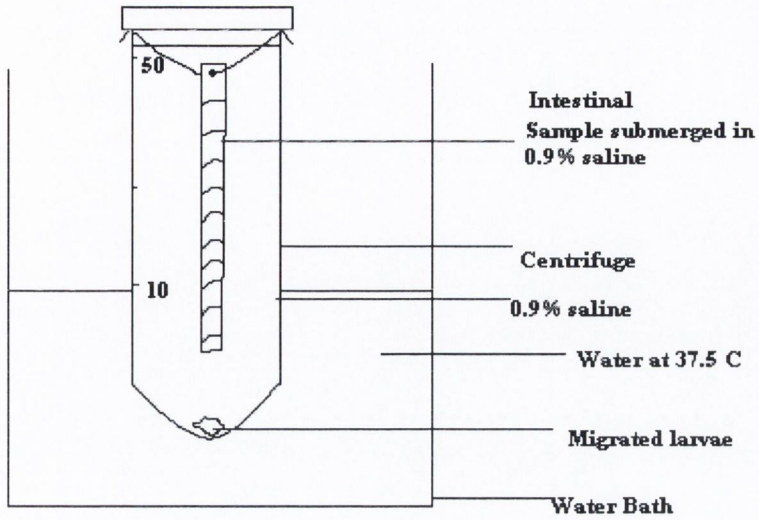


Figure D.1: Modified Baermann apparatus for the recovery of larvae from the intestinal wall of mice inoculated with 500 *A. suum* in chapter 5.

APPENDIX E: PUBLICATIONS

The development of a mouse model to explore resistance and susceptibility to early *Ascaris suum* infection

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SUMMARY

Ascaris suum and *Ascaris lumbricoides* exhibit an over-dispersed frequency distribution in their host populations in both the adult and larval stages. The impact of host factors on this observed distribution is still poorly understood and difficult to investigate in the natural host populations. The use of a mouse model has been supported by the observations that the larval migratory pattern, in this host, mimics the pattern observed in the pig. We explored the extrinsic factors that might affect the quantitative recovery of larvae during this migration in order to standardize a model system facilitating accurate future assessment of host genetic variation on this phase of the infection. In Exp. 1 larvae accumulated in the livers of both C57BL/6j and BALB/c mice up to and including days 4–5 p.i. and then declined in both strains until day 9. Loss of larvae from the livers corresponded to arrival in the lungs and maximum accumulation on day 7 p.i. but recovery was considerably higher in C57BL/6j mice. It was concluded that day 7 recoveries gave the best indication of relative resistance/susceptibility to this parasite. In Exp. 2 A/J, BALB/c, CBA/Ca, C57BL/6j, C3H/HeN, DBA/2, NIH, SJL, and SWR mice were compared. C57BL/6j mice were identified as the most susceptible strain and CBA/Ca mice as having the most contrasting phenotype, but with a similar kinetic pattern of migration. Finally, in Exp. 3, a strong positive correlation between the size of the inoculum and the mean worm recovery from the lungs was found in CBA/Ca and C57BL/6j mice, but the difference between these strains was highly consistent, 66·6–80%, regardless of the initial dose. These results demonstrate that, using our protocols for infection and recovery, between-experiment variation in *A. suum* worm burdens is minimal, and that C57BL/6j mice are highly susceptible to infection compared to other strains. The mechanistic basis of this susceptibility in relation to the resistance of other strains is unknown, but the possibilities are reviewed.

Key words: *Ascaris suum*, *Ascaris lumbricoides*, mouse model, pigs, C57BL/6j, CBA/Ca, susceptibility, lungs, migration.

INTRODUCTION

Ascaris lumbricoides and *A. suum* are important parasites of humans and pigs respectively (O'Lorcain and Holland, 2000; Crompton, 2001). Their importance lies primarily in the heavy adult worm burdens that can accumulate in predisposed subjects living in communities where transmission is intense (Holland and Boes, 2002) and similarly in pigs where husbandry procedures permit transmission (Boes *et al.* 1998). Additionally, both parasites also cause liver and lung pathology when hosts are exposed to large numbers of infective eggs simultaneously, as a result of the synchronized migration of the larvae and temporary development in these organs on their migration route to the intestine. Pulmonary symptoms can be pronounced and even life threatening and are known as Loeffler's syndrome (Loeffler, 1932, 1956; Vogel and Minning, 1942).

Recently there has been renewed interest in the consequences of *Ascaris* larvae migrating through the lungs in the context of the hygiene and related hypotheses (Cooper *et al.* 2000), and yet to-date very little is known about this phase of the parasite's life-cycle. Recent comparative studies on larval migration in pigs and mice have led to the suggestion that the mouse is a suitable model for exploring susceptibility in the early phase of *A. suum* infection (Murrell *et al.* 1997; Slotved *et al.* 1998) as the pattern of migration of the larvae is analogous. The recovery of larvae from mice has been shown to be higher when compared to other abnormal hosts such as rabbits, guinea-pigs and rats (Douvres and Tromba, 1971) and even pigs (Roepstorff *et al.* 1997). Relative host size and parasite size contribute to this higher recovery.

Like most other helminths *Ascaris* show an aggregated distribution in their hosts, and although recent studies have suggested a genetic basis to this phenomenon in humans (Williams-Blangero *et al.* 2002), behavioural, immunological and physiological factors are also thought to play key roles in causing predisposition (McSharry *et al.* 1999; Holland and Boes, 2002). The mechanistic basis of predisposition is not understood, but it may well be initiated during the early phase of infection. Indeed, Cooper *et al.* (2000) have suggested that pulmonary migrations of

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Ascaris larvae could create a highly polarized Th2 immune environment in the lung mucosa, and genetic variation in the host response at this level may influence the course of subsequent infection. Since mice are known to vary in their susceptibility to infection with *Ascaris* (Mitchell *et al.* 1976), they provide a convenient model for investigating the basis of variation in establishment and in the success of subsequent migration of larvae through the liver and lungs.

In this paper we report a series of experiments which explore the kinetics of early *Ascaris* infection in the small intestine, liver and lungs of inbred mice. We first optimized the methodology for worm recovery and, on the basis of earlier publications, chose two inbred mouse strains to confirm that pronounced differences in migration kinetics exist. We then tested the hypothesis that genetic factors play a key role in influencing the success of larval migration, by comparing migration in a panel of mouse strains, incorporating some of those used by earlier workers but focusing particularly on the strains that have been highlighted for the mouse genome projects. We also explored the degree to which migration success is influenced by the dose of larvae administered and hence the size of the migrating population of worms. Finally, the effects of strain, day post-infection and dose on worm length were assessed.

MATERIALS AND METHODS

Experimental animals

Male mice were purchased from Harlan, UK at 7 weeks of age and were 8 weeks old upon commencement of the initial infections. Seventy inbred mice (35 C57BL/6j and 35 BALB/c) were purchased for Exp. 1. A further 180 inbred mice were acquired for Exp. 2, 20 each of; A/J, BALB/c, C57BL/6j, C3H/HeN, CBA/Ca, DBA/2, NIH, SJL and SWR. Finally, in Exp. 3, 180 inbred mice, 90 of the selected susceptible strain C57BL/6j and 90 of the resistant strain CBA/Ca, were purchased. Animals were maintained in conditions that were standard and constant; approximately 22 °C with a daily 12 h photoperiodicity. Water and pelleted commercial food were provided *ad libitum*. The cages were cleaned and sawdust changed regularly.

Parasite

Approximately 4 000 000 embryonated ova (batch no.: 8/2002) were provided by the Danish Centre for Experimental Parasitology (CEP), Copenhagen. The eggs were removed from an isolate of *A. suum* taken from pigs at a Danish slaughterhouse. The eggs were isolated from the worm uteri and cultured to infectivity in 0·1 M H_2SO_4 for 3 months (in the dark at room temperature), conditions that have been shown to result in fully infective eggs (Oksanen *et al.* 1990). Upon arrival in Dublin, these eggs were diluted to a concentration of 25 eggs/μl as this is the maximum egg density which does not inhibit the infectivity or viability of the eggs (Eriksen, 1990). Prior to inoculation, the doses were adjusted to the desired number of fully embryonated eggs.

Experimental protocol

The preparation of a typical inoculum of 500 eggs was prepared as follows. A standard dose of 100 μl of egg suspension containing 5 eggs μl^{-1} was drawn from the stock egg suspension during magnetic stirring and gentle agitation by hand, into an automatic pipette fitted with a disposable tip. The contents were emptied into a 1·5 ml Eppendorf tube. Higher egg doses were concentrated from the stock solution. Using a modified version of the method described by Slotved (1997) we evaluated the number of eggs in the prepared dosages and thus standardized the number of eggs in an inoculation dose. Briefly, the numbers of eggs containing larvae were counted under ×10 magnification in 10 × 10 μl samples. The procedure was repeated 6 times and the tip of the automatic pipette used to dispense the eggs onto the slide was changed between each replication.

Mice were inoculated by gastric intubation between 8 and 10 a.m. The inoculation apparatus, which consisted of a 1 ml graduated syringe containing the relevant dose with a gastric intubation needle, was guided into the stomach of the mouse and the appropriate dose was administered.

All mice were euthanased by cervical dislocation on the relevant post-mortem day. Mice were weighed pre-infection and on the day of post-mortem and in experiments 2 and 3 the weight of the liver and spleen was also recorded at post-mortem. The days on which larvae were recovered post-inoculation (p.i.) were days 3–9 (Exp. 1), days 6–9 (Exp. 2) and days 6–8 (Exp. 3).

Experiment 1. Mice were inoculated with 500 fully embryonated eggs by stomach intubation. Five mice were euthanased from each strain daily between days 3 and 9 post-inoculation. At post-mortem examination, the liver and the lung were removed for examination of larval burdens. This experiment provided data on the larval kinetics in the lungs and liver of these reference strains, which were believed to represent susceptible and resistant phenotypes. It also tested the modified Baermann method that had been developed for recovering larvae from macerated organs.

Experiment 2. Mice were inoculated with 500 embryonated eggs and 5 mice of each strain were euthanased daily between days 6 and 9 p.i. to determine larval burdens in the lungs. This experiment was designed to determine which 2 strains showed

the most contrasting phenotypes with respect to susceptibility to *A. suum* larval infection. Susceptibility was assessed by the number of larvae recovered from the lungs. The experiment provided additional data on the consistency of the recovery protocol.

Experiment 3. Mice were inoculated with 1 of 4 single inoculum doses (3000, 1000, 500, or 100) and 2 doses of 1000 ova administered over consecutive days in split doses (250 × 4 and 500 × 2). Groups of 5 mice were euthansed daily between days 6 and 8 p.i. The lungs were examined for larval burden. The objective of this experiment was to determine the influence of dose on the larval burden not only within a strain, but also between different strains, and to provide further data on the consistency of the protocol.

The modified Baermann method (adapted from Eriksen, 1981)

The modified Baermann apparatus was constructed using a mesh comprising a double layer of gauze (sterile absorbent type 13 light gauze [Boots Pharmacy]) fixed with a rubber band to a plastic tube (20 ml universal sample tube) from which the bottom was removed. This gauze-tube apparatus was suspended in a 50 ml centrifuge tube with the gauzed end inserted into the tube. Saline (0·9%) was added to this apparatus up to the 35 ml mark. The centrifuge tube apparatus was suspended up to the 15 ml mark in a water bath at 37·5 °C, overnight prior to post-mortem. On the day of post-mortem the liver and lungs were macerated using fine scissors. The macerated organ was placed on top of the gauze net and warm 0·9% saline was added to ensure the sample was completely submerged. The centrifuge-tube apparatus was capped to prevent loss of liquid. This apparatus was kept at a constant 37·5 °C in the water bath for 24 h.

Recovery of worms

After overnight incubation the gauze mesh was removed from the centrifuge tubes and the tubes capped. These were then centrifuged for 5 min at 1000 ***g***. After centrifugation, the solution (supernatant) was extracted carefully, so as not to disturb the pellet, using an aspirator, until 10 ml of solution with the pellet beneath remained. Formalin (6%) was added and the solution was allowed to settle for 24 h at room temperature. This was then re-centrifuged for 5 min at 1000 ***g*** and reduced to 4 ml.

Larval counts

The remaining 4 ml solution and pellet in the centrifuge tube was agitated using a vortex mixer. Five 200 μl samples were then placed on a slide and the number of larvae were counted with a phase-contrast stereomicroscope at ×40 magnification. The sum of the larvae counted in the 5 aliquots was then calculated using the appropriate calculations.

Measurement of larval length

During larval counts, larvae that were suitable for measuring were photographed using an Olympus camedia digital camera C5050 (5.0 megapixels with a F1.6 lens) at ×10 magnification. These images were transferred to a computer (OS windows XP) and each individual was measured using the programme ImageJ 1.32j. Larvae were measured by drawing a segmented line through the centre of the body from the anterior to the posterior end.

Statistical analysis

Larval recovery was assessed for normality visually and statistically. Values for means, standard deviations, ranges, skewness, kurtosis and standard errors were obtained for each subset of data. The influence of the various factors on larval burden and size were analysed by means of analysis of variance (ANOVA in SPSS 12.0.1), using 1-, 2- or 3-way ANOVAs depending on the number of explanatory factors and covariates involved. Full-factorial models were simplified by step-wise deletion of non-significant terms beginning with the 3-way interactions, and minimum sufficient models in which only significant terms remained were used to assess significance of fitted factors. In some cases least squares difference (LSD) *post-hoc* tests were applied to tease out the major sources of variation within factors. Continuous variables were examined by Spearman's rank correlation. Statistical analysis was carried out at a confidence limit of 95% ($\alpha = 0{\cdot}05$).

RESULTS

Mean larval burdens

In Exp. 1 the numbers of larvae in the liver and lungs were observed on days 3–9 post-inoculation (p.i.) to identify the migration patterns of larvae through these organs (Fig. 1). Larval counts in the liver changed significantly with time in both mouse strains (2-way ANOVA with time and strain as factors, model $R^2 = 0{\cdot}75$, main effect of time $F_{6,54} = 22{\cdot}0$, $P < 0{\cdot}0001$), and the strains differed significantly (main effect of strain $F_{1,54} = 4{\cdot}5$, $P = 0{\cdot}038$ and 2-way interaction strain*time $F_{6,54} = 2{\cdot}3$, $P = 0{\cdot}05$), larvae moving into the livers of C57BL/6j at a faster rate than in BALB/c. This was particularly evident by the larger worm burdens on day 3 p.i., although by day 4 p.i. the mean larval burdens were similar in both strains. After this, worm burdens stabilized in BALB/c mice but continued to rise in C57BL/6j mice until day 5, after which there was a steady decline to

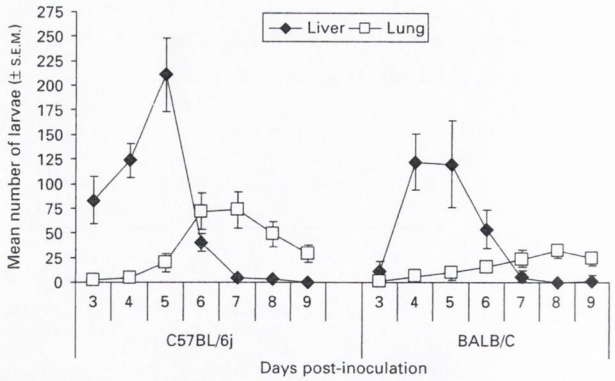


Fig. 1. The mean number (±S.E.M.) of larvae recovered from the liver (◆) and the lung (□) in the susceptible (C57BL/6j) and resistant (BALB/c) strains of mice on days 3–9 p.i.

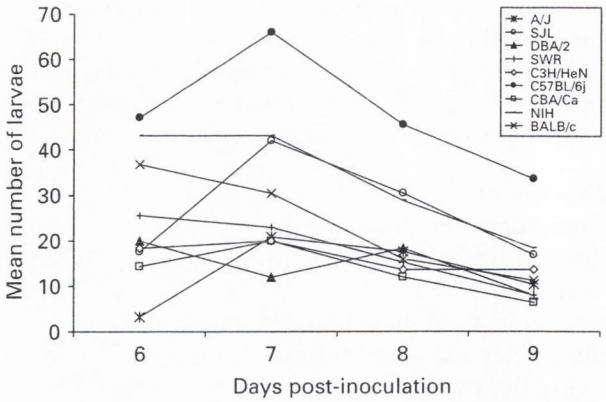


Fig. 2. The mean number of larvae recovered from the lungs of 9 strains of mice on days 6–9 p.i.

zero by day 9 p.i. in both strains (Fig. 1). The highest percentage recovery of the initial inoculum from the liver was 42·2% in C57BL/6j on day 5 and 24·4% in BALB/c on day 4.

The reduction in larval numbers in the liver coincided with the movement of larvae into the lungs on days 5–6 p.i. in both strains, and again this was reflected in significant changes in worm burdens with time (2-way ANOVA with strain and time as factors on lung worm burdens, model $R^2 = 0{\cdot}743$, main effect of time $= F_{6,54} = 8{\cdot}99$, $P < 0{\cdot}0001$). Overall, in both strains, there was a reduction in the number of larvae that successfully migrated to this organ in comparison to the numbers observed in the liver. By day 7 p.i., in C57BL/6j, the majority of larvae were found in the lungs (14·7% of the inoculated dose). However, in respect of lung worm burdens there was a more marked difference between strains compared with liver counts (main effect of strain, $F_{1,54} = 15{\cdot}8$, $P = 0{\cdot}0002$ and 2-way interaction strain*time, $F_{6,54} = 3{\cdot}58$, $P = 0{\cdot}005$). The greatest difference in the mean number of larvae recovered between the 2 strains was observed in the lungs ($\geqslant 67{\cdot}4\%$) on days 6 and 7 p.i. This is in contrast to the liver, which although displaying higher larval numbers showed less of a percentage difference in worm burdens on the days of peak recovery (=43·1%).

Six standard inoculation dosages of 500 eggs, chosen randomly, were examined prior to inoculation and gave a mean of 493·5 eggs (±3·8) confirming the consistency of administered doses and showing that variation in the administered dose was unlikely to make a significant contribution to the observed variance in larval burdens.

Strain differences

Exp. 2 employed 9 strains of mice to compare their relative susceptibilities to infection with *A. suum* and to identify for future work, 2 strains that show the greatest contrast of resistance/susceptibility phenotype. The results are illustrated in Fig. 2 and show that for the reference strain C57BL/6j the mean number of larvae recovered and the migratory kinetics in the lungs on the 4 days examined were indistinguishable from those observed in Exp. 1. Across all strains worm burdens in the lungs changed significantly with time (2-way ANOVA with day and strain as explanatory factors, model $R^2 = 0{\cdot}61$, on the lung worm burdens, main effect of time, $F_{3,140} = 14{\cdot}2$, $P \leqslant 0{\cdot}0001$). As can be seen, the strains differed markedly in lung worm burdens throughout the period after infection (main effect of strain, $F_{8,140} = 17{\cdot}95$, $P \leqslant 0{\cdot}0001$) but in general the pattern of changes in worm burdens with time did not differ significantly between strains (2-way interaction strain*day $F_{24,176} = 1{\cdot}517$, $P =$ N.S.), indicating consistency in the daily changes in worm burdens across mouse strains.

Post-hoc analysis identified those strains that differed significantly from each other. Thus, C57BL/6j differed significantly from all strains ($P \leqslant 0{\cdot}0001$), with a mean larval burden of 66±8·2 (13·2% of the administered inoculum recovered) on day 7 p.i. and are classified as the most susceptible strain. NIH, SJL and BALB/c mice, in which mean larval recoveries on day 7 ranged from 6 to 8%, were significantly different from the 6 other strains, but not each other and are classified as intermediate susceptible strains. Finally A/J, C3H/HeN, CBA/Ca, DBA/2 and SWR, with worm burdens of less than 4%, were not significantly different from each other but were significantly different from the other 2 groupings of intermediate susceptible and most susceptible (C57BL/6j), and are classified as resistant strains.

The greatest significant difference between strains (1-way ANOVA, confined to day 7 p.i., model $R^2 = 0{\cdot}61$, effect of strain, $F_{8,34} = 6{\cdot}8$, $P \leqslant 0{\cdot}0001$) was observed on day 7 post-inoculation. This was also the day on which larvae accumulated to their maximum number in the lungs of the susceptible C57BL/6j mouse strain and the day on which this strain differed

most markedly from the other strains. In 4 of the other strains, A/J, SJL, C3H/HeN and CBA/Ca worm burdens were also maximum on day 7 p.i., but not in DBA/2, SWR, NIH and BALB/c mice. However, while in the latter strains worm recoveries were higher on day 6, the difference between day 6 and day 7 counts was marginal.

The most marked overall difference was detected between CBA/Ca and C57BL/6j (*post-hoc* test, $P=0{\cdot}000014$), which despite the large difference in numbers of larvae recovered on each day, showed the same pattern of worm migration during the 4-day period (Fig. 2). The percentage difference in the mean number of larvae recovered from these 2 strains on the 4 days was also highly consistent ranging between 69 and 81%. These recoveries contrast with the next most susceptible strain C3H/HeN, the recovery of larvae from this strain being as consistent but with a lower percentage difference relative to C57BL/6j mice, 59·5–70·2%. It must be noted that although DBA/2 mice appeared to be most resistant on day 7 p.i., accumulation of larvae occurred in the lungs on day 6 in this strain (Fig. 2). While A/J mice appeared to be highly resistant overall, there was a great deal of variation among individuals of this strain with a range of values on the day of peak recovery, from 0 to 40 larvae, contrasting with CBA/Ca mice where the range of individual burdens was considerably more restricted (12–28 larvae).

As an additional precaution, an analysis was undertaken that took host body weight into consideration. Lung worm burdens were expressed as worms per gram of body weight (total worm burden from the lungs/mouse body weight; Table 1). Once again, the two-way ANOVA for the number of larvae recovered per gram of mouse body weight (model $R^2=0{\cdot}59$) revealed that the main effects of strain ($F_{8,140}=15{\cdot}0$, $P\leqslant 0{\cdot}0001$) and day ($F_{3,140}=14{\cdot}7$, $P\leqslant 0{\cdot}0001$) were highly significant explanatory factors for variation in larval burdens but in this case there was a marginally significant 2-way interaction (strain * day, $F_{24,140}=1{\cdot}61$, $P\leqslant 0{\cdot}0468$). Interestingly, *post-hoc* analysis confirmed a comparable outcome to that cited above, with the relative ranking of mouse strains identical to that concluded on the basis of total worm burdens.

Finally, we assessed whether there were any significant differences in body weight between the mouse strains that might have confounded our analysis. The analysis revealed that the body weight of mouse strains varied significantly (2-way ANOVA, with strains and time as factors, on mouse weight, main effect of strain, $F_{8,140}=31{\cdot}9$, $P\leqslant 0{\cdot}0001$), but not with time (day p.i.) and the 2-way interaction between strain and day was not significant. *Post-hoc* analysis showed that 1 strain differed from all of the other strains; NIH mice were significantly heavier than all the other strains. Thus no relationship emerged that related host body weight to parasite burdens. For example BALB/c and NIH differed significantly in their body weight (Table 1) but not in the number of worms recovered per gram of body weight.

The influence of dose on mean larval numbers

The outcome of this experiment was analysed in 2 stages. First we analysed the dose response with single-pulse infections (100, 500, 1000 and 3000 larvae) administered to C57BL/6j and CBA/Ca mice. As previously, there was a highly significant difference between the strains with time and dose taken into consideration (Fig. 3, 2-way ANOVA with strain * time + dose as a covariate, model $R^2=0{\cdot}70$, main effect of strain $F_{1,114}=30{\cdot}4$, $P<0{\cdot}001$) but no significant interaction between strain and time. Because this experiment was restricted to just 3 days, and given the variation in recovery within subgroups of mice, overall worm burdens did not vary significantly with time (main effect of days $F_{2,114}=2{\cdot}4$, $P=$ N.S.). Nevertheless, as in both of the earlier experiments, the arithmetic mean worm burdens were highest on day 7 in C57BL/6j mice at all 4 doses. This was also the case in the lower 3 doses (100, 500 and 1000 eggs) of CBA/Ca mice. There was an exception in CBA/Ca mice inoculated with 3000 eggs with the highest level of infection observed on day 6.

There was a highly significant positive relationship between the dose of eggs administered and the worm burdens in the combined data set with strain and time taken into account (effect of dose $F_{1,114}=242{\cdot}1$, $\beta=0{\cdot}129$, $t=15{\cdot}6$, $P<0{\cdot}001$) and *post-hoc* within each of the 2 strains when analysed separately (1-way ANOVAs with time as a factor day, main effect of dose, $\beta=0{\cdot}179$, $R_{\text{adj}}{}^2=0{\cdot}848$, $t=13{\cdot}98$ C57BL/6j mice and $\beta=0{\cdot}079$, $R_{\text{adj}}{}^2=0{\cdot}903$, $t=18{\cdot}68$, for CBA/CA and $P<0{\cdot}0001$ in both cases) suggesting a very strong correlation between the administered dose size and the number of larvae recovered within each strain. However, unexpectedly, the percentage of the initial inoculum recovered, on the day of peak accumulation, increased with increasing dose size (Fig. 4). In C57BL/6j mice percentage recoveries rose from 7·2% in mice given just 100 larvae to 21·6% in those given 3000. Similarly in CBA/Ca mice there was also a trend of increasing worm recoveries from 2·4% to 7·2%. However, the percentage difference in worm burdens between C57BL/6j and CBA/Ca mice was independent of doses of larvae administered, and ranged from 66·6 to 78·9% (Fig. 4; Spearman's rank correlation on day 7 p.i., $R_S=0{\cdot}179$, $n=20$, $P=$ N.S.), implying that as the percentage of the initial inoculum recovered increased within the strains, the percentage difference between the 2 strains was constant and did not vary with increasing dose size and thus the disparity between the strains was maintained across the range of infection doses examined.

Table 1. The mean number of larvae recovered from the lungs per gram of mouse body weight (±S.E.) from each strain: C57BL/6j, NIH, SJL, BALB/c, SWR, C3H/HeN, CBA/Ca A/J and DBA/2 on days 6–9 p.i. and the mean percentage of the initial inoculation dose of 500 *Ascaris suum* eggs recovered in this organ

(Also shown is the mean weight of the group on the day of post-mortem.)

Strain	*n*	Days p.i.	Post-mortem weight	Lung	Percentage
C57BL/6j	5	6	24·86 (±0·81)	1·90 (±0·40)	9·4
	5	7	23·27 (±0·37)	2·83 (±0·33)	13·2
	5	8	22·98 (±0·38)	1·98 (±0·46)	9·1
	5	9	22·74 (±0·43)	1·48 (±0·16)	6·7
NIH	5	6	26·94 (±0·58)	1·63 (±0·32)	8·6
	5	7	25·58 (±0·63)	1·71 (±0·34)	8·6
	5	8	27·51 (±0·82)	1·08 (±0·28)	5·8
	5	9	25·61 (±0·63)	0·71 (±0·11)	3·7
SJL	5	6	23·35 (±0·28)	0·75 (±0·09)	3·5
	4	7	23·55 (±0·69)	1·78 (±0·43)	8·4
	5	8	21·65 (±0·30)	1·40 (±0·21)	6·1
	5	9	22·33 (±0·44)	0·74 (±0·14)	3·4
BALB/c	5	6	21·45 (±0·50)	1·71 (±0·30)	7·4
	5	7	21·19 (±0·41)	1·44 (±0·26)	6·1
	5	8	20·81 (±0·85)	0·75 (±0·26)	3·2
	5	9	21·66 (±1·09)	0·51 (±0·09)	2·2
SWR	5	6	20·99 (±0·57)	1·22 (±0·10)	5·1
	4	7	19·36 (±1·11)	1·20 (±0·36)	4·6
	5	8	19·44 (±1·03)	0·78 (±0·13)	3·0
	5	9	18·84 (±0·93)	0·41 (±0·11)	1·6
C3H/HeN	5	6	23·11 (±0·28)	0·81 (±0·44)	3·7
	5	7	23·40 (±0·66)	0·87 (±0·15)	4
	5	8	21·94 (±1·21)	0·62 (±0·17)	2·7
	5	9	22·77 (±0·33)	0·59 (±0·14)	2·7
CBA/Ca	5	6	19·62 (±0·46)	0·74 (±0·22)	2·9
	5	7	21·01 (±0·49)	0·95 (±0·12)	4
	5	8	21·24 (±0·73)	0·58 (±0·10)	2·4
	5	9	19·97 (±0·33)	0·32 (±0·08)	1·3
A/J	5	6	21·68 (±0·17)	0·14 (±0·07)	0·6
	5	7	21·86 (±0·17)	0·92 (±0·29)	4·2
	5	8	21·07 (±0·12)	0·83 (±0·11)	3·5
	5	9	21·46 (±0·05)	0·47 (±0·18)	2·1
DBA/2	4	6	21·08 (±0·77)	0·95 (±0·23)	4
	5	7	21·20 (±0·91)	0·57 (±0·21)	2·4
	5	8	19·25 (±1·14)	0·94 (±0·19)	3·7
	4	9	22·27 (±1·33)	0·35 (±0·07)	1·6

The second stage of the analysis was confined to mice that had received 1000 eggs but either as a single dose, as 2 doses or as 4 doses. The results are illustrated in Fig. 5. These show that under the split dose regime, the difference between the strains was preserved (3-way ANOVA, with infection regime, strain and time as factors on worm burdens in the lungs, model $R^2 = 0{\cdot}547$, main effect of strain, $F_{1,70} = 44{\cdot}7$, $P < 0{\cdot}001$), the 2 strains carrying significantly different worm burdens on different days after infection (2 way interaction strain * time, $F_{2,70} = 3{\cdot}1$, $P = 0{\cdot}05$), especially on day 6 rather than on day 7. Only in the single pulse infection was the peak accumulation of larvae in the lungs evident on day 7 in C57BL/6j mice. Nevertheless, the percentage difference between strains was 69% (500 × 2 dose group) and 76% (250 × 4) on day 6, which is a similar percentage difference when compared to the single inoculum groups. The 3 infection regimes clearly produced significantly different patterns of worm recovery overall (main effect of regime, $F_{1,70} = 12{\cdot}7$, $P < 0{\cdot}001$) and on different days after infection (2-way interaction regime * time, $F_{4,70} = 3{\cdot}17$, $P = 0{\cdot}019$). The 2 strains of mice behaved differently (2-way interaction strain * regime, $F_{2,70} = 3{\cdot}93$, $P = 0{\cdot}02$ and 3-way interaction, $F_{4,70} = 2{\cdot}8$, $P = 0{\cdot}032$) with worm burdens being more consistent over the 3-day period in CBA/Ca mice compared to C57BL/6j, where worm burdens were considerably higher on day 7 (single dose of 1000 eggs) or day 6 (the other two regimes) compared to day 8. There was also a highly significant main effect of time ($F_{2,70} = 6{\cdot}8$, $P = 0{\cdot}002$),

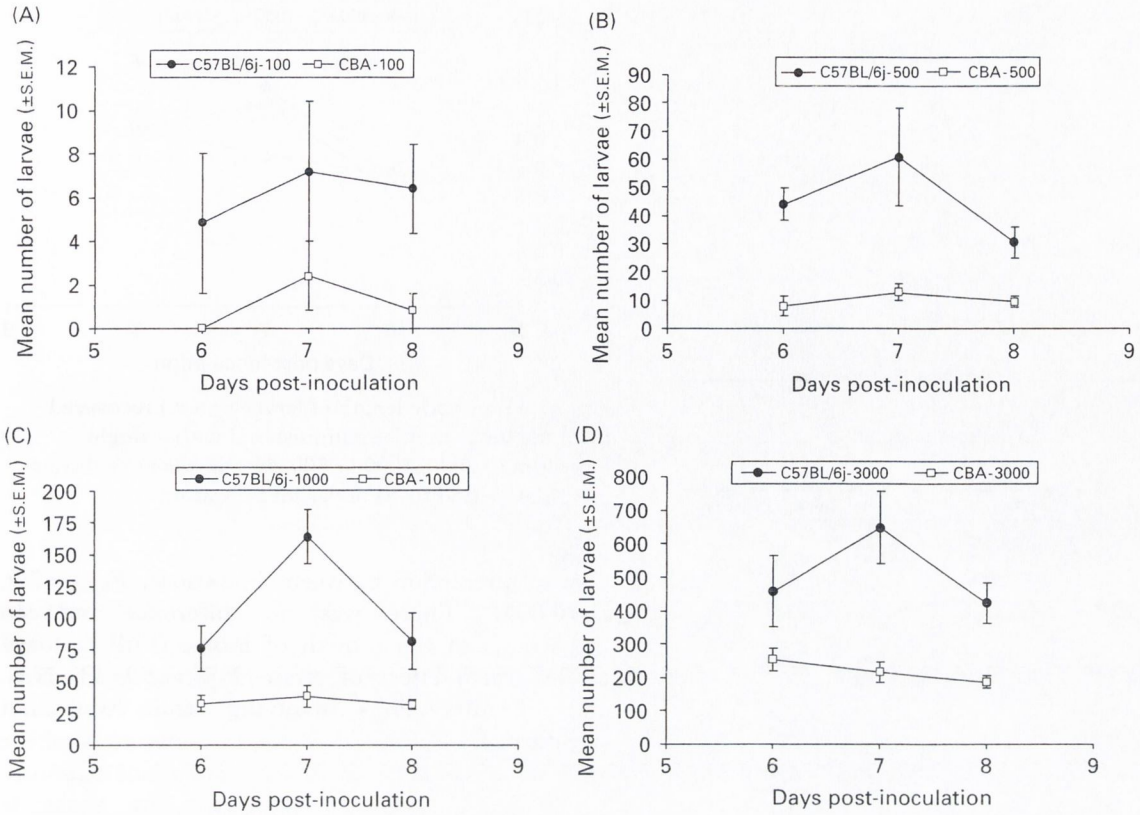


Fig. 3. The mean number (±S.E.M.) of larvae recovered from the lungs of both the C57BL/6j and CBA/Ca mice administered 1 of 4 single-pulse inoculations; (A) 100, (B) 500, (C) 1000 and (D) 3000 *Ascaris suum* ova on days 6–8 p.i.

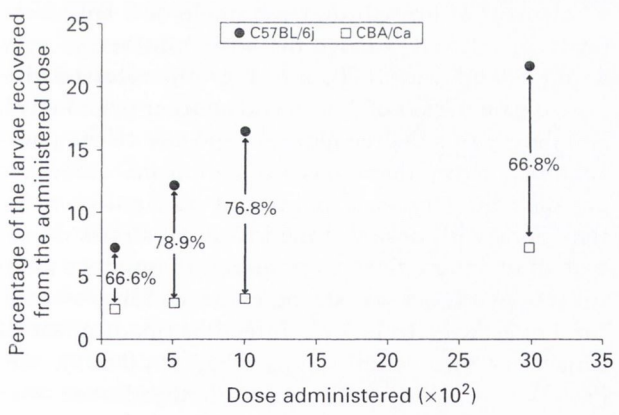


Fig. 4. The percentage of larvae recovered on day 7 p.i. after the initial inoculation doses of 100, 500, 1000 and 3000 from the lungs of the mouse strain C57BL/6j (●) and the strain CBA/Ca (□) and the percentage difference between the 2 strains. (Percentage difference calculated as: Total number of larvae recovered from C57BL/6j − Total number of larvae recovered from CBA/Ca/Total number of recovered from C57BL/6j).

with worm burdens generally declining across the 3-day period of the experiment.

The effect of strain, time and dose on growth of larvae

An analysis of the body length of larvae and the factors resulting in variation in growth were investigated. The 3 explanatory factors used to determine variation in larval length were: strain of mouse, dose of larvae and day after infection. The infection protocol comprised animals given single pulse infection and split dose infections and these were analysed separately.

Effect of increasing doses of eggs on the growth of larvae

The mean sizes of larvae recovered from the lungs of mice exposed to 100, 500, 1000 or 3000 eggs, reflecting growth over the 3-day period examined, are illustrated in Fig. 6. There were insufficient numbers of viable larvae recovered from the 100-dose group to assess growth confidently.

As expected, there was a highly significant increase in larval length during the three days that larvae were observed (initially 3-way ANOVA, with strain and time as factors and dose as covariate, full factorial model reduced to a minimum sufficient model (adjusted model $R^2 = 0{\cdot}329$) comprising only time, dose and the 2-way interaction between time and dose, main effect of time, $F_{2,185} = 27{\cdot}95$, $P \leqslant 0{\cdot}00$). The minimum individual larval length was observed on day 6 (474 μm) and the maximum length observed was on day 8 (1687 μm). Growth was influenced by dose of eggs administered (main effect of dose, $F_{1,185} = 17{\cdot}7$, $P \leqslant 0{\cdot}001$) reflecting density-dependent constraints on growth (Fig. 6), with faster growth in

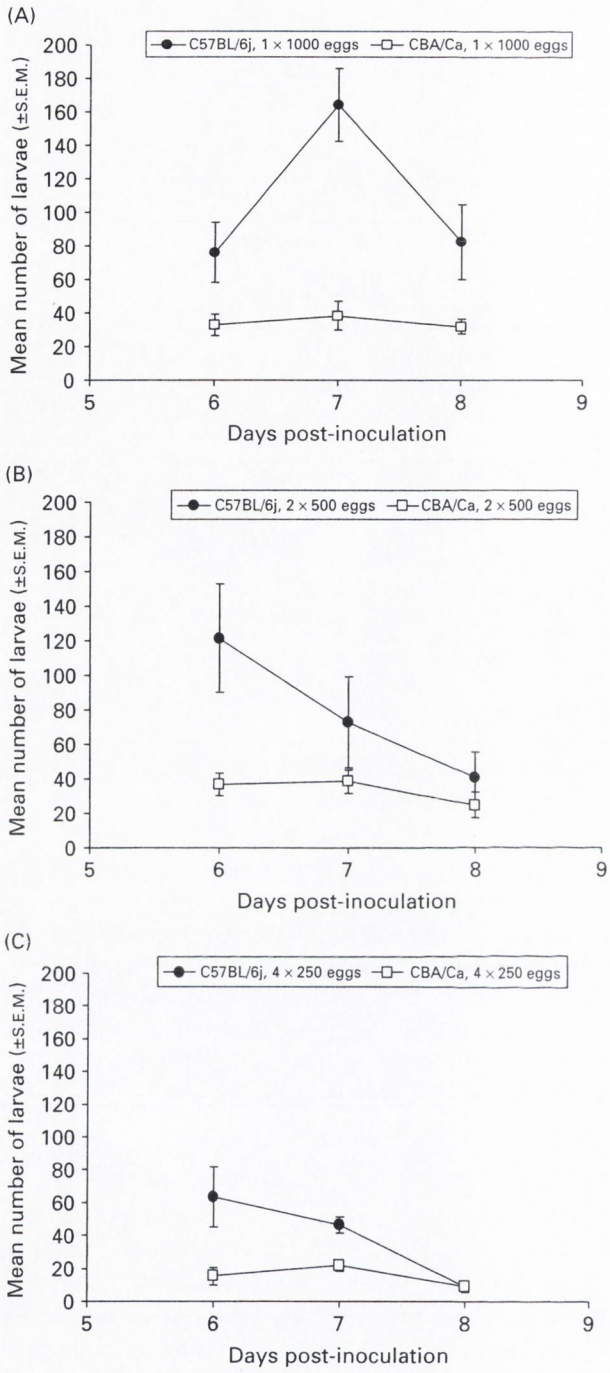


Fig. 5. The mean number (±S.E.) of larvae recovered from the lungs of both C57BL/6j and CBA/Ca mice administered with a single inoculum of 1000 ova (A) and those inoculated with a trickle dose of either 250 × 4 (B) or 500 × 2 (C) of *Ascaris suum* ova during the infection period (day 6–8 p.i.).

mice exposed to the lowest inoculum, and slowest in the mice given 3000 eggs. This is most evident by pattern of growth between day 6 and 7 after infection (Fig. 6). Larval length in the animals exposed to 1000 eggs was an intermediate length but was less consistent than in the other groups, larvae failing to increase in length between days 7 and 8

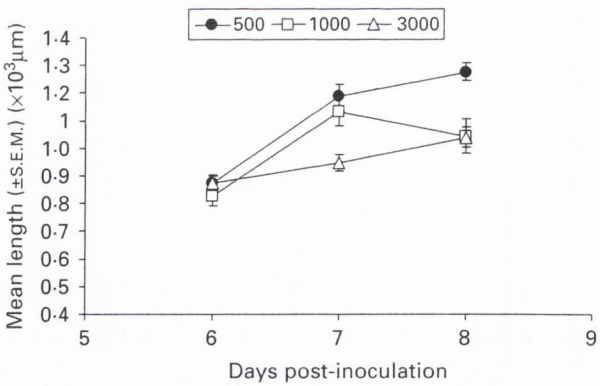


Fig. 6. Mean body length of larvae (±S.E.) recovered from the lungs of mice administered with a single inoculum of 3000, 1000 or 500 *Ascaris suum* ova during the 3-day period (6–8) under investigation.

(2-way interaction between time*dose, $F_{2,185}=7{\cdot}9$, $P=0{\cdot}001$). There was no difference between the strains in the growth of larvae (Full factorial model, main effect of strain $F_{1,179}=2{\cdot}9$, $P=$N.S. and all interactions involving strain were not significant).

Effect of a split-dose of 1000 eggs on the growth of larvae

The second component of this experiment was assessment of growth under a single or 2 split-dose protocols. In all 3 cases the total number of eggs administered was 1000, but 1 group received this dose over a period of 2 days and another over 4 days, simulating to a degree natural exposure to the parasite. As above, there was no significant difference between the strains of mice, and no significant interactions with dose or time involving strain. Strain and all its interactions were therefore removed from the model. There was strong evidence for growth of the larvae with time after infection (main effect of time on worm length $F_{2,146}=9{\cdot}5$, $P<0{\cdot}001$), and growth was clearly affected by whether larvae were given in one dose or split into several doses (main effect of dose regimen $F_{2,146}=3{\cdot}4$, $P=0{\cdot}037$). Initially on day 6, assuming that only the larvae from the first inoculum would have arrived in the lungs, a dose-dependent effect was apparent (Table 2), the longest larvae being from the mice infected with 4 × 250 larvae, but thereafter there was little growth in this group. Best growth was observed in mice exposed to 2 × 500 eggs. An LSD *post-hoc* analysis between the dose groups showed that the doses of 1000 and 500 × 2 were most different and that larval length differed significantly between day 6 and the 2 other post-inoculation days. Clearly the overall picture was not as clear-cut as in the mice exposed to single pulse infections because it was confounded by the arrival of larvae from the eggs given on days 2, 3 and 4.

Table 2. Mean larval length ± S.E. (*n*) on days 6, 7, and 8 post-inoculation in the lungs of the susceptible (C57BL/6j) and resistant (CBA/Ca) strains of mice inoculated with either 1000, 500 × 4 or 250 × 2 infective eggs of *Ascaris suum*

Dose	Day p.i.	C57BL/6j Length (μm) ± S.E. (*n*)	CBA/Ca Length (μm) ± S.E. (*n*)
1000	6	838 ± 27 (10)	818 ± 64 (12)
	7	1133 ± 66 (10)	1130 ± 92 (7)
	8	1088 ± 71 (12)	940 ± 135 (5)
500 × 2	6	918 ± 64 (17)	1092 ± 66 (9)
	7	1203 ± 64 (8)	1090 ± 97 (8)
	8	1240 ± 51 (9)	1244 ± 158 (6)
250 × 4	6	1093 ± 91 (12)	909 ± 10 (2)
	7	1086 ± 70 (12)	1111 ± 143 (6)
	8	1197 ± 267 (3)	872 ± 66 (3)

DISCUSSION

This study provides strong evidence that inbred strains of mice differ markedly in their susceptibility to early *A. suum* infection as measured by larval burdens in the lungs on day 7 post-infection. Although there was some disparity in the movement of larvae into the lungs of a number of strains (BALB/c, DBA/2), this variation can be accounted for in part by the smaller number of larvae recovered in these strains compared with the most susceptible C57BL/6j mice and the associated variation generating apparent changes in the timing of migration. However, this does not impact on the significance of the analysis. The maximum accumulation in the susceptible strain C57BL/6j was consistently observed on day 7 and as such this day post-infection is suggested as the best day for assessing differences in susceptibility between strains of mice. However, it is recommended that larval migration is observed on days 6–8 also, to ensure that peaks of accumulation either side of day 7 are not missed when comparing new strains of mice. C57BL/6j were uniquely the most susceptible strain, exhibiting consistency in larval burden in the lung over 3 experiments and maintaining their susceptibility at lower and higher doses of infective ova. Other inbred strains fell into 2 categories – the intermediate susceptible group and the resistant group. CBA/Ca were selected as the most resistant strain based upon the similarity in the kinetics of their migration, with the susceptible strain, over the 4-day period of assessment and the highly significant difference in larval numbers between the two strains on day 7. Crucially, the differences in the larval burden were not attributable to host body weight (a possible confounding factor), since heavier mice were not more susceptible to increased larval burdens.

Earlier work by Mitchell *et al.* (1976) demonstrated variability in susceptibility to *Ascaris* in the lungs of inbred strains of mice but variations in infectivity of batches of eggs and doses resulted in lack of comparability between groups. For example, the average recovery of larvae from the lungs of C57BL/6j varied from 14·5% to 0·73% in 2 separate experiments with varying dosage administered. Johnstone, Leventhal and Soulsby (1978) reported large variation in the number of larvae recovered from the lungs of C57BL/6j mice as a result of using 2 different protocols for the recovery of larvae (8·2% to 5·3% between 2 procedures). However, the larval burdens observed here in the BALB/c mice are comparable to the recoveries achieved by Slotved *et al.* (1997) with higher percentages of the initial inoculum recovered (4·3% (Slotved *et al.* 1997) compared to 7·3% and 6·6% in the current experiments). It is pertinent that their recoveries of worms do not correspond to the observations of Mitchell *et al.* (1976) who concluded that BALB/c were the most resistant strain, with a mean recovery of 7 (±1) or 0·04% of the administered dose. The methodology employed in the present study with resultant consistency between separate experiments in worm recoveries from uniformly treated mice, and consistent between-strain variation as well as a significantly higher proportion of larvae being recovered compared to previous studies (Johnstone *et al.* 1978; Slotved *et al.* 1997, 1998), all represent marked improvements on earlier published studies.

The dose did not influence the relative susceptibility of the 2 strains. Therefore it can be assumed that there are underlying intrinsic host factors resulting in the observed phenotypes, as the difference in the migrating larval population between the 2 strains remained constant irrespective of the initial dose size. Within strains there was a correlation between the inoculum size and the mean number of larvae recovered. There was also a positive density-dependent relationship between dose and the percentage of the initial inoculum that was recovered, as

percentage larval recovery increased with increasing inoculum size. This pattern was observed in both the susceptible and resistant strains of mice, and is consistent with observations by Johnstone *et al.* (1978) on *A. suum* in the lungs of C57BL/6 mice, but in other host-parasite systems it is more usual to observe a reduction in percentage establishment with increasing parasite density (see for example Norozian-Amiri and Behnke, 1993 and below for further discussion).

In most cases, the protective immune response against helminths residing in the intestinal tract is a Th2 response. When immune responses are effective at eliminating tissue stages of these parasites, there is abundant evidence that this can be orchestrated by the Th1 arm of the immune system (Mulcahy *et al.* 2005). A common genetic variant involved in Th-2 immune signalling, STAT6 (signal transducers and activators of transcription), a mediator of Th2 responses in helminth infections, predicts increased resistance to adult *A. lumbricoides* infections (Peisong *et al.* 2004). Such Th2 responses result in increased mucosal eosinophil and mast cell activity, mucus hypersecretion and muscle hyperactivity in the gut and they represent conditions favourable for the expulsion of helminths. In contrast the opposing effect of STAT6 has been observed in relation to susceptibility to larval stage of *Taenia crassiceps*. STAT6 deficiency is known to impair IL-4 signalling and thus the ability to generate a Th2 response. Wild type BALB/c mice developed a strong Th-2 like response and remained susceptible to *T. crassiceps* larval stages. However, similarly infected STAT6$^{-/-}$ mice mounted a strong Th-1 like response and controlled infection (Rodriguez-Sosa *et al.* 2002). This variation in Th1 and Th2 responses may play a role in variations in susceptibility to early-stage *A. suum* infection between mouse strains. Individuals that naturally upregulate the Th2 response during larval migration may cause suppression of a Th1 immune response and thus show an increased susceptibility to infection. Furthermore there is the possibility that increased administration of infective ova, and thus successfully migrating larvae, resulted in greater preferential stimulation of the Th2 response, aiding parasite evasion of host defences or increasing their survival by further down-regulation of the Th1 parasite specific responses, especially in the susceptible mouse strain.

Larval burdens that established in the lungs as the result of an inoculation divided over time (split doses) exhibited lower numbers compared to an equivalent single dose and this was particularly pronounced in the C57BL/6j mice. The underlying reasons may relate to a differential immune response to gradual inoculation or the influence of stress due to increased handling. Some evidence for a differential immune response is provided by the reduced larval burden in the 1000 ova dose administered over 4 days in contrast to the dose administered over a period of 2 days and the single pulse administration of 1000 eggs. Mice have been shown to be resistant to an *Ascaris* challenge infection shortly after primary inoculation or when repeatedly inoculated at short intervals (Mitchell *et al.* 1976; Eriksen, 1981). Increased cellular response in the liver of re-inoculated abnormal hosts resulted in increased encapsulation of larvae (Sprent and Chen, 1949; Taffs, 1965). Thus mice exposed to *A. suum* ova over a period of time would be expected to have an increased reaction to larvae in the early stages of migration and this would be observed as fewer larvae successfully penetrating the lungs during the latter stages. Overall, it was concluded that the 500 or 1000 single doses represented the optimum dose range for further experiments exploring the basis of the differential susceptibility between the 2 selected strains without generating unnecessarily heavy worm burdens in the mice and placing undue stress upon the mice.

Larval length was not influenced by strain suggesting that the environment within both strains was equally suitable for the development of the worms and that the observed differences in relative susceptibility are unlikely to be attributable to differences in larval fitness but rather to innate barriers to migration or differences in the early phase of the host response to the parasite. Johnstone *et al.* (1978) demonstrated that in immunised mice, resistance affected the stage to which *A. suum* larvae develop and also the length to which they grow. An expected observation was the increase in larval length over the three days post-inoculation examined (Douvres and Tromba, 1971; Johnstone *et al.* 1978). There was a correlation between dose administered and larval lengths, lower doses resulting in an increase in the average larval length, especially on day 7 p.i. The mean larval length was consistently higher among worms recovered from mice exposed to 500 eggs, indicating that dose exerts an important influence on the growth of worms. The observation that as dose increased, more worms were successful in completing the migration to the lungs, even though they did not grow as well as when fewer were present, suggests that there may be an active host component that underlies the difference between strains. One possibility is that with increasing worm burden damage (e.g. lesions in the gut mucosa, liver, blood vessels) to host tissues provided greater opportunities for onward migration for relatively more worms, and at the same time stimulated greater innate resistance which impeded worm development, and hence resulted in slower growth.

Perhaps the most intriguing question to emerge from these experiments is why C57BL/6 mice are so much more susceptible than other strains of mice. The C57BL/6 strain has also been described as susceptible to *Brachylaima cribbi*, *Plasmodium*

berghei, *Strongyloides ratti*, and *Toxoplasma gondii* (Dawkins *et al.* 1980; Butcher, Palethorpe and Grove, 2002; de Souza and Riley, 2002; Lee and Kasper, 2004). It is known that the ancestors of C57BL/6 (and also C57BL10 and C58BL) diverged from the lineage that gave rise to other laboratory strains early on during domestication and selection for traits for laboratory work (Beck *et al.* 2000). These 3 strains form a clade of their own. Equally interesting are recent data that indicate that C57BL/6j mice do not express the gene for intelectin-2, which is believed to play an important role in resistance to GI nematodes such as *Trichinella spiralis* (Pemberton *et al.* 2004). The authors suggest that the presence of intelectin-2 in resistant BALB/c mice, its absence from susceptible C57BL/10 strain and the kinetics of its up-regulation during *T. spiralis* infection are all consistent with this novel lectin serving a protective role in the innate immune response to parasite infection.

At this stage, we cannot be certain about the mechanism underlying the greater susceptibility of C57BL/6j mice to infection with *Ascaris* but it is likely to be genetic and to consist of innate or molecular/structural/physiological differences between the strains with one set of alleles enabling *Ascaris* to migrate in large numbers and the other impeding this migration. The early variation in the larval burdens observed in the liver between C57BL/6j and BALB/c in experiment one and the differences in the timing of the accumulation in this organ suggest that larvae may be lost early in infection. One possibility is a difference in larval penetration of the large intestine between the two strains (Slotved *et al.* 1998). Mouse strains possibly vary in the activation signals that they provide for embryonated eggs to hatch, and eggs may pass through the intestine without hatching. Equally it is conceivable that more larvae are entrapped in the livers of the resistant strain, or that their onward migration from the liver is less successful than in the susceptible strain. Finally it is possible that on arriving in the lungs more larvae fail to establish in resistant mice, or are expelled possibly through the bronchial airways by coughing.

We are now confident that our experimental procedures can yield consistency in *Ascaris* larval lung burdens between 2 inbred strains of mice. This model system will enable exploration of the mechanisms underlying the observed susceptibility and resistance to early *Ascaris* infection, an aspect of the host-parasite relationship that has proved very difficult to investigate in human and porcine hosts for financial, logistical and ethical reasons. Furthermore, successful identification of the gene or genes underlying the different (resistance/susceptibility) phenotypes of CBA/Ca and C57BL/6j might provide an avenue for finding homologous genes in pigs and humans in the future.

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