A Murine Model for Cerebral Toxocariasis: Characterisation of Susceptibility, Behaviour and Immune Response

Clare M. Hamilton
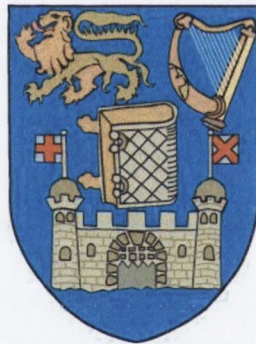
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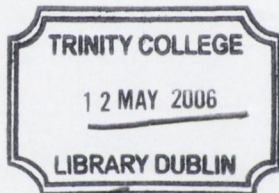
DECLARATION

I hereby declare that this thesis, submitted to the University of Dublin, Trinity College, is entirely my own work, unless otherwise stated, and that it has not been submitted previously to this or any other university. I also give my permission to the library to lend or photocopy this thesis on request.

Clare M. Hamilton

SUMMARY

Toxocara canis, the parasitic roundworm of dogs, can infect a number of paratenic hosts, such as mice and humans, due to the widespread dissemination of its ova in the environment (Glickman and Schantz, 1981). In the murine host, larvae have been shown to exhibit a predilection for the central nervous system, resulting in an increasing number of parasites in the brain as infection progresses (Sprent, 1955; Dunsmore *et al*, 1983; Skerret and Holland, 1997). In previous research, cerebral larval burdens have been correlated with various alterations in murine behaviour (Burright *et al*, 1982; Dolinsky *et al*, 1985; Cox and Holland, 1998; 2001a,b), and burdens have been shown to vary between individual outbred mice receiving the same inocula (Cox and Holland, 2001a; Skerret and Holland, 1997), suggesting a role of immunity and host genetics in the establishment of cerebral infection. Though *T. canis* has been shown to induce a Th2 systemic immune response (Del Prete *et al*, 1991; Buijs *et al*, 1994; Cuéllar *et al*, 2001), the cerebral immune response has received no attention. The thesis presented here represents an investigation of the differential brain involvement of *T. canis* in mice of contrasting genetic background – with characterisation of the humoral and cerebral immune response in two inbred strains of mice, and the simultaneous assessment of cerebral larval accumulation and behavioural alterations.

In order to identify a susceptible and resistant strain of mice, seven inbred strains were infected with *T. canis*, and sacrificed on days 7, 14, 35 and 42 post-infection. Cerebral larval burdens on all days post-infection were recorded, and results revealed that BALB/c mice carried a significantly higher larval burden in the brain compared with NIH mice, on days 7, 35 and 42 post-infection. These strains were therefore chosen as susceptible (BALB/c) and resistant (NIH) to cerebral toxocariasis. The choice of strains was supported by results of previous studies (Abo-Shehada and Herbert, 1989; Bardón *et al*, 1994; Epe *et al*, 1994). The two strains of mice were infected with *T. canis*, and larvae recovered from the brain and visceral organs on days 3, 7, 14, 35, 42 and 97 post-infection. Cerebral larval burdens in both strains differed to the first experiment, and the strains no longer appeared divergent. However, when larval burdens were expressed as a percentage of the total number of larvae recovered,

BALB/c mice had a significantly lower cerebral burden than NIH mice, on days 14, 35 and 97 post-infection, suggesting that these remained more susceptible.

Behavioural testing took place between days 35 and 42 post-infection, and involved the assessment of baseline activity in the homecage, exploratory behaviour and response to novelty using a 'T'-maze, and learning and memory using a water-finding task. The most striking result was that infected BALB/c mice took significantly longer to drink from a water-source (following a period of deprivation) than control mice, indicating a degree of memory impairment. This result correlated with the significantly higher cerebral larval burdens observed in these mice, suggesting that higher larval burdens may lead to behavioural alterations.

Analysis of sera by ELISA revealed a dominant Th2 humoral immune response in both strains of mice, with the production of IgG1 and IgE. Low levels of IgG2a in BALB/c mice, however, indicated Th1-cell activation. Significantly higher levels of IgE in NIH mice suggested a role for this antibody in parasite control, since these mice had significantly lower cerebral larval burdens. Relative quantification of cytokines in the brain using real time-PCR revealed a mixed Th1/Th2 cerebral immune response to *T. canis* infection in both strains of mice, with up-regulation of IL-5, IL-10, IFN-γ and iNOS, relative to control mice. BALB/c mice displayed significantly higher levels of all cytokines and iNOS, compared with NIH mice, on days 35 and 42 post-infection, suggesting a role of immunopathology in the observed behavioural alterations.

Overall, *T. canis* infection led to larval accumulation in the brains of both BALB/c and NIH mice, with significantly higher burdens correlating with behavioural alterations, in particular, memory impairment. A dominant Th2 humoral immune response had little control over migrating larvae, although there may be a role for IgE. The mixed Th1/Th2 cerebral immune response appeared to do little to control cerebral larval establishment, and instead may be linked with pathology and the induction of behavioural alterations.

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1.1 INTRODUCTION

Toxocara canis, the parasitic roundworm of dogs, can infect a number of paratenic hosts, such as mice and humans, due to the widespread dissemination of its ova in the environment (Glickman and Schantz, 1981). Following ingestion of infective eggs, second-stage larvae hatch in the intestine, and undergo a somatic migration through various organs of the body, though they do not develop to adulthood in the intestine. Toxocariasis may manifest as several clinical syndromes, involving visceral organ invasion and ocular disease (Beaver, 1952; Wilder, 1950; Taylor *et al*, 1987, Good *et al*, 2004). A phenomenon of ecological significance in mice, and of potential public health significance in man, is that larvae exhibit a predilection for the central nervous system, resulting in an increasing number of parasites in the brain as infection progresses (Sprent, 1955; Lee, 1960; Dunsmore *et al*, 1983; Skerrett and Holland, 1997). Smith (1991) reported that the migration pathway of larvae in humans and mice is very similar, and that lesions elicited in experimental mouse models and humans are also comparable. Due to these parallels, the mouse model has been widely used to study toxocariasis. Furthermore, rodents caught in the wild have been shown to harbour *Toxocara* larvae in their tissues and so have been hypothesised to contribute to parasite transmission if consumed by an appropriate definitive host (Dubinsky *et al*, 1995).

The mouse model has been used to study the impact of *Toxocara* infection on different aspects of murine behaviour, including baseline activity, social behaviour, and learning and memory. In general, infected mice appear less active and explorative, less responsive to novelty, and less aversive to open areas and predator stimuli than uninfected animals (Burright *et al*, 1982; Dolinsky *et al*, 1981; Cox and Holland, 1998; 2001a,b). The extent of some behavioural changes could be correlated with larval burdens in the brain, although the magnitude of the changes did not always attain statistical significance (Cox and Holland 1998; 2001a,b).

Previous studies have reported variation in the number of *T. canis* larvae recovered from the brains of both inbred and outbred mice (Cox and Holland, 2001; Skerrett and Holland, 1997; Epe *et al*, 1994), suggesting a role of immunity and host genetics in the

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establishment of cerebral infection. During migration, *T. canis* larvae release excretory/secretory (E/S) antigens, and shed surface components stimulating an inflammatory response (Buijs *et al*, 1994). *T. canis* infection has been reported to induce a Th2 systemic immune response, with production of the antibodies IgG1 and IgE, and the cytokines IL-4, IL-5 and IL-10 (Del Prete *et al*, 1991; Wang *et al*, 1995; Kuroda *et al*, 2001). The immune response against *T. canis* larvae in the brain, however, has received much less attention, with previous studies only reporting the lack of cellular reaction surrounding larvae in the brain (Sprent, 1955; Dunsmore *et al*, 1983; Epe *et al*, 1994).

In light of the above studies, it was decided to develop a murine model for cerebral toxocariasis, with the specific aims of characterising susceptibility and resistance, and the immune and behavioural responses. By using inbred mice it was hoped that any infection-induced behavioural alterations that could be potentially masked in heterogeneous outbred mice, may appear more pronounced in these strains. Also, since there can be great heterogeneity between inbred strains, their use allows for the comparison and analysis of a number of parameters of infection in completely divergent strains. The aims of this study, therefore, were:

❖ To investigate the differential brain involvement of *T. canis* in mice of contrasting genetic background, by infecting a range of inbred mouse strains, and selecting a strain susceptible and resistant to larval establishment in the brain.

❖ To compare the susceptible and resistant strain of mice in terms of:
  - larval accumulation in the brain, and course of larval migration.
  - behavioural alterations, including baseline activity, exploratory behaviour, response to novelty, and learning and memory.
  - humoral and cerebral immune responses, using ELISAs and real time PCR.

1.2 LITERATURE REVIEW

1.2.1 History of *Toxocara canis*

Toxocara canis, the intestinal roundworm of dogs, was first described by Werner (1782), and, along with *Toxocara cati* (Schrank, 1788), is one of the main species of the *Toxocara* genus recognized as a causative agent of human disease. When *T. canis* was first described, it was thought to be a parasite of dogs only. However, over a century later, Ransom and Foster (1920) recovered *T. canis* larvae from the lungs of rats infected with embryonated eggs, and confirmed that the parasite was capable of infecting hosts other than dogs. In a comprehensive study on the migratory behaviour of larvae of various *Ascaris* species in mice, Sprent (1952) described the presence of *T. canis* larvae in the intestines, liver, lungs, kidneys and skeletal tissue following infection. A number of later studies demonstrated the ability of *T. canis* to establish infection in many mammalian and bird species (reviewed in Levine, 1980). In the early 1920s and 1930s it was suspected that *Toxocara* spp. larvae were infective to humans (Fülleborn, 1921; Schwartz, 1932, cited in Taylor and Holland, 2001), but diagnosis of a clinical syndrome attributed to infection with ascarid larvae was not made until 20 years later (Perlingiero and Gyorgy, 1947; Mercer *et al*, 1950; Behrer, 1951). In these reports, however, the infective agent was proposed to be *Ascaris lumbricoides*, but this was disputed by Beaver and colleagues who suggested *T. canis* as a more likely causative agent. Beaver *et al* (1952) went on to describe the presence of *T. canis* larvae in liver biopsy specimens taken from 3 infected humans, confirming the zoonotic potential of this parasite. The disease caused by migrating *T. canis* larvae is known as toxocariasis, and there are 3 main clinical syndromes associated with human infection: (1) visceral larva migrans, (2) ocular larva migrans and (3) covert toxocariasis.

Visceral larva migrans was originally described by Beaver *et al* (1952), as mentioned above, and refers to the migration of larvae through the somatic tissues, and the associated pathogenesis. This syndrome is mainly diagnosed in children between 1 and 7 years of age (mean age of 2 years), and is characterised by persistent eosinophilia, leukocytosis and hypergammaglobulinemia (Synder, 1961; Huntley *et al*, 1965). Clinical symptoms generally include malaise, fever, abdominal pains (associated with

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hepatomegaly), wheezing or coughing. Pulmonary involvement, which may appear as acute bronchiolitis, asthma or pneumonitis, is common, but severe respiratory distress is rare (Glickman and Schantz, 1981). Chronic urticaria (irritating skin condition characterised by hives on the skin) has also been strongly associated with *Toxocara* infection in children and adults, and is suggested to be caused by the shedding of larval excretory/secretory antigens (Wolfrom *et al*, 1995).

The second clinical syndrome, ocular larva migrans (OLM), was first described by Wilder (1950), who reviewed children's eyes enucleated because of endophthalmitis and/ or suspected retinoblastoma. In 23 of 46 eyes examined, *T. canis* larvae, or the capsular remnants of larvae, were identified, and in many eyes, a granulomatous inflammation characterised by numerous eosinophils was noted. OLM results from the migration of as few as a single larva in the eye, and is characterised by loss of visual acuity, squint and 'seeing lights' (Overgaauw, 1997). In severe cases, total blindness in one or both eyes can occur, but this is rare (Girdwood, 1986). The mean age of patients with OLM is 8 years, but it occurs in adults also (Schantz, 1989). The syndrome is thought to result from infection with a low number of *T. canis* eggs. The resulting low numbers of migrating larvae are thought to be insufficient to stimulate a strong immune response, and thus can persist in the tissues for as much as 10 years, periodically resuming migration (Overgaauw, 1997). This longer 'incubation period' has been suggested as a possible explanation for the higher mean age of patients, in comparison to those with VLM. Time taken from the onset of clinical signs to a definitive diagnosis of OLM and VLM has been reported as 22.6 and 5.6 months, respectively (Overgaauw, 1997).

The third and most recently described clinical syndrome of human toxocariasis is known as covert toxocariasis, and was first put forward by Taylor *et al* (1987) to describe a series of non-specific symptoms which did not fall within the categories of VLM or OLM, yet were recognisable as *Toxocara* infection. That such a disease might exist had previously been suggested by Bass *et al* (1983). Symptoms such as hepatomegaly, splenomegaly, coughing, sleep disturbances, abdominal pains, anorexia, nausea, headaches and behavioural changes were associated with raised *Toxocara* antibodies, particularly in children beyond the toddler stage (Taylor *et al*, 1987). Glickman *et al* (1987) described similar clinical symptoms in a group of French adults,

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including weakness, pruritus (skin itch), rash, difficult breathing, abdominal pain, and allergic manifestations such as eosinophilia and increased IgE levels. Taylor *et al* (1988) reported covert toxocariasis to be more common in a study of 137 people (from 30 families), than VLM or OLM, and suggested that the diagnosis of toxocariasis should be considered in children presenting with cough, wheeze and with a history of headache and abdominal pain.

1.2.2 Taxonomy, morphology and life cycle of *Toxocara canis*

Toxocara spp. are roundworms belonging to the Phylum Nematoda, Class Rhabditea, Order Ascaridida, Family Ascarididae and Genus *Toxocara* (Schmidt and Roberts, 1996). Belonging to the order Ascaridida, these intestinal worms are among the largest nematodes, with some species reaching lengths of 18 inches and more. Their characteristics include a prominent 3-lipped mouth, and a bulbed or cylindrical pharynx. Female worms have an elongated vagina, and males generally have a ventrally curled tail and two spicules (Schmidt and Roberts, 1996).

T. canis adult worms reside in the proximal small intestine of dogs, where female worms measure 6-18cm, and males 4-10cm (Glickman and Schantz, 1981). Adult worms can live for as long as 4 months, although nearly all worms are expelled from the intestine before the host reaches 6 months of age (Douglas *et al*, 1965, cited by Glickman and Schantz, 1981). Individual female worms are known to produce as many as 200,000 eggs per day, leading to widespread environmental contamination when they are passed in the faeces of infected animals – particularly since intestinal worm burdens can range from one to several hundred (Douglas *et al*, 1965, cited by Glickman and Schantz, 1981). When eggs are passed in the faeces, they are unembryonated and, therefore, not infective. The rate of embryonation depends on the temperature and humidity of the environment. At temperatures of 25-30°C, and humidity levels of above 85%, *T. canis* eggs will become infective within 9-15 days (Parsons, 1987). At lower temperatures (16.5°C), embryonation takes longer, and eggs become infective after 35 days. Due to their characteristic thick shells, *T. canis* eggs are long-lived, and resistant to many environmental conditions. They have been shown to remain viable in temperatures as low as -11.5 (Velichkin and Radun, 1975, cited in Lloyd, 1993), and can survive composting for at least one year (Pegg and Donald, 1978, cited in Lloyd,

1993). The infective second stage (L$_2$) larvae of *T. canis* are approximately 400µm in length and 20µm in width (Plate 1.1).

Dogs and other canids can become infected via several routes of transmission: (1) ingestion of infective eggs from the environment, (2) ingestion of larvae in the tissues of infected paratenic hosts, (3) transplacental migration of larvae from an infected bitch to her unborn foetuses, (4) transmammary transmission of larvae in the milk of an infected lactating bitch to her nursing pups, and (5) ingestion of late stage larvae or adult worms in the vomit or faeces of infected pups (Glickman and Schantz, 1981). Once infected, the life cycle of *T. canis* within the canine host is quite complex and involves 2 different routes of migration, generally dependent on the age of the host. The life cycle of *T. canis* was first described by Sprent (1958), but the following description is adapted from Glickman and Schantz (1981) (Figure 1.1).

Following ingestion of infective eggs, second-stage (L$_2$) larvae hatch in the stomach and small intestine of the dog, and invade the intestinal mucosa. They enter lymph and blood vessels, and most reach the liver within 24 hours. They then migrate to the heart and lungs through the vascular system, with peak numbers in the lungs being observed on days 3-5 post-infection. From the lungs, the larvae can follow 2 routes of migration, dependent on the age of the host: (1) generally, in dogs under the age of 5 weeks, larvae pass through the bronchioles to the trachea and pharynx where they are then swallowed. These larvae undergo further moults and develop into adult worms in the small intestine. Female worms deposit eggs, which pass out in the faeces 4-5 weeks post-infection; (2) in dogs older than 5 weeks of age, larvae in the lungs penetrate the pulmonary vein and are carried to the heart, where they are distributed throughout the body by the systemic circulation. Larvae penetrate small blood vessels, and invade various somatic tissues, primarily the lungs, kidneys, liver and muscles, where they can remain dormant for years. Larvae can also invade the central nervous system, but this is rare. If the dog is pregnant, puppies can become infected either transplacentally, or via transmammary transmission. Transplacental migration of larvae occurs in the last trimester of pregnancy and is thought to be attributed to hormonal changes in the pregnant bitch. At parturition, larvae migrate from the liver of infected pups to the lungs and trachea, where they are swallowed to reach adulthood in the intestine. Eggs appear in the faeces of the puppies 4 weeks after birth. Transmammary transmission

can occur soon after birth, and peaks during the second week of lactation. Larvae ingested this way undergo development without tracheal migration. Both transplacental and transmammary transmission ensures that almost all dogs are infected around the time of birth. Patent infection can also develop in the pregnant bitch, resulting in the excretion of eggs and infection of newborn puppies. Older dogs may also develop patent infection when infected paratenic hosts, such as mice, are consumed (Sprent, 1958).

The lack of patent infection in dogs older than 5 weeks of age is not an 'all or nothing' phenomenon but rather a gradual decrease in the recovery rate of adult worms as the age of the dog increases (Oshima, 1976). The phenomenon of 'age resistance' is thought to be due to acquired immunity as the dog matures, with the lungs playing an important role (Glickman *et al*, 1981; Barriga, 1988).

Plate 1.1: Second stage infective *Toxocara canis* larvae (x400 magnification).
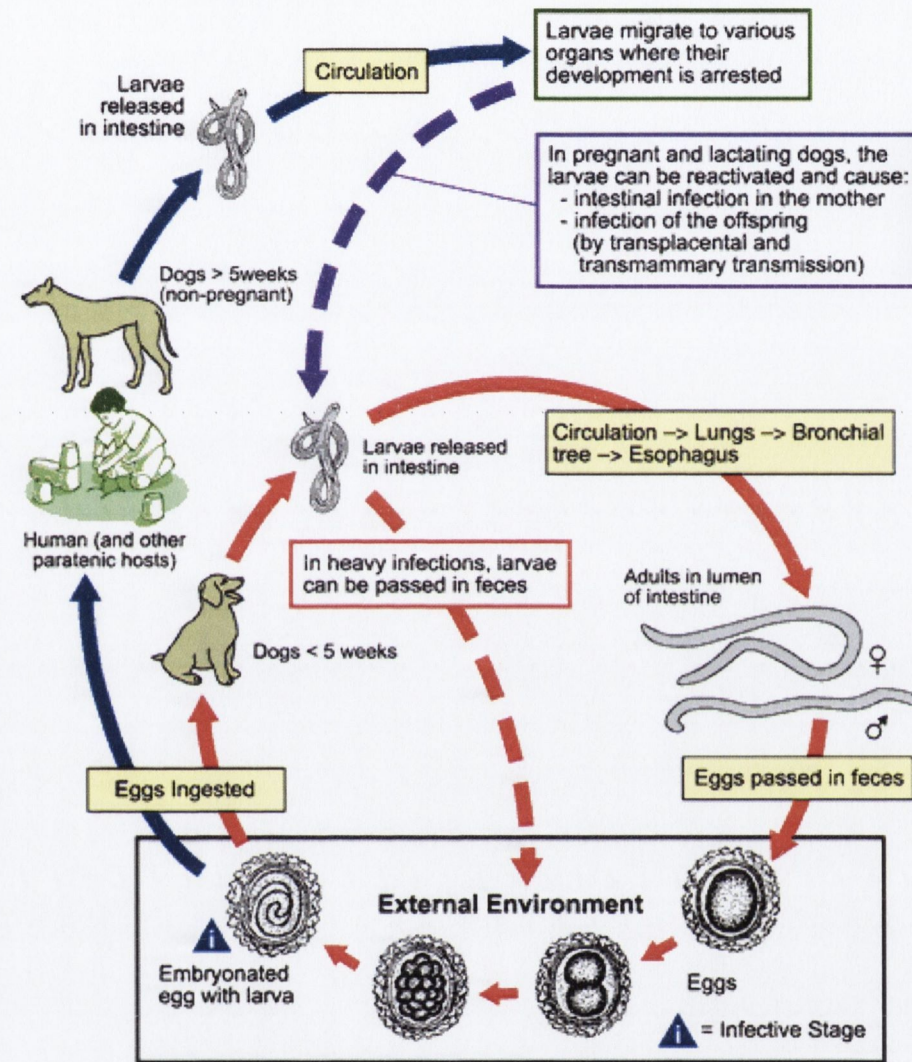
Figure 1.1: Life cycle of *Toxocara canis*. Taken from the Centers for Disease Control and Prevention, Division of Parasitic Diseases, website (http://www.dpd.cdc.gov/DPDx/HTML/Toxocariasis.htm).

1.2.3 *Toxocara canis* infection in the paratenic host

Due to the widespread dissemination of *T. canis* eggs in the environment, many non-canid accidental, or paratenic hosts, can become infected. Known paratenic hosts of *T. canis* include mice, rats, chickens, pigeons, earthworms, lambs, pigs and, most significantly, humans (Glickman and Schantz, 1981). In these abnormal hosts, second-stage larvae hatch from the eggs, and follow a similar route of migration to that seen in older dogs – with migration to the visceral organs, and failure to develop and reach maturity as adults in the intestine.

1.2.3.1 Human infection

As mentioned above, Beaver *et al* (1952) were the first to confirm toxocariasis in humans when they identified *T. canis* larvae in liver biopsy specimens. Humans can become infected with *T. canis* through the ingestion of eggs directly from the soil, or indirectly through soil-contaminated hands or food (Glickman and Schantz, 1981; Glickman and Shofer, 1987). Pica (the ingestion of non-food substances) occurs in around 25% of children aged between 1 and 6 years old, and often more than one type of non-food substance is ingested (Cooper, 1957, cited by Dolinksy, 1981). Geophagia refers specifically to the ingestion of earth, and is classically associated with *T. canis* infection in children (Glickman, 1993; Holland *et al*, 1995). Studies of the prevalence of *T. canis* eggs in the soil from public parks and private gardens have revealed varying results, ranging from 6-66% (Thompson *et al*, 1986; Snow *et al*, 1987; Abo-Shehada, 1989; Gillespie, 1991). The range in prevalence could be influenced by a number of factors, such as the rate of dog ownership, the number of stray dogs, and the number of dogs unwormed. In Dublin, 38% of samples taken from private gardens were contaminated with *T. canis* eggs, compared to 6% of samples taken from public parks (Holland *et al*, 1991). It was suggested that the higher prevalence of contamination in gardens may reflect a tendency in Irish people to let dogs out unaccompanied for exercise rather than to take them for walks in the park (Taylor, 1993). The high prevalence of *T. canis* ova in private gardens is a potentially significant source of infection for children – especially when previous studies have shown that 40% of patients with OLM had a history of pica (Schantz *et al*, 1979).

Another source of infection for humans is through the ingestion of larvae in undercooked organ and muscle tissue of infected animals, such as chicken, pigs, snails and sheep (Nagakuru *et al*, 1989; Taira *et al*, 2004; Romeu *et al*, 1991; Salem and Schantz, 1992). A more significant potential source of transmission has come to light recently. In a preliminary study, Woolf and Wright (2003) reported the presence of *T. canis* eggs in fur samples taken from 60 dogs. Of those eggs recovered, 4.2% were embryonated and 23.9% were embryonating, indicating the potential for a direct route of transmission from dogs to man.

Once humans are infected, *T. canis* larvae hatch in the stomach, migrate into the mucosa of the proximal small intestine, and migrate to the liver via the portal circulation (Glickman and Schantz, 1981). From the liver, they migrate to the lungs and heart, and are then carried throughout the body in the systemic circulation. When the diameter of the larvae is larger than that of the blood vessel, their migration is impeded, and they actively bore through the wall of the blood vessel, invading the surrounding tissue (Glickman and Schantz, 1981). Larvae migrate extensively throughout the body, and have been recovered from the liver, lungs, heart and, more significantly, the brain (Dent *et al*, 1956).

Due to the arrested development of larvae within the human host, infection can only be assessed indirectly by means of serology (Holland *et al*, 1995). Interpretation of seroprevalence studies, however, is hampered by the variation in cut-off titres used in different studies, and the difficulty in interpreting the relationship between antibody titres and levels of infection, since seropositivity does not necessarily equate to disease. Based on results of existing seroprevalence studies, it is apparent that *T. canis* infection in humans varies throughout the world. The highest seroprevalence has been recorded in St Lucia, where 86% of 82 children aged 0.5-6 years had antibody titres to *T. canis* of $\geq$ 1:32 (Thompson *et al*, 1986). In a study of 78 Irish children (0-15 years) with a history of asthma, 53.2% of those screened had antibody titres to *T. canis* of 1:50 (Taylor *et al*, 1988). In more recent studies, 52% of 140 Irish adults and children, and 31% of 2129 Irish school children (4-19 years of age), exhibited antibody titres of $\geq$ 1:50 (Holland *et al*, 1991; 1995). In the study of 2129 school children, some of the risk factors associated with seropositivity were dog ownership, geophagia, thumbsucking, location of school, and age and sex of host. Children from rural schools showed

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significantly higher seropositivity rates to *T. canis* than those from urban schools, and the highest antibody titres ($\geq$ 1:800) were found in children of 7 years. Male children exhibited a significantly higher seroprevalence compared with female children, and this was thought to perhaps relate to differences in play and social behaviour, although the incidence of geophagia did not differ between the sexes.

1.2.3.2 Murine infection

The distribution of *T. canis* larvae in various tissues of experimentally-infected rodents was described in the early 1920s by Fülleborn (1921, cited by Sprent 1952). In a more extensive study, Sprent (1952) described the migration of *T. canis* larvae in the mouse, noting that they remained alive in the tissues for at least 4 weeks. Following infection with 2500 eggs, the author reported the presence of larvae in the carcass as early as 2 days post-infection, and the early disappearance of larvae from the intestinal wall, liver and lungs. The absence of larvae in the intestinal contents was thought to indicate an exclusively somatic migration. Larvae were demonstrable in the kidneys from 2 days post-infection until 7 days, by which time most had left. Encapsulated larvae were evident from 12 days post-infection, and were most abundant on the subcutaneous tissues of the back, legs and chest. There was no investigation of the brain in the study. However, in follow-up studies, the same author reported an abundance of *T. canis* larvae in the brains of infected mice, compared with other ascarid larvae, as late as 6 months post-infection (Sprent, 1953; 1955).

Abo-Shehada *et al* (1984) described the early path of migration in outbred mice infected with 5000 *T. canis* eggs, recovering larvae from various organs at 1-8, 14, 16 and 24 hours post-infection. Larvae were recovered in appreciable numbers from the stomach contents as early as one hour post-infection, although hatching had been seen to occur within 30 minutes of infection in preliminary studies. Hatched larvae were found within the small intestine and colon, at 2 and 3 hours post-infection, respectively. The number of larvae found free in the lumen of the small intestine peaked between 4 and 6 hours post-infection, and then decreased over the remaining hours.

In a follow-up study, the same authors carried out a series of different experiments to investigate the post-intestinal migration of *T. canis* in primary infections (Abo-Shehada

and Herbert, 1984). In one experiment, inbred NIH mice were orally infected with 2000 eggs, and sacrificed on a number of days post-infection, ranging from 2 to 120. The authors noted that larval numbers peaked in the liver and lungs on days 2 and 3 post-infection, respectively, and then declined rapidly as infection progressed. From the 7th day of infection, the majority of larvae were recovered from the brain and musculature – marking the end of the hepato-pulmonary phase and the beginning of the myotropic-neurotropic phase. Overall, the total number of larvae recovered from mice decreased over the course of infection, although (in a second experiment) larvae were still demonstrable in some tissues as late as 360 days post-infection. In mice infected intracerebrally, larvae were recovered from the musculature, liver and lungs up to 7 days post-infection, demonstrating their ability to leave the brain and migrate to the visceral organs.

The presence and distribution of *T. canis* larvae within the central nervous system of the murine host is an area of increasing interest to parasitologists, since larvae appear to show a predilection for this part of the body. Sprent (1955) investigated the presence and distribution of larvae in the brains of *T. canis*-infected mice, over a series of experiments. In a 2 week experiment, larval numbers increased in the brain, with highest penetration occurring between days 2 and 3 post-infection. In a longer experiment, lasting 6 months, the length and width of larvae recovered from the brains of infected mice were noted to be similar throughout infection – indicating no development. The distribution throughout the brain was scattered, with larvae being recovered from a range of areas. However, the majority of larvae were recovered from the cerebral hemispheres, followed by the cerebellum. A study of brain sections showed that larvae had begun to invade the brain as early as 2 days post-infection, at which time free larvae were observed in the tissues, and haemorrhages were evident mostly near the dorsal surface of the brain. Burren (1971) reported the presence of *T. canis* larvae in the brains of infected mice up to 138 days post-infection.

The first evidence of larval accumulation in the brain, as opposed to larvae simply being found there, was provided by Dunsmore *et al* (1983). In their study, outbred (Canberra) and inbred (C57BL) strains of mice were infected with 1000 *T. canis* eggs, and larvae were recovered from the brain and visceral organs. The authors noted that the numbers of larvae increased over the course of infection in both strains, with as

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much as 92.2% of the larval burden recovered being found in the brain at the end of a long term infection (122 days). Moreover, as the larvae increased in the brain, they decreased in the other tissues – providing evidence of accumulation in this organ. The relative stability of *T. canis* larvae in the brain was demonstrated by Bardón *et al* (1994), who recovered 67.5 % of total larvae, as much as one year post-infection.

1.2.4 Animal models for cerebral toxocariasis

As mentioned above, *T. canis* is capable of infecting a wide range of paratenic hosts, and over the decades, many of these animals have been experimentally infected with *T. canis*, and the route of larval migration determined. Larvae have been recovered from the brains of experimentally-infected monkeys (Glickman and Summers, 1983), rabbits (Church *et al*, 1975), chickens (Taira *et al*, 2003), quail (Nakamuru *et al*, 1991), gerbils (Akao *et al*, 2003), mice (Sprent, 1952; Dunsmore *et al*, 1983; Abo-Shehada and Herbert, 1984; Epe *et al*, 1994; Skerrett and Holland, 1997; Cox and Holland, 1998), pigs (Done *et al*, 1960; Helwigh *et al*, 1999; Taira *et al*, 2004), and rats (Olson and Rose, 1966; Lescano *et al*, 2004). Some of these hosts are highly unlikely to serve as natural paratenic hosts in the wild, so the ecological significance of their infection is questionable. Although evidence for larval accumulation was not sought in the majority of those experiments cited above, it is of interest to note that where quantitative data were available, the largest numbers of larvae were found in the murine brain compared with other hosts (Holland and Hamilton, 2005).

Recently, the pig and Mongolian gerbil have received more attention, and their relative merits as models for toxocariasis have been evaluated. The pig has been suggested to be a suitable model for a number of human infections due to the many immunological, physiological and biochemical similarities between the two (Willingham and Hurst, 1996; Boes and Helwigh, 2000). Helwigh *et al* (1999) reported the recovery of *T. canis* larvae from various organs of infected pigs, including the liver, lungs and brain. Unlike in mice, however, the numbers of larvae recovered from the brain were very small, ranging from only 0-0.7 per gram of tissue, and no larvae were recovered from the eyes. Taira *et al* (2004) recovered larvae from various organs of pigs fed swine or poultry viscera contaminated with *T. canis* eggs, and reported that very few larvae were recovered from the brain and eyes. The lack of CNS involvement indicates that pigs

are a less suitable model for human toxocariasis. Furthermore, unlike mice, detection of larvae in their tissues is time consuming, and they do not act as paratenic hosts under natural conditions. The fact that infection can be established from contaminated viscera after preservation at 4°C for one week, however, does highlight the potential zoonotic risk for humans if raw or undercooked meat is consumed (Taira *et al*, 2004).

Takayanagi *et al* (1999) reported that Mongolian gerbils exhibited a high susceptibility to ocular toxocariasis following a single oral inoculation of *T. canis*, compared with mice, rabbits, guinea pigs and monkeys. Furthermore, the dark grey fundi of these animals made the observation of motile *T. canis* larvae much easier. Alba-Hurtado *et al* (2000) also recommended the use of the gerbil as a model for ocular toxocariasis, although towards the end of infection animals were displaying clinical signs of illness such as weight loss, reduced co-ordination, and palpebral oedema. The use of the gerbil as a model for cerebral toxocariasis was assessed by Akao *et al* (2003). Authors reported that 49% (6/13) of gerbils infected with 1000 *T. canis* eggs developed neurological abnormalities such as a swinging gait, walking in circles, paralysis of the hind limbs and urinary incontinence, from day 50 of infection. These symptoms were found to coincide with severe degenerative changes and lesions in the cerebellum. The authors suggest that since mice do not display such severe neurological symptoms, the gerbil is a more appropriate model to study progressive neurological toxocariasis. However, since the effects of cerebral toxocariasis in humans are much more subtle (see below), the mouse is likely to be a more appropriate model. Gerbils are unlikely to act as a natural paratenic host of *T. canis*, whereas mice in the wild have been shown to harbour *T. canis* larvae in their tissues (Dubinsky *et al*, 1995). Since patent infections can be established in dogs and foxes fed *T. canis*-infected mouse tissue (Sprent, 1958), it is likely that mice act as important reservoirs of infection in the environment.

1.2.5 Cerebral toxocariasis in humans

In a recent article, Moreira-Silva *et al* (2004) reviewed all the literature available from the 1950s to 2004, and reported 29 cases of brain involvement in toxocariasis, as determined by the finding of *Toxocara* larvae in the cerebrospinal fluid (CSF) or inside the brain or meninges, and/or by immunodiagnosis of serum or CSF. Following a second search of the literature, and excluding 2 previously reported cases where

cerebral toxocariasis was suspected but no definite clinical signs were noted (Sumner and Tinsley, 1967; Kapur *et al*, 1976), I estimate that there have been at least 34 reported cases of cerebral toxocariasis to the present date (Table 1.1). Of these cases, 9 were discovered at autopsy following death from a different cause (e.g. non-accidental injury, poliomyelitis, fire victim, epileptic seizure), and 25 reported different clinical and laboratory manifestations such as eosinophilic meningitis, encephalitis or meningo-encephalitis, arachnoiditis (inflammation of the arachnoid membrane), epilepsy, spinal cord lesions, and, in some cases, cognitive disorders. Although the occurrence of cerebral toxocariasis in humans would appear relatively uncommon, and the clinical evidence is patchy, there are still some indications that larval involvement in the human brain may have subtle public health implications.

Magnaval *et al* (1997) sought to characterise a recognisable cerebral or neurological syndrome associated with *Toxocara* infection among adults in France. Seropositive cases, with neurological symptoms in the absence of an aetiological diagnosis, were compared with 2 control groups matched for age, sex, ethnicity and travel history outside the European community. A questionnaire was used to assess each individual's living habits and social status, and a variety of clinical, radiological and laboratory investigations were carried out. The authors concluded that although low doses and repeated infections of *T. canis* can result in the migration of larvae to the CNS and the induction of antibody, it does not induce a recognisable neurological syndrome. However, infection was correlated with several risk factors including exposure to dogs, rural residence and dementia. The authors suggested that a longer follow-up study would be needed to fully evaluate the significance of *T. canis* larvae in the brain. They also highlighted the fact that all participants in their survey were adults, and that perhaps possible differences between cases and controls would be more obvious in children, given their greater exposure potential.

An earlier American study focused upon the relationship between *Toxocara* seropositivity and neuropsychologic parameters in young children (Marmor *et al*, 1987). Findings from this study suggest a role for toxocariasis in subtle effects on cognition. Sera from 4652 children aged 1-15 years were obtained from blood samples submitted to the New York City Department of Health Lead Screening Programme over 15 months. The study was carried out on 155 case-control pairs, matched by age,

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sex, lead category and date of blood sampling. The pairs were compared in terms of a range of parameters, including various risk factors, and a battery of neuropsychological tests. The authors found that more parents of cases reported hyperactivity in their children than did parents of controls, and furthermore, in all elements of the neuropsychological test battery, cases performed less well than controls. These results are in contrast to those obtained by Worley *et al* (1984), who failed to demonstrate a relationship between *Toxocara* seropositivity and cognitive abnormalities, after controlling for social class. Although authors of both studies highlight the difficulties in the interpretation of their results, if the evidence of impaired neurological function is correct, the extent of *Toxocara*-induced pathology may be much wider than currently documented (Maizels *et al*, 2000).

Further evidence concerning neurological involvement during *T. canis* infection was provided by a recent study of ocular toxocariasis. The prevalence of consultant-diagnosed ocular toxocariasis was determined in over 120,000 Irish schoolchildren ranging in age from 3 to 19 years (Good *et al*, 2004). A case-control study (with each case matched by 4 controls for age, sex, and rural/urban status by school and county) revealed a strong association between having had a convulsion and ocular toxocariasis, supporting the hypothesis that ocular toxocariasis may be part of a more widespread neurological invasion. Some reports have also suggested a possible association between *Toxocara* infection and epilepsy, with those patients with epilepsy having a higher seroprevalence than apparently healthy individuals (Woodruff *et al*, 1966; Glickman *et al*, 1979; Arpino *et al*, 1990). However, children with epilepsy of known aetiology are not more commonly infected with *Toxocara* than children with epilepsy of unknown cause (Glickman *et al*, 1979).

The evidence of cerebral toxocariasis in humans is clearly fragmentary and indicates that if a recognisable neurological syndrome exists, its effects are likely to be cryptic and difficult to detect in human subjects. In my view, this strengthens the choice of the mouse as the most appropriate animal model to explore the significance of cerebral toxocariasis. As described earlier, the severe lesions and sequelae associated with cerebral infection in the gerbil would not appear to reflect the characteristics of human infection.

Table 1.1: Summary of cases reporting brain involvement in toxocariasis (adapted from Moreira-Silva *et al* (2004)). Age of each case is in years.

Age (sex) of case	Observations	Reference
Cases reported at autopsy		
1 (M)	Granulomas with larvae in the CNS[*]	Dent *et al* (1956)
6 (M)	Granulomas with larvae in the CNS	van Theil (1960)
2 (M)	Granulomas with larvae in cerebellum and medulla	Moore (1962)
5 (F)	Multiple granulomas and larvae in CNS	Schoenfield *et al* (1964)
6 (F)	Granulomas and larvae in thalamus	Beautyman *et al* (1966)
2 (M)	Multiple granulomas with larvae in CNS	Schochet (1967)
1 (M)	Multiple granulomas with larvae in CNS	Mikhael *et al* (1974)
2 (F)	Died from "non-accidental" injury; cried excessively; larvae found in the pons, cerebrum and cerebellum	Hill *et al* (1985)
3 (M)	Larvae found in cerebrum; granulomas in liver	Nelson *et al* (1990)
Cases with clinical data		
32 (F)	Headache and convulsions; positive *Toxocara* skin test; eosinophilia	Brain & Allan (1964)
25 (M)	Meningomyelitis; 17% eosinophils in CSF[**]; precipitating anti-*Toxocara* antibodies in serum	Engel *et al* (1971)
1 (F)	Progressive weakness of right arm and leg; 80% eosinophils in CSF; serum and CSF positive for *Toxocara*	Anderson *et al* (1975)
43 (F)	Neck rigidity, lower limb weakness; larvae compatible with *T. canis* detected in CSF	Wang *et al* (1983)

[*]CNS = central nervous system; [**]CSF = cerebrospinal fluid

Table 1.1: Cases with clinical data continued.

Age (sex) of case	Observations	Reference
11 (F)	Pronounced meningism; CT[***] scan normal; 30% eosinophils in CSF; positive serology for *Toxocara* in serum	Gould *et al* (1985)
55 (F)	Severe paraparesis; myelography; lesion visible on CT; positive serology for *Toxocara* in serum	Russeger & Schmutzhard (1989)
26 (F)	Epileptic seizures; multiple hyperintense lesions in CNS; positive serology for *Toxocara* in serum and CSF	Ruttinger & Hadidi (1991)
1 (M)	Recurrent seizures, truncal ataxia and lethargy; positive serology for *Toxocara*	Fortenberry *et al* (1991)
24 (F)	Paresthesis of legs; 10% eosinophils in CSF; positive serology for *Toxocara*	Sellal *et al* (1992)
53 (F)	History of spastic tetraparesis; CT scan revealed intradural and extra spinal cord expansive process; histology on fibrotic tissue from arachnoidea revealed remnants of *Toxocara* larvae	Villano *et al* (1992)
48 (M)	Ataxia, rigor and neuropsychological disturbances; CT scan and MRI[****] revealed diffuse lesions in the white matter; positive serology for *Toxocara* in the blood	Sommer *et al* (1994)
Unknown	Positive serology for *Toxocara*; abnormal CT and MRI findings	Zachariah *et al* (1994)[*****]

[***]CT = computed tomography; [****]MRI = magnetic resonance imaging; [*****]Information collected from abstract only.

Table 1.1: Cases with clinical data continued.

Age (sex) of case	Observations	Reference
23 (F)	CSF eosinophilia and myelopathy; MRI revealed a spinal cord lesion; positive serology for *Toxocara*	Kumar and Kimm (1994)[*****]
21 (F)	Frontal headache, fever and convulsion; meningeal irritation and cerebellar ataxia; MRI revealed lesions in cerebrum and cerebellum; 30% eosinophils in CSF; positive serology for *Toxocara* in blood and CSF; lesions on cervical cord and optic nerve.	Ota *et al* (1994)
58 (M)	Severe sensory disturbance to all modalities in the left arm; spinal MRI revealed swollen areas; positive serology for *Toxocara* in the blood	Duprez *et al* (1996)
49 (M)	Subacute weakness of quadriceps muscles of the thighs; paraparesis with discrete hyperaethesia and hypalgesia; 33% eosinophils in CSF; positive serology for *Toxocara* in blood and CSF	Strupp *et al* (1999)
40 (F)	Weakness of right leg; spinal MRI revealed hyperintense foci; 40% eosinophils in CSF; positive serology for *Toxocara* in blood and CSF	Goffete *et al* (2000)
61 (M)	Paralysis of one side of face; CT scan revealed hypodense areas in right posterior temporal lobe; positive serology for *Toxocara* in blood	Ardilles *et al* (2001)
65 (F)	Depressive symptoms and cognitive deficits; CSF eosinophilia; positive serology for *Toxocara* in blood and CSF	Richartz & Buchkremer (2002)[*****]

Table 1.1: Cases with clinical data continued.

Age (sex) of case	Observations	Reference
Unknown	Meningoradiculitis; CSF eosinophilia; positive serology for *Toxocara* in serum and CSF	Robinson *et al* (2002)[*****]
2 (M)	Mental confusion and motor weakness; 58% eosinophils in CSF; positive serology for *Toxocara* in serum and CSF; hyperintense subcortical lesion	Vidal *et al* (2003)
54 (F)	Left-arm clumsiness, gait disturbance and behavioural disorders; 36% eosinophils in serum; subcortical lesions in frontal and occipital lobes; positive serology for *Toxocara* in serum	Xinou *et al* (2003)
5 (F)	Paralysis of right cranial nerves; 57% eosinophils in CSF; positive serology for *Toxocara* in serum and CSF; lesions in the spine-bulbar transition and pedunculus cerebellaris	Moriera-Silva *et al* (2004)
5 (M)	Palsy of the inferior limbs and urinary retention; 57% eosinophils in CSF; positive serology for *Toxocara* in serum and CSF	Moriera-Silva *et al* (2004)
11 (F)	Generalised epileptic seizure; hypodense lesion in right parietal lobe; positive serology for *Toxocara* in serum; lesion contained large numbers of eosinophils; no seizures following surgery and antihelminthic treatment	Bächli *et al* (2004)

[*****]Information collected from abstract only.

1.2.6 Behavioural alterations in *Toxocara canis*-infected paratenic hosts

Parasites are well known for their capacity to alter their hosts' behaviour in a number of different ways (Moore, 2002), and over the years, researchers have shown a particular interest in whether or not changes in behaviour are an adaptive manipulation by the parasite or a mere side-effect of pathology. For the most part, attention has focused on those parasites with an indirect life cycle, where any changes in behaviour are seen as an "adaptive" evolutionary strategy adopted by the parasite in order to increase transmission from an intermediate host to a definitive host (Bethel and Holmes, 1973, 1974; Moore and Gotelli, 1992; Berdoy *et al*, 1995). In other cases, the change in behaviour is viewed as an adaptation by the host as a means of ridding itself of the parasite, or compensating for its effects (Hart, 1990; Milinski, 1990). It is not only parasites with indirect life cycles, however, that have the ability to influence host behaviour. Parasites with direct life cycles, and parasites which can infect accidental (paratenic) hosts, have also been shown to induce behavioural alterations (Freeland, 1981; Dolinsky *et al*, 1981; Kavaliers and Colwell, 1995; Kavaliers *et al*, 1995) - although the data available on paratenic host behavioural alterations is not as abundant as for those parasites with indirect life cycles.

The simplest explanation for parasite-induced behavioural alterations is that they are a side-effect of the pathological changes caused by the infection itself (Poulin, 1995). However, there are some examples where the change in behaviour is so direct that it is unlikely to be a consequence of pathology, and more likely an adaptive manipulation by the parasite. Such examples include the grass-climbing behaviour of the ant intermediate host of *Dicrocoelium dendriticum* (Trematoda), which must be ingested by grazing sheep to complete its life cycle (Carney, 1969), and the bright, pulsating tentacles of snails infected with *Leucochloridium* (Trematoda), which must be ingested by birds for the parasite to complete its life cycle (Kagan, 1951). Poulin (1995) stated that in order for parasite-induced behaviour to be called adaptive, it must first satisfy 4 criteria – complexity, purposiveness of design, convergence and fitness effects. After reviewing a number of cases where parasite manipulation was suspected, the author reported that very few actually met with all criteria. In a recent study, Webster *et al* (2000) demonstrated that *Tenebrio* beetles (intermediate hosts of *Hymenolepis diminuta*), thought to be predisposed to predation by rats (definitive hosts) through

altered behaviour, were no more at risk of being eaten than uninfected beetles – highlighting the need for caution when assessing parasite-induced behavioural changes.

There have been a number of investigations over the years on the effects of *T. canis* on paratenic host behaviour, particularly in the mouse. Hay and Aitken (1984) demonstrated that *T. canis*-infected mice were more active compared with uninfected controls, crossing significantly more squares in the open-field. They also demonstrated that infected mice were significantly more active in a 'Y'-maze, and spent significantly more time in the novel arm, compared with controls. In a similar study on activity levels, Hay *et al* (1985) demonstrated that *T. canis*-infected mice were significantly more active on a running wheel compared with control mice. Cox and Holland (2001a) investigated the influence of *T. canis* infection on the baseline activity of an outbred (LACA) and inbred (NIH) strain of mouse. They reported that the activity of the mice differed both pre- and post-infection, with LACA mice spending more time immobile compared with NIH mice. Hyperactivity was reported in *T. canis*-infected mice, as measured by the bout length of a range of baseline activities – with infected mice displaying a significantly greater number of short bouts of activity, lasting 0-2 seconds, than control mice (Hay *et al*, 1986). The effect of *T. canis* infection on activity levels is of interest with regard to human infection, particularly in seropositive children where episodes of hyperactivity have been reported (Magnaval *et al*, 1997; Varga *et al*, 1998).

Changes in exploratory behaviour have also been investigated. Cox and Holland (2001b) reported that *T. canis*-infected mice were less explorative and less responsive to novelty than uninfected control mice. In an earlier study, Burright *et al* (1982) examined exploratory behaviour as a function of change in a familiar environment, in *T. canis*-infected mice in the open field and in a two-tier cage. The authors noted that none of the infected mice ascended to the upper tier of the cage, after habituation to the lower, compared with 4 out of 5, 4 out of 6, and 3 out of 6 control mice in the groups infected with 1000, 250 and 500 *T. canis* eggs, respectively. The control mice also spent significantly more time in the upper tier compared with all other groups. In the open field test, although the number of squares crossed was similar in all groups, those mice in the highest *T. canis* dose group performed significantly fewer hole-explorations compared with control mice. In a similar study of exploration, Dolinksy *et al* (1981) reported that *T. canis* infected mice ascended to a novel cage, placed on top of their

familiar cage, significantly fewer times than control mice. Furthermore, the mean latency to ascent was considerably longer in the infected mice.

The effect of *T. canis* on the learning capacity of rodents was some of the first behavioural research undertaken with this parasite. Olson and Rose (1966) reported a dose-dependent effect of *T. canis* infection on the ability of rats to solve complex maze problems. Rats infected with 5000 eggs performed similarly to control rats in all maze trials, whereas when the dose was increased to 20,000 eggs, infected rats made significantly more errors than controls. The authors hypothesised that *T. canis* infection in children may play a role in altering their learning capacity, and recommended further studies to investigate this. Dolinksy *et al* (1981) used a passive avoidance test and 'T'-maze to assess the effect of *T. canis* infection on the learning capacity of outbred mice. The authors noted that infected mice took longer to avoid the shock than control mice, and took more trials to acquire and retain spatial discrimination, indicating impairment of learning, although differences were not significant. More recently, Cox and Holland (2001b) demonstrated that outbred mice with moderate and high *T. canis* infections displayed a greater latency to enter an alcove, locate a water source and drink from it (following a period of water-deprivation), compared with control mice, indicating a degree of memory loss, although the differences were not statistically significant. The effects of *T. canis* infection on learning and memory may be of particular interest with respect to human infection, and also as regards the use of the mouse model to explore the effects of chronic helminth infection on cognitive development.

1.2.7 Distribution of *Toxocara canis* in the brain and the associated neuropathology

Early work by Sprent (1955) and Bisseru (1969) described how *T. canis* larvae can penetrate the mouse brain as early as 2-3 days post-infection. In both studies, haemorrhages on the brain surface indicated that larvae penetrate out of the arteries near the brain surface, possibly due to subdivision of the arteries, making them too narrow for larvae to traverse. Sprent (1955) observed larvae in many sections of the brains of mice killed on day 112 post-infection, including the hippocampus, midbrain, pons and medulla. Most larvae, however, were recovered from the cerebral

hemispheres and cerebellum. Bisseru (1969) also reported that larvae were most abundant in the cerebellum. Burren (1971) examined the brain and eyes of outbred mice infected with 1000 *T. canis* eggs, and noted that 4.5% of mice examined had larvae in either one or both eyes. The brains were segmented into cerebral hemispheres, brain stem and cerebellum, and larvae were enumerated by means of brain squashes. The distribution of larvae in the brain was not random, with the cerebellum containing more larvae than the cerebral hemispheres, despite weighing less than the latter by a factor of 3.6. The fewest larvae were located in the brain stem. Larvae were also noted most abundantly in the cerebellum in a study by Hay and Aitken (1984), where mice were infected with 1000 *T. canis* eggs. In a more recent study, Good *et al* (2001) reported that the majority of larvae recovered from the brains of *T. canis*-infected outbred mice were from the telencephalon and cerebellum. The authors also noted that, for the telencephalon and diencephalon only, more larvae were detected on the right-hand side of the brain compared with the left, indicating non-random distribution. The frequency of larvae within particular areas of the brain may be of interest with regard to the behavioural alterations observed in *T. canis*-infected mice.

The active migration of larvae in the brain has been associated with varying degrees of pathology. Summers *et al* (1983) noted necrosis, cavitation and perivascular cuffing in the brains of mice infected with 1000 *T. canis* eggs. Moreover, foci of parasites were often found in close proximity to the lesions. The lesions described were most frequently noted in the heavily myelinated tracts of the brain, including the corpus callosum, internal and external capsules, cerebellar peduncles and the cerebellar medulla. Inflammatory cells were also observed in the areas surrounding parenchymal necrosis, with the predominant cells being lymphocytic, although plasma cells, eosinophils and neutrophils were also noted. Despite the cellular activity, the larvae appeared to be viable at the time of sacrifice. As part of a larger study, Dolinsky *et al* (1985) noted the neuropathological effects of *T. canis* larvae in the brains of infected mice over the course of infection. Those mice sacrificed at 10 days post-infection had numerous focal hemorrhages on the dorsal surface of the cerebrum and cerebellum. Although fibre degeneration and the presence of larvae were noted in the brain on day 51 post-infection, the most severe pathological changes were noted on day 86 post-infection, when there was marked degeneration of fibre pathways, including the corpus

callosum, cerebellar peduncles and dorsal cerebellar medulla. It is interesting that in both studies, larvae showed an affinity for the white matter tracts, which may reflect a selective tropism for myelin (providing nutritional advantages), or simply the pathway of least resistance through the brain (Summers *et al*, 1983). In a study of several different inbred strains and one outbred strain of mouse, Epe *et al* (1994) reported demyelisation, focal malacia and mixed-cell infiltration in the brains of infected mice. Larvae could be demonstrated in the absence of an inflammatory reaction in all areas of the brain except the bulbi olfactorii.

In humans, the frequency and localisation of *T. canis* larvae in the brain is largely unknown. Of the few cases reported, larvae have been recorded in the leptomeninges (Dent *et al*, 1956), gray and white matter of the cerebrum and cerebellum (van Theil, 1960; Moore, 1962; Hill *et al*, 1985; Nelson *et al*, 1990), and the thalamus (Beautyman *et al*, 1966) and spinal cord (Dent *et al*, 1956).

1.2.8 Immunological response to infection

Vertebrates have a specialised system of "non-self" recognition that results in an increased resistance to specific foreign substances or invaders on repeated exposures. The protection afforded by the immune system is divided into two functional divisions – the innate immune system and the adaptive immune system. Innate immunity serves as the first line of defence against infectious agents, and involves biochemical and physical barriers such as the skin, mucous, stomach acid, and enzymes in tears and saliva. However, this line of defence is non-specific and there is no lasting protective immunity (Weir, 1993). Adaptive immunity on the other hand, is a highly sophisticated and specialised antigen-specific response, and provides a long lasting immunity which ensures a more rapid response in secondary infection. Adaptive immunity can be divided into two arms, known as the cellular or cell-mediated immune response, also referred to as the T-helper type 1 response (Th1), and the humoral immune response, also called the Th2-type response (Weir, 1993) (Figure 1.2).

The main cells involved in adaptive immunity are known as T-cells, in particular the subset of CD4$^+$ T-helper cells. These cells can be further divided into T-helper type 1 (Th1) cells, and Th2 cells (Mosmann and Coffman, 1987). The differentiation into

either Th1 or Th2 cells determines whether humoral or cell-mediated immunity will predominate, and is influenced by a number of factors, the most potent of which seems to be the cytokine environment a T-cell experiences at the time of antigen presentation (Else and Finkelman, 1998). Antigen presentation is a process required for Th-cells to recognise and bind to foreign antigen, and is carried out by antigen presenting cells (APCs). APCs, such as macrophages and dendritic cells, partially digest foreign antigen and incorporate portions into their own cell surface (Schmidt and Roberts, 1996). The portion of antigen presented on the surface of the APC (known as the epitope) is recognised by Th-cells, along with a class of proteins known as the major histocompatibility complex (MHC) proteins. MHC proteins allow Th-cells to distinguish "self" from "non-self", and there are two classes: MHC class I molecules generally present peptides derived from endogenous antigens, generated from within a cell (e.g. viral proteins), and are recognised by cytotoxic T-cells (CD8^{+}); and MHC class II molecules generally bind peptides derived from exogenous antigens (ingested or inhaled etc) living in macrophage vesicles or internalised by phagocytic B-cells, and are recognised by Th1 or Th2 cells (Weir, 1993). Both the MHC protein and the epitope must be presented by the APC for Th-cell recognition – neither is effective alone.

The balance between the Th1 and Th2 arms of the immune system is modulated by a highly integrated network of molecular and cellular interactions driven by cytokines - small secreted proteins that act as intercellular chemical messengers (Weir, 1993). Cytokines can be classified as either Th1 or Th2, depending on their role (Table 1.2). The main Th1 cytokines are interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-β, and the main Th2 cytokines are IL-4, IL-5, IL-6 and IL-10 (Mosman and Coffman, 1987). The induction of Th1 cytokines stimulates the cell-mediated response, with the activation of macrophages and production of various cytokines including TNF-α and IFN-γ. Macrophages also produce other biological mediators such as nitric oxide (NO), which has shown to be cytotoxic to a number of parasites (James, 1991). Production of IFN-γ stimulates the immunoglobulin class switch to IgG2a (Mosmann and Coffman, 1987) (Table 1.3). This type of response is known as a delayed-type hypersensitivity response, and generally occurs in response to intracellular pathogens, such as protozoan parasites. Induction of Th2 cytokines on the

other hand, stimulates the humoral response, with predominant production of the antibodies IgG1 and IgE (Table 1.3), and the involvement of mast cells and eosinophils (Mosmann and Coffman, 1987). This type of response is known as an immediate hypersensitivity response, and is generally associated with the control of extracellular pathogens, such as helminths. Although Th2-type responses are predominantly associated with little cellular activity, the production of IL-4 may be involved in the activation of macrophages. Classically, macrophages are activated in response to proinflammatory cytokines such as IFN-γ, or in response to microbial infection or bacterial products, such as lipopolysaccharide (Dalton *et al*, 1993; Gordon, 2003). However, there is growing evidence for an alternative route of activation, via an IL-4/IL-13-dependent signal pathway (Donnelly *et al*, 2005), inducing macrophages to express products such as arginase 1, Fizz1 and Ym1 – associated with asthma, and eosinophil infiltration (Holcomb *et al*, 2000; Welch *et al*, 2002).

It is important to bear in mind, however, that the Th1/Th2 paradigm is not a strict set of rules (Allen and Maizels, 1997) and that there may be switches in the patterns of Th1 and Th2 cytokines during the course of an infection (Bancroft *et al*, 1994; Taylor-Robinson and Phillips, 1998). Also, there is now accumulating evidence to suggest that a functionally distinct population of CD4$^+$ T-cells, known as regulatory T (Tr) cells, may play an important role in regulating Th1/Th2 responses, subsequently controlling pathology (Groux *et al*, 1997; McGuirk and Mills, 2002). Several subsets of Tr-cells have been identified, and they include: type 1 Tr-cells (Tr1), which secrete high levels of IL-10 and moderate levels of transforming growth factor (TGF)-β; type 3 T cells (Th3), which secrete TGF-β; and CD4$^+$CD25$^+$ T cells, which inhibit immune responses through cell-cell contact (Shevach, 2000). Various studies on these subsets have revealed their immunosuppressive properties, with Tr1 cells being shown to suppress inflammatory Th1-type responses, and Th3 and CD4$^+$CD25$^+$ T cells being involved in autoimmunity suppression and the modulation of Th2-type responses (Groux *et al*, 1997; Sakaguchi, 2000; Jaffar *et al*, 2004).
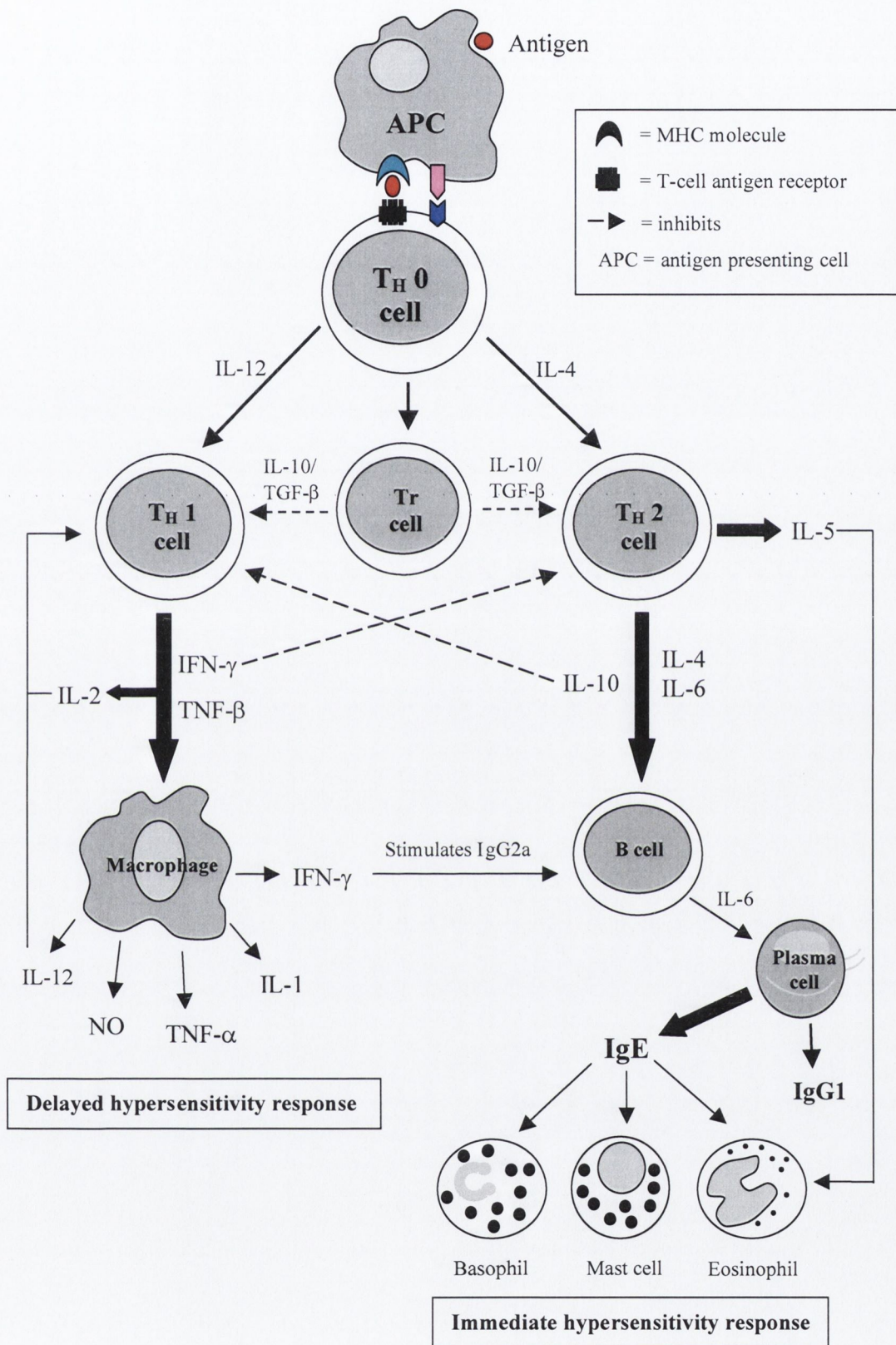
Figure 1.2: Th1 versus Th2 immune response during infection.

Table 1.2: Murine Th1 and Th2 cytokines, and their roles in immunity.

Cytokine	Producing cell	Function
IL-2	Th1	Stimulates growth of T cells Costimulates B-cell differentiation
IFN-γ	Th1	Activates macrophages Induces Ig[1] class switch to IgG2a Inhibits proliferation of Th2 cells
TNF-β	Th1	Mediator of inflammation Promotes phagocytosis
IL-4	Th2	Stimulates proliferation and differentiation of B-cells Enhances IgG1 and IgE synthesis Suppresses Th1 differentiation
IL-5	Th2	Stimulates activation of eosinophils Stimulates the growth and differentiation of B-cells
IL-6	Th2	Stimulates differentiation of B-cells into plasma cells Promotes antibody production by plasma cells
IL-10	Th2	Inhibits cytokine production by Th1 cells, natural killer cells and macrophages
iNOS [2] (enzyme)	Th1	Needed for production of NO[3] by macrophages NO involved in pathology and the killing of some intracellular parasites

[1]Immumoglobulin; [2]inducible nitric oxide synthase; [3]nitric oxide

Table 1.3: Main classes and subclasses of antibodies, and their roles in immunity.

Antibody	Function
IgA	Main Ig in colostrum and milk; protects mucosal surfaces; involved in neutralisation[1]
IgD	Present in minute concentrations in serum; functions are unknown
IgE	Present in very low concentrations; produced mainly in response to helminth parasites and in allergic reactions; binds avidly to mast cells
IgG	Most abundant Ig
IgG1	Main Ig in serum; good opsoniser[2]; long lasting
IgG2a	Subclass found in mice; involved in neutralisation
IgM	First response antibody; short biological half-life; activates complement[3]

[1]Antibodies bind to the pathogen, preventing it from entering cells.
[2]Antibodies bind to the surface of the pathogen, coating it, and promoting engulfment by phagocytic cells.
[3]Either complement proteins bind to the pathogen, coating it, and enhancing phagocytosis, or a cascade of proteolytic reactions results in the formation of holes on the surface of the pathogen.

1.2.9 Immunological response to *Toxocara canis* infection

When *T. canis* larvae migrate through the tissues of the paratenic host, they secrete large amounts of excretory-secretory (E/S) antigens (de Savigny, 1975). Five major TES (*Toxocara canis* excreted-secreted antigens) macromolecules have been defined and are described as TES-32, TES-55, TES-70, TES-120 and TES-400 kDa (Maizels *et al*, 1984). These glycosylated antigens are rapidly recognised by the immune system and provoke strong antibody responses (Taylor and Holland, 2001). Maizels and Robertson (1991) have suggested, however, that this type of response may not serve as a protective function, and that one advantage of producing large quantities of E/S antigen is to divert the immune system into the synthesis of ineffective antibody. The ability of larvae to survive for considerable periods of time in the tissues of paratenic hosts prompted investigations into the mechanisms by which they are able to evade the hosts' immune response. Smith *et al* (1981) demonstrated that bound anti-TES antibodies were shed by larvae within 3 hours at 37°C, unless they were metabolically arrested, suggesting a mechanism for evading both antibody-dependent and antibody-mediated cellular cytotoxicity *in vivo*. Subsequently, it has been shown that *T. canis* larvae are able to resist killing by eosinophils from guinea pig (Badley *et al*, 1987) and human (Fattah *et al*, 1986) serum, by sloughing off the adherent cells within a matter of hours, leaving free, undamaged parasites.

Infective *T. canis* larvae elicit a characteristically strong and persistent immune response in infected paratenic hosts. The level of antibodies produced in infected mice has been shown to be directly proportional to the number of eggs used in the inoculum - with antibody titres, eosinophilia and splenomegaly all increasing with numbers of larvae (Kayes *et al*, 1985; Havasiová-Reiterová *et al*, 1995). Kayes *et al* (1985) demonstrated that although mice infected with a low dose of only 5 *T. canis* eggs had significantly detectable eosinophilia by day 14 post-infection, antigen-specific antibody responses were not measurable until day 28 post-infection. This was in contrast to those mice infected with larger inocula, in which significant antibody responses were detected on day 14 post-infection. These results suggest an immunological threshold below which there is not enough antigen to drive the immune system into action (Kayes, 2005).

Overall, *T. canis* has been reported to induce a Th2 systemic immune response in the murine host, which is generally accepted as being the mechanism responsible for two of the most striking clinical features of larva migrans – eosinophilia and an increase in IgE (Del Prete *et al*, 1991; Grieve *et al*, 1993). *In vitro*, the Th2 cytokines IL-4 and IL-5 have been shown to be involved in the switching of an IgM response to IgG1 and IgE, and the activation of eosinophils, respectively (Mosmann and Coffman, 1987; Snapper and Mond, 1993). Previous studies have reported the production of antibodies in response to *T. canis* infection. Cuéllar *et al* (2001) reported more pronounced levels of IgG1 than IgG2a and IgM over the course of a 480-day infection, in mice inoculated with 1000 *T. canis* eggs. Buijs *et al* (1994) reported peak levels of IgE in the serum of *T. canis*-infected mice on day 14 post-infection, and Pinelli *et al* (2001) reported significantly higher levels of total serum IgE as early as day 8 post-infection, in infected mice compared with controls. Kurodo *et al* (2001) investigated cytokine production by macrophages from the spleens and peritoneal cavities of mice infected with 100 *T. canis* eggs. On day 10 post-infection, levels of IL-1 and IL-6 were similar to those of control mice, but there was enhanced production of IL-10 and TGF-β, along with diminished IFN-γ and TNF-α production, indicating a Th2 response to infection, or perhaps a Tr-type response.

The tissue response to migrating larvae often matures into a granuloma, with large numbers of lymphocytes, neutrophils and eosinophils (Kayes and Oaks, 1978; Smith, 1991). Microscopic lesions have been observed in the liver and lungs of *T. canis* infected mice. Bisseru (1969) demonstrated that larvae which had not left the liver by 72 hours post-infection were found to be encysted, as observed as milky white spots in an enlarged, congested liver. Examination of lung tissue revealed haemorrhagic areas, and granulomas containing inflammatory cells. The liver is now well recognised as an important site for controlling the migration of *T. canis* larvae (Sugane and Oshima, 1983). Parsons and Grieve (1990) demonstrated that a large portion of larvae remained in the livers of mice previously infected with *T. canis*, compared to naïve mice, in which larvae followed the normal route of migration passing from the liver to the lungs, and other sites such as the brain.

In contrast to the inflammatory response induced by *T. canis* in some parts of the body, there appears to be little reaction to the presence of larvae in the CNS. Sprent (1955)

reported the presence of haemorrhages on the dorsal surface of the brains of *T. canis*-infected mice, and in some cases, larvae were associated with these areas, but there was no evidence of meningeal or perivascular cellular infiltration, or of granuloma formation in any of the brain sections. Dunsmore *et al* (1983) also reported the lack of larval encapsulation in the brains of *T. canis*-infected mice, noting that larvae were not surrounded by any cellular reaction and that they remained alive, as verified by observing their movements in the tissue-squash preparations in which they were counted. In a study involving four different strains of *T. canis*-infected mice, Epe *et al* (1994) reported that although there was demyelisation and mixed-cell infiltration in the brain, there was no evidence of granuloma or capsule formation. Smith (1991), however, reported low grade astrocytosis, microglial enlargement and a diffuse accumulation of macrophages in the white matter of the brains of *T. canis*-infected mice. Although no cellular infiltrate could be demonstrated in proximity to a parasite, immunohistochemical studies revealed both immunoglobulin and complement around apparently inactive larvae. There have been reports of *T. canis* larvae entrapped in granulomas in the brains of children who died of other causes (see Table 1.1), indicating that the brain is perhaps not as an immune privileged site as first thought.

Investigations into the cerebral immune response to other CNS-invading parasites have revealed that, indeed, the brain is not a site of immune privilege, and that in some cases, the quality and quantity of immune response elicited contributes to cerebral pathology. Cerebral toxoplasmosis has been shown to induce increased expression of the Th1 cytokines IL-2 and IFN-γ, along with decreased expression of the Th2 cytokines IL-4 and IL-5 in the brains of infected mice (Gazzinelli *et al*, 1993). Infection also sees an increase in expression of inducible nitric oxide synthase (iNOS), IL-10 and TNF-α, indicating a slight mix in Th1/Th2 response. There is compelling evidence that proinflammatory cytokines mediate cerebral dysfunction in murine cerebral malaria. Grau *et al* (1989) demonstrated that treatment with anti-TNF-α antibodies significantly prolonged the lives of infected mice and prevented the development of neurological signs. The same author also reported that mice deficient in type 2 TNF receptors were protected against cerebral malaria. Brown *et al* (1999) reported the presence of IL-1β in the brains of mice with cerebral malaria – a cytokine known to play a role in neurodegeneration (Rothwell, 1997). Cytokine production in

the brains of mice infected with *Taenia solium* (neurocysticercosis) and *Trypanosoma brucei brucei* (African sleeping sickness) has also been reported (Restrepo *et al*, 1998; Hunter *et al*, 1991).

1.2.10 Extending the *Toxocara* mouse model

As stated earlier, a number of studies have reported the accumulation of *T. canis* larvae in the brains of infected mice (Sprent, 1955; Burren, 1971; Dunsmore *et al*, 1983). In a more recent paper, Epe *et al* (1994) reported clinical signs of the CNS, such as lethargy, paresis, lack of co-ordination and tremor, in *T. canis*-infected mice, and demonstrated that as many as 80% of mice were affected by week 17 post-infection, and that increasing numbers of mice died at this time. Earlier studies, cited in the same paper, reported the onset of clinical signs of the CNS in *T. canis*-infected mice generally coincide with the beginning of chronic infection, and that they may be attributed to tissue damage caused by migrating larvae in the brain. Given the evidence that the immune response elicited against other CNS-dwelling parasites can induce pathology, and in light of the abundance of behavioural alterations cited earlier, it seems a logical progression to investigate the cerebral immune response to *T. canis* infection in concert with behavioural responses.

This study represents an investigation into the differential brain involvement of *T. canis* in mice of contrasting genetic background. Previous studies on behavioural alterations have mainly utilised outbred mice, with inbred mice receiving less attention (Dolinksy *et al*, 1981; Hay and Aitken, 1984; Cox and Holland, 1998; 2001a, b). By using inbred mice in this study, it is hoped that any infection-induced behavioural alterations which could be potentially masked in heterogeneous outbred mice, may appear more pronounced. Characterisation of the immune response in the brain is important for understanding the host-parasite relationship, and the associated pathology. Elucidating what components of the immune response are associated with susceptibility and resistance to cerebral larval establishment is of particular novelty, as there is no literature on this area already. Furthermore, no previous study has focused upon the simultaneous assessment of the influence of host genetics and immunity on cerebral toxocariasis in addition to the outcome of infection on host behaviour.

CHAPTER TWO: General materials and methods

2.1 Ordering of mice

Following a thorough search of the literature, seven strains of inbred mice were chosen for experiment one, where resistance and susceptibility to cerebral toxocariasis was investigated. Male inbred mice, 6-8 weeks old, were purchased from Harlan UK. For experiment one, a total of 140 inbred mice were purchased - 20 each of the following strains:

- A/JOlaHsd
- C57BL/6JOlaHsd
- BALB/cOlaHsd
- SWR/OlaHsd
- CBA/CaOlaHsd
- NIH/OlaHsd
- C3H/HeNHsd

Following the results of experiment 1, a further 190 inbred mice, 6-8 weeks old, were purchased for experiment two - 95 each of the following strains:

- BALB/cOlaHsd
- NIH/OlaHsd.

All experiments were conducted under licence following approval by the ethics committee at Trinity College Dublin. The numbers of mice assigned to each group were determined statistically in order to minimise the total number of animals used.

2.2 Maintenance of mice

Mice were housed in the animal maintenance room of the Bioresources Unit (Trinity College Dublin) for the duration of the experiments, in standard plastic cages (35 x 15 x 13 cm) with sawdust bedding. The room was maintained at approximately 22°C, and operated on a 12-hour light/dark photoperiod (8am lights on – 8pm lights off). Water and pelleted commercial food were supplied *ad libitum*, and cages were cleaned on a regular basis. The mice were individually weighed on arrival, and randomly assigned to groups of 5 per cage. In order to identify individual mice within a cage, each subject had its ear punched in such a way that all five mice were distinguishable from each

other (Figure 2.1). Where any mice were fighting during the course of experiments, they were transferred to individual cages.
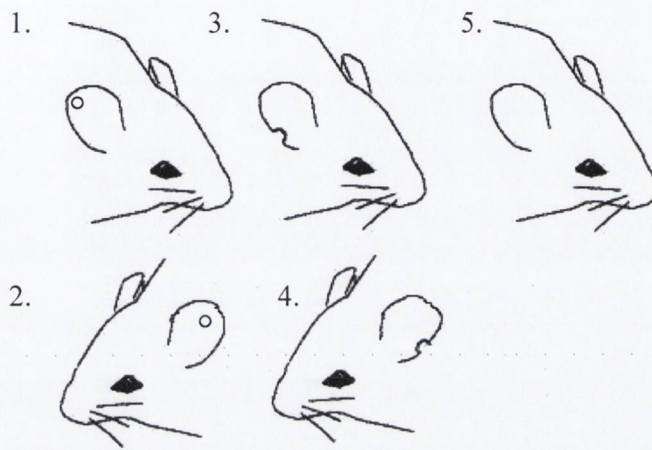

Figure 2.1: Ear-punch marking system used to identify individual mice. 1 = full circle (0.5-1 mm diameter) in right ear; 2 = full circle in left ear; 3 = half circle in right ear; 4 = half circle in left ear; 5 = no markings. Adapted from Case Western Reserve University homepage: http://ko.cwru.edu/services/musfrming.html#hous

2.3 Preparation of *Toxocara canis* ova for inocula

Toxocara canis ova were supplied by colleagues at the National Institute of Public Health and the Environment (RIVM), The Netherlands. In brief, *Toxocara canis* adult worms were collected from the faeces of naturally infected dogs, after routine de-worming with anthelmintics. The posterior end of the female worms (containing the uterus) were cut, and transferred to a conical flask containing PBS. The contents of the flask were stirred overnight (using a magnetic stirrer), to release the eggs from the uterus. The following morning, contents of the flask were filtered through a double layer of gauze, removing any worm material and allowing the eggs to pass through and be collected in a clean conical flask containing 0.05M H_2SO_4. The flasks were covered with parafilm, (pierced, to allow air to enter), and kept at room temperature in a

darkened cupboard for 4-6 weeks, to allow embryonation of the eggs, and development of larvae to the L_2 stage. Following this time, egg viability was determined by examining 10μl aliquots of the suspension on glass slides, under a Nikon light microscope, at x40 objective. Viable eggs were those in which the larva could be seen moving within the eggshell. Eggs were stored in 0.1M H_2SO_4 at 4°C until required.

2.4 Preparation of inocula

Inocula were made up from the stock egg suspension received from the RIVM, The Netherlands. To calculate the number of eggs in suspension, and the percentage of embryonated eggs, 10μl of suspension was transferred to a glass slide, cover-slipped, and examined under a Nikon light microscope, using a x10 objective. Eggs were enumerated by scanning the entire slide, starting from the top left-hand corner. A tally was kept of both embryonated and non-embryonated eggs (Plate 2.1), so that the total number of eggs and percentage embryonated eggs per 10μl could be calculated. This was repeated for five 10μl aliquots, then the average number of eggs and percentage embryonated eggs was calculated, and the appropriate calculations carried out to determine the number of infective eggs in suspension. Using these calculations, the appropriate volume of suspension to provide a dose of 2000 infective eggs, was aliquoted into clean 1.5ml microcentrifuge tubes. If the volume of egg suspension needed was more than 200μl, the microcentrifuge tubes were left overnight, to allow eggs to sediment, and excess solution was aspirated down to the appropriate volume. Any aspirated solution was transferred to clean 1.5ml microcentrifuge tubes, and checked for egg loss (Appendix 1). During each preparation of inocula, five extra doses were always prepared to enable checking of dosage prior to infection (Appendix 1). Where control animals were used, 200μl distilled water was aliquoted into the appropriate number of microcentrifuge tubes.
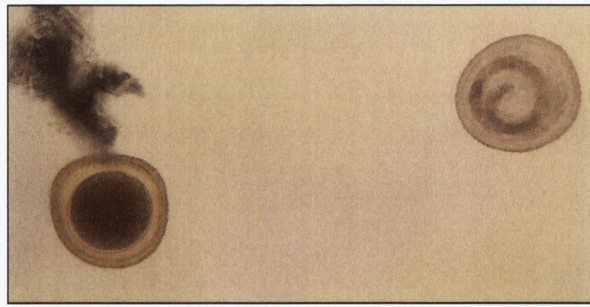
Plate 2.1: Embryonated (right) and unembryonated (left) *Toxocara canis* egg (x 100 magnification).

2.5 Infection of mice

In experiment one, infection of mice took place one week after their arrival, and in experiment 2, infection took place 5 days after pre-infection behavioural testing had been carried out. On the day of infection, all mice were weighed prior to inoculation. Previously prepared doses were drawn up into a graduated 1ml syringe attached to a specially designed needle with a bulb at the tip, used for intragastric intubation. Mice were 'scruffed' in preparation for inoculation – this involved holding the scruff of the neck between forefinger and thumb, while holding the tail with the small finger. The needle was then very carefully inserted into the mouth, down the oesophagus and into the stomach – taking care not to enter the trachea. If any resistance was felt, the needle was removed and repositioned. Once in the stomach, the syringe was depressed slowly, dispensing the inocula. The needle was then carefully removed, and the mouse returned to its homecage. Control mice (in experiment 2) were handled in the same manner, but inoculated with $200\mu l$ distilled water rather than infective ova. All mice were monitored hourly after infection, for one day, for post-inocula trauma or ill effects.

2.6 Experimental design

2.6.1 Experiment 1

In order to investigate the differential brain involvement of *T. canis* in mice of contrasting genetic background, a range of inbred strains of mice were infected so a resistant and susceptible strain to cerebral larval establishment could be selected. It has previously been reported that *T. canis* larvae stabilise in the brain between days 35 and 42 post-infection (Burren, 1971) and Good (1998) demonstrated that larval numbers peaked by day 35 post-infection in outbred CD1-ICR mice. On this basis it was decided that days 35 and 42 p.i would be included in the post mortem dates. Earlier dates of day 7 and 14 p.i were also included to monitor migration of larvae in the various organs. The experimental design is outlined below, and includes the tissues collected at post mortem.

Experiment one - experimental design

- Strains of mice: A/J, BALB/c, CBA/Ca, C3H/HeN, C57BL/6J, SWR and NIH.
- Tissues collected at post mortem: left and right cerebral hemispheres of brains, left and right lungs, liver, and right hind-leg muscle.

Days post-infection

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        |     |     |     |     |
              7    14    35    42
    ▲
    |
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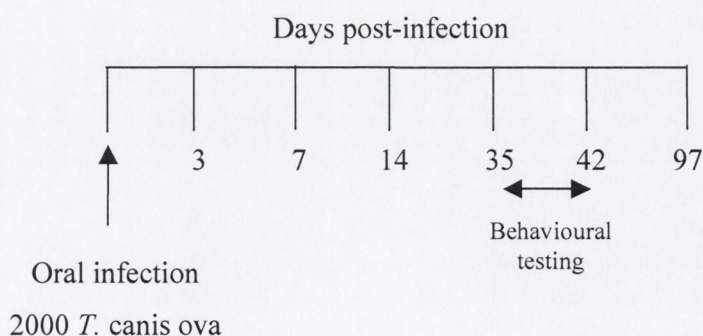
Oral infection
2000 *T. canis* ova
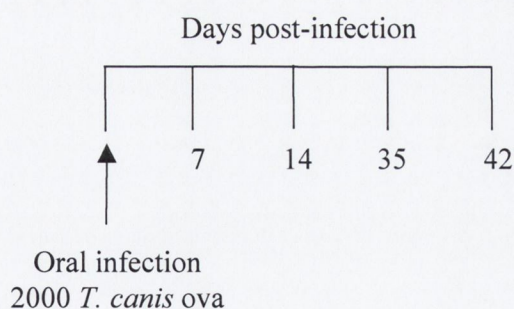
2.6.2 Experiment 2

Following the choice of a susceptible and resistant strain of mouse, based on cerebral larval burdens, mice were infected, and investigated in terms of course of infection, larval accumulation, and behavioural and immune response. The same dates were chosen as for experiment 1, with the inclusion of an earlier and later date – day 3 and day 97 p.i. These dates were included to allow further investigation into the migration pattern of the larvae, and also to investigate whether burdens in the brain remain stable or decrease in the later stages of infection. The experiment was originally proposed to include day 120 p.i., however by day 97 some of the mice were exhibiting central nervous symptoms (i.e. lack of co-ordination and balance), so for ethical reasons the mice were sacrificed on this day. Behavioural testing was carried out between days 35 and 42 p.i, since it was thought that any changes in behaviour would be more pronounced on the days larvae stabilise in the brain.

Experiment two – experimental design

- Strains of mice: BALB/c and NIH.
- Tissues collected at post mortem: left and right cerebral hemispheres of brains, left and right lungs, liver, and right hind-leg muscle.
- Blood was collected on all days, and sera extracted for later serological work.

Days post-infection

CHAPTER 3: Cerebral larval burdens and course of migration in two inbred strains of mice infected with *Toxocara canis*

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3.1 INTRODUCTION

Migration of *Toxocara canis* larvae through definitive and paratenic host tissues is well documented. Sprent (1958) reported the migration of *T. canis* larvae in dogs of different ages, and demonstrated that in young dogs less than 5 weeks old, different developmental stages of *T. canis* larvae were visible in the stomach and intestine by day 9 post-infection. Some 4th stage larvae and immature adults were present in the intestine by day 16 – or in very young dogs (1-3 weeks old), by day 11 post-infection. In contrast, in older dogs (over 5 weeks of age), only 2nd stage larvae were recoverable from the tissues, and there was no development of these larvae and none were found in the alimentary tract. Migration in the murine host is similar to that of older dogs, in that the larvae migrate through the visceral organs, but undergo no further development.

Smith (1991) reported that the migration pathway of larvae in humans and mice is very similar, and that lesions elicited in experimental mouse models and humans are comparable. Furthermore, rodents caught in the wild have been shown to harbour *Toxocara* larvae in their tissues and so have been hypothesised to contribute to parasite transmission if consumed by an appropriate definitive host (Dubinský *et al*, 1995). Due to these parallels, and the evidence for cerebral larval accumulation, the mouse is thought to be a more suitable model for toxocariasis than other animals (Holland and Hamilton, 2005), and has been widely used to study the disease.

Early studies reported the migration of larvae through various organs of the murine host. Bisseru (1969) reported *T. canis* larvae in the livers of mice within 24 hours after infection, and noted that those larvae which had not left the liver by 72 hours post-infection showed signs of encystment (i.e. were seen as milky white spots in an enlarged liver). Scattered foci of inflammatory cells (e.g. neutrophils, lymphocytes and eosinophils) around the portal tracts were seen as an indication that the larvae had entered the liver from the gut through the portal vein. Larvae were also found in the lungs of infected mice by day 2 post-infection, and in the brain from day 2 or 3. In two comprehensive studies by Abo-Shehada *et al* (1984) and Abo-Shehada and Herbert (1984), *T. canis* larval migration was recorded in the murine host from 1 hour post-

infection to 360 days post-infection. In the very early stages of infection (1-6 hours), larvae were recovered from the stomach contents and small intestine, and as infection progressed, peak larval numbers were recovered from the liver on day 2 post-infection, the lungs on day 3 post-infection, and in the brain and musculature from day 7 post-infection. The authors described two phases of migration followed by *T. canis* larvae – the hepato-pulmonary phase and the myotropic-neurotropic phase, with larvae usually entering the latter around day 7 post-infection.

The ability of *T. canis* larvae to penetrate the central nervous system of their murine paratenic hosts has been an area of particular interest for researchers over the years. In early work, encompassing numerous experiments, Sprent (1955) reported the presence of larvae in the brains of *T. canis*-infected mice as early as 2 days post-infection, and noted that their distribution was scattered, although the majority of larvae were recovered from the cerebral hemispheres. In a number of later studies, larvae were recovered from the brains of *T. canis*-infected mice from 2 days post-infection up to as much as one year post-infection (Burren, 1971; Kayes and Oaks, 1976; Bardón *et al*, 1994) – revealing the stability of larvae in this organ. Dunsmore *et al* (1983) were the first to report the accumulation of *T. canis* larvae in the brains of infected mice, as opposed to simply being found there. The authors noted that as larvae increased in the brain over the course of infection, they disappeared from other tissues. This phenomenon had previously been suggested by Lee (1960), but the author provided no quantitative data to support the claim.

Differences in infection profiles among individual animals is influenced by the ability (or inability) to mount an effective immune response against the invading parasite (Wakelin, 1978) – generally with those individuals mounting an inadequate response, and succumbing to infection, being termed 'susceptible'. The susceptibility of different inbred strains of mice to infection with intestinal nematodes is well documented. B10 (congenic) and C57BL/10 (inbred) mice have been reported to be more susceptible to infection with *Trichinella spiralis*, compared with NIH mice – with the former strains taking longer to expel adult worms from the intestines (Wakelin, 1978; Alizadeh and Wakelin, 1983). Lee and Wakelin (1982) reported the susceptibility of CBA mice to infection with *T. spiralis* and *Trichuris muris* compared with NIH mice. The susceptibility to infection, and resistance to reinfection, was assessed in mice infected

with *Strongyloides ratti*, and out of 11 inbred strains, CBA, C57BL/6 and BALB/c mice were the most susceptible, taking a longer time to excrete larvae compared with the other strains (Dawkins *et al*, 1980).

Variations in the numbers of larvae recovered from the brains of *T. canis*-infected mice have been reported in both outbred and inbred strains. Epe *et al* (1994) reported differences in the numbers of larvae recovered from the brains of *T. canis*-infected inbred and outbred strains on different days post-infection – with BALB/c mice generally carrying a higher burden than any of the other strains. Cox and Holland (2001a) and Skerrett and Holland (1997) have reported variation in the numbers of *T. canis* larvae recovered from the brains of individual outbred mice receiving the same infective dose – suggesting a role of immunity or host genetics in the establishment of cerebral infection.

Since little is known of the significance of cerebral larval involvement during *T. canis* infection, the aims of this chapter were (1) to identify a susceptible and resistant strain of mice, with regard to cerebral larval establishment, from several different strains of *T. canis*-infected mice, and (2) to compare these chosen strains in terms of larval accumulation and course of migration.

3.2 MATERIALS AND METHODS

3.2.1 Collection of tissues at post mortem

3.2.1.1 Experiment 1: Optimisation of a murine model for cerebral toxocariasis identifying two divergent phenotypes

One hundred and forty mice (20 each of seven inbred strains) were infected with 2000 *T. canis* ova, as described in Chapter 2, and post mortems took place on days 7, 14, 35 and 42 post-infection. On each post mortem day, five mice from each strain were weighed, and sacrificed by cervical dislocation. Liver, right and left lungs (treated as separate samples), and right hind-leg muscle were removed and transferred to glass flat-bottomed tubes. The tissues were then macerated in a small volume of either 1% Trypsin (adjusted to pH 7 with 0.1N NaOH – Appendix 2) or 1% pepsin (adjusted to pH 1 with conc. HCl – Appendix 2), depending on day post-infection. Trypsin can be used to digest samples taken on or before day 7 post-infection, whereas pepsin is more suitable for samples taken after day 7 (Sprent, 1952). The brain was carefully removed and sagitally bisected into right and left hemispheres, and macerated in a small volume of 0.85% saline (Appendix 2) using fine scissors. All tissue samples were then transferred to tubes for Baermann procedure.

3.2.1.2 Experiment 2: Course of *Toxocara canis* infection and cerebral larval burden in two inbred strains of mice

One hundred and twenty mice (60 each of BALB/c and NIH) were infected with 2000 *T. canis* ova, and 70 mice (35 each of BALB/c and NIH) were sham inoculated with distilled water, as described in Chapter 2. On days 3, 7, 14, 35, and 97 post-infection, fifteen mice from each strain (10 infected and 5 controls) were weighed, and sacrificed by cervical dislocation. On day 42 p.i., 20 mice were sacrificed (10 infected and 10 controls) following behavioural testing. Five infected mice per strain were randomly assigned to the Baermann technique, and from these mice, brain, liver, lungs and right hind-leg muscle were taken and processed exactly as above. From the 10 remaining

mice per strain (5 infected and 5 controls – or 10 controls on day 42 p.i.), organs were taken for immunological analysis, but this will be described in Chapter 5.

3.2.2 Recovery of *Toxocara canis* larvae from mouse tissues using the Baermann technique

This technique was modified from previous descriptions (Sprent 1952), in order to reduce evaporation and improve larval recovery.

A double layer of gauze (sterile absorbent Type 13 light gauze) was fixed to the bottom of an open-ended small tube (universal tube cut in half) using an elastic band. The small tube was placed into a conical 50ml centrifuge tube, filled with 37ml of either saline or enzyme solution (depending on tissue sample), and adjusted so it reached the 35ml mark. The centrifuge tube was then placed in a water bath set to $37^{o}C$, and the water level was topped up to the 15ml mark on the tube. The macerated tissue sample (in either saline or enzyme solution) was then poured into the small tube so that it lay on the gauze. The flat-bottomed tubes were rinsed to ensure the entire sample was removed. The small tube was then capped to avoid evaporation, and the samples were left in the water bath for 24 hours (Plates 3.2.1 and 3.2.2).

Each tissue sample was processed separately using this method. This procedure works on the basis that any live larvae present in the tissue sample will migrate through the gauze towards the warm saline/enzyme solution heated by the water bath.

Following the 24-hour incubation period, all centrifuge tubes were collected from the water baths. The small tubes were removed, and the gauze (with the tissue sample) was discarded. The centrifuge tubes were capped and spun at 2000 G for 5 minutes. The supernatant was aspirated down to 10mls, and an equal volume of 6% formalin (Appendix 2) was added to fix any larvae present in the sample. Samples were stored at room temperature until enumeration.

3.2.3 Larval counts

To aid enumeration, sample volume was reduced to 5mls following centrifugation at 2000 G for 5 minutes. Each sample was then vortexed thoroughly for 15-20 seconds to ensure an even mix of solution. A volume of 200μl was transferred onto a clean glass slide, and systematically screened for larvae under a light microscope (on the phase contrast setting) using the x10 objective. Where brain material was being screened, this procedure was repeated until the entire sample was examined. For the visceral organ material, the procedure was repeated five times so that a total volume of 1ml was examined - this was due to time constraints, given the sheer volume of samples. In these cases, relevant calculations were made to obtain the total number of larvae in each 5ml sample.

In experiment 1, larvae were enumerated in the brains of all strains of mice in order to identify a susceptible and resistant strain. Once these strains had been chosen (BALB/c and NIH), larvae were enumerated in their liver, lungs and musculature. In experiment 2, larvae were enumerated in the brains and visceral organs of BALB/c and NIH mice.

3.2.4 Statistical analysis

All statistical analysis was carried out at the 95% confidence limit, and data were checked for normality prior to testing.

3.2.4.1 Experiment 1

Overall effects of day and strain on larval recoveries from the brain were investigated across all 7 strains of mice by means of a 2-way ANOVA, and F-ratios, with degrees of freedom, and *P* values are given in the text. Separate strain comparisons were made using least significant difference (LSD) post-hoc tests, in order to ascertain a resistant and susceptible strain of mouse (with regard to cerebral larval establishment), and *P* values are reported in the text.

Once the susceptible and resistant strains had been chosen, the effects of day and strain on larval recoveries from the brain and visceral organs were investigated using a

multivariate analysis of variance (MANOVA), which allowed for more than one observed variable to be analysed at once – since the number of larvae in the organs may not be independent of each other. Since the MANOVA does not allow for the analysis of between-group differences (by both day and strain), separate 2-way ANOVAs and LSD post-hoc tests were carried out on data for each organ. Appropriate statistics are quoted where necessary.

Effects of day and strain on the percentages of total larvae recovered from each organ were investigated using a MANOVA (data were arcsine square root transformed prior to analysis), and F ratios, with degrees of freedom, and P values are given. Separate 2-way ANOVAs with LSD post-hoc tests were performed for each strain to investigate the effects of organ and day on larval recoveries, and appropriate statistics are reported.

3.2.4.2 Experiment 2

A MANOVA was used to investigate effects of day and strain on larval recoveries from the brain and visceral organs of BALB/c and NIH mice, and F ratios, with degrees of freedom, and P values are given in the text. Between-group differences were then investigated as for experiment 1, by means of a 2-way ANOVA for each organ to determine the effects of day and strain on larval recoveries. As for experiment 1, effects of day and strain on the percentages of total larvae recovered from each organ were investigated using a MANOVA (on transformed data), and statistics are given in the text. Separate 2-way ANOVAs with LSD post-hoc tests were performed for each strain to investigate the effects of organ and day on larval recoveries, and appropriate statistics are reported.

In both experiments, the effects of day and strain on the numbers of larvae recovered from the left and right cerebral hemispheres and lungs of BALB/c and NIH mice were investigated using 2-way ANOVAs and post-hoc tests, and statistics are given in the text.

50ml conical centrifuge tube

Saline or enzyme solution within centrifuge tube

Small, capped tube (within centrifuge tube), with double layer of gauze attached with elastic band.

Water (37°C)
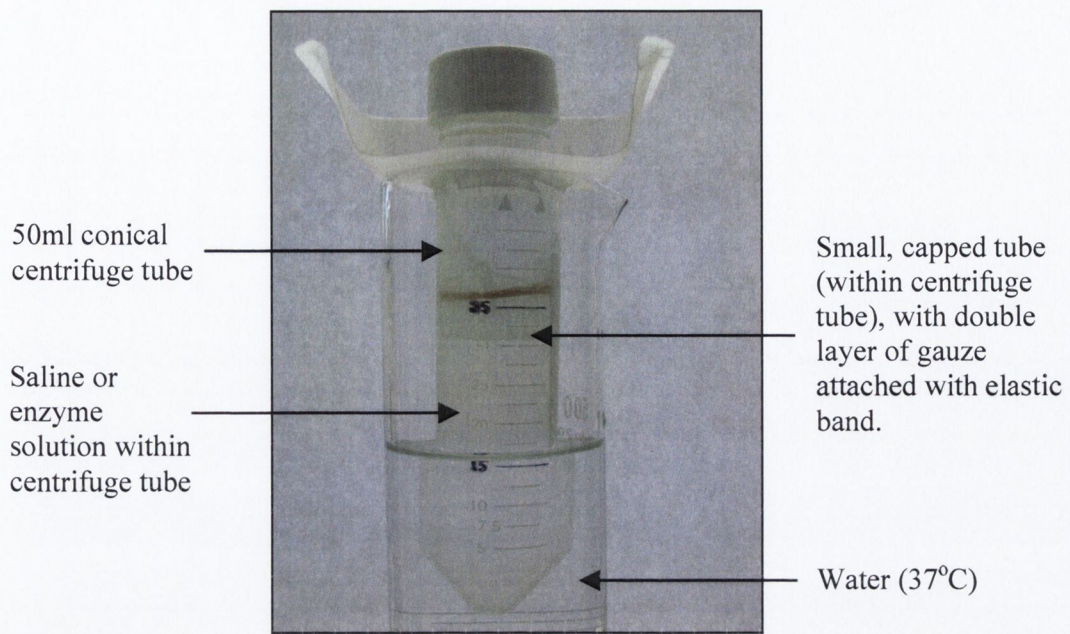
Plate 3.2.1: Demonstration of modified Baermann apparatus, used to recover *T. canis* larvae from mouse tissues.

Plate 3.2.2: Baermann tubes set up in water baths in laboratory

3.3 RESULTS

3.3.1 Experiment 1: Optimisation of a murine model for cerebral toxocariasis - identifying two divergent phenotypes

3.3.1.1 Larval recoveries from the brains of different strains of inbred mice

Toxocara canis larvae were recovered from the brains of all strains of mice, on each post mortem date in experiment 1, and recoveries, as a percentage of inoculum, ranged from 3-19% (Table 3.3.1). In all strains, larvae had reached the brain in appreciable numbers by as early as day 7 p.i – the highest numbers being observed in the brains of A/J mice (mean ± SD: 211 ± 31.50). The general pattern of accumulation in the brain mirrored that of previous studies, i.e. increasing over the course of infection, with numbers peaking around days 35 and 42 p.i. – although this pattern was not evident in A/J mice, where larval numbers remained similar between days 7, 14 and 35 p.i. before increasing on day 42 p.i.

A 2-way ANOVA revealed significant effects of day and strain on the numbers of larvae recovered from the brain (day: $F_{3,107} = 17.0$, $P \leq 0.0001$; strain: $F_{6,107} = 7.3$, $P \leq 0.0001$). Since NIH mice appeared to carry lower larval burdens overall, comparisons were made with each other strain in order to identify the most divergent phenotypes. These are illustrated in Figures 3.3.1 to 3.3.6.

Figure 3.3.1 shows that larval recoveries differed significantly at day 42 p.i. only, between NIH and SWR mice (2-way ANOVA post-hoc tests: $P = 0.02$). Figures 3.3.2, 3.3.3 and 3.3.4 show that larval recoveries differed significantly between: NIH and A/J mice on day 7 p.i. ($P = 0.04$) and day 42 p.i. ($P = 0.004$); NIH and C57BL/6j mice on day 14 p.i. ($P = 0.03$) and day 42 p.i. ($P \leq 0.0001$); and NIH and C3H/HeN mice on day 35 p.i. ($P = 0.035$) and day 42 p.i. ($P = 0.002$). Figure 3.3.5 shows that larval recoveries from CBA/Ca mice differed significantly from NIH mice on day 14 p.i. ($P = 0.03$), day 35 p.i. ($P = 0.01$), and day 42 p.i ($P \leq 0.0001$). Figure 3.3.6 illustrates that larval recoveries from BALB/c mice differed significantly from NIH mice on day 7 p.i. ($P = 0.05$), day 35 p.i. ($P = 0.02$), and day 42 p.i ($P = 0.0001$).

Table 3.3.1: Mean larval burdens, with standard deviations, and percentage of inocula recovered from the brains of different inbred *T. canis*-infected mice over the course of infection. ([a] recoveries expressed as percentage of 2000 *T. canis* ova inocula).

Strain		Days post-infection			
		7	14	35	42
A/J	Mean	211	215	222	294
	SD	31.5	39.4	68.4	24.8
	%[a]	10.5	10.7	11.1	14.7
BALB/c	Mean	205	340	371	356
	SD	76.9	143.7	148.4	117.7
	%	10.2	17.0	18.5	17.8
CBA/Ca	Mean	189	366	380	361
	SD	55.8	102.1	81.6	122.0
	%	9.5	18.3	19.0	18.0
C3H/HeN	Mean	195	246	361	305
	SD	80.2	65.7	32.3	121.9
	%	9.7	12.3	18.0	15.2
C57BL/6j	Mean	142	372.5	326	382.5
	SD	62.9	137.5	36.1	123.3
	%	7.1	18.6	16.3	19.1
NIH	Mean	93	242	238	124
	SD	40.4	90.4	145.3	89.3
	%	4.6	12.1	11.9	6.2
SWR	Mean	163.7	188.7	287	272.5
	SD	33.5	92.8	66.4	105.4
	%	8.2	9.4	14.3	13.6
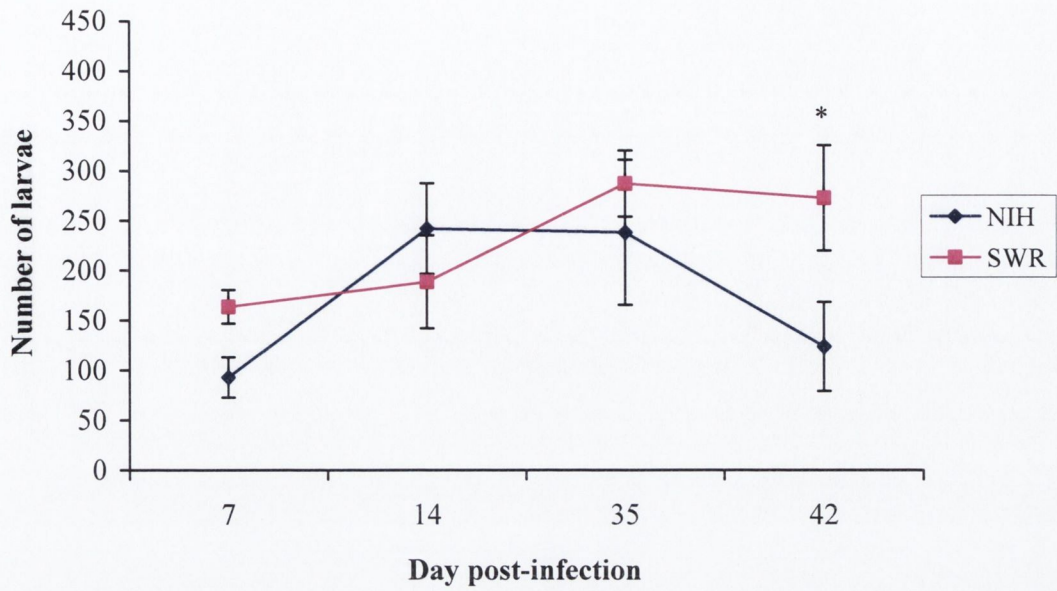
Figure 3.3.1: Mean (± SEM) number of larvae recovered from the brains of *T. canis*-infected NIH and SWR mice. (Between strain comparisons: * $P \leq 0.05$).
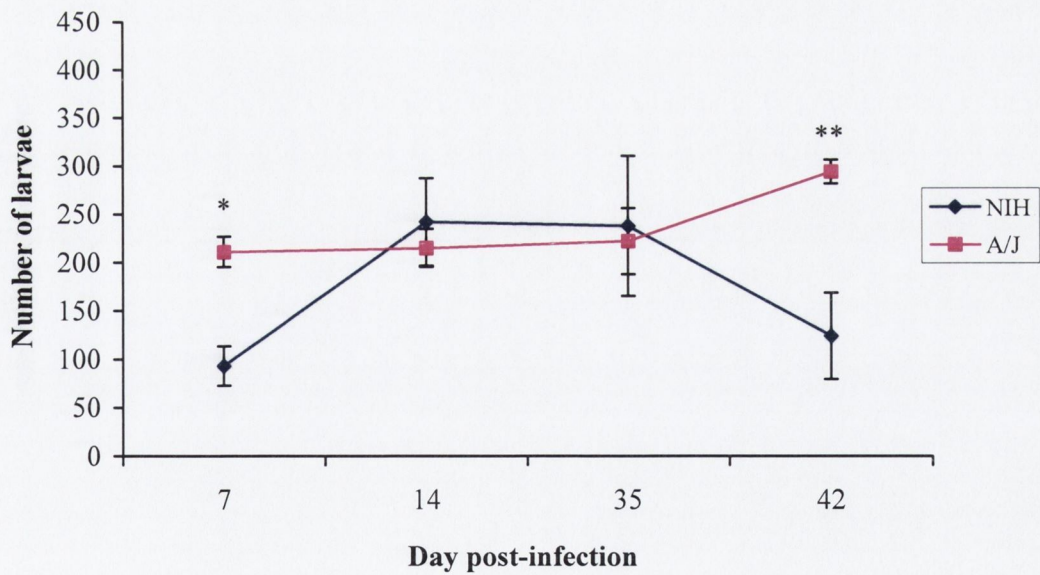
Figure 3.3.2: Mean (± SEM) number of larvae recovered from the brains of *T. canis*-infected NIH and A/J mice. (Between strain comparisons: * $P \leq 0.05$; ** $P \leq 0.01$).
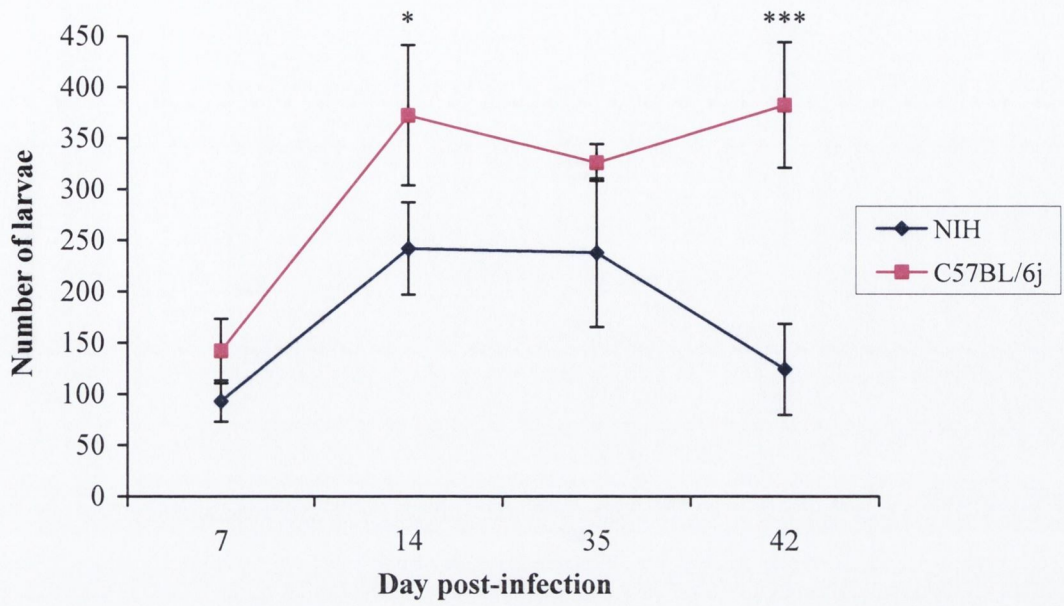
Figure 3.3.3: Mean (± SEM) number of larvae recovered from the brains of *T. canis*-infected NIH and C57BL/6j mice. (Between strain comparisons: * $P \leq 0.05$; *** $P \leq 0.0001$).
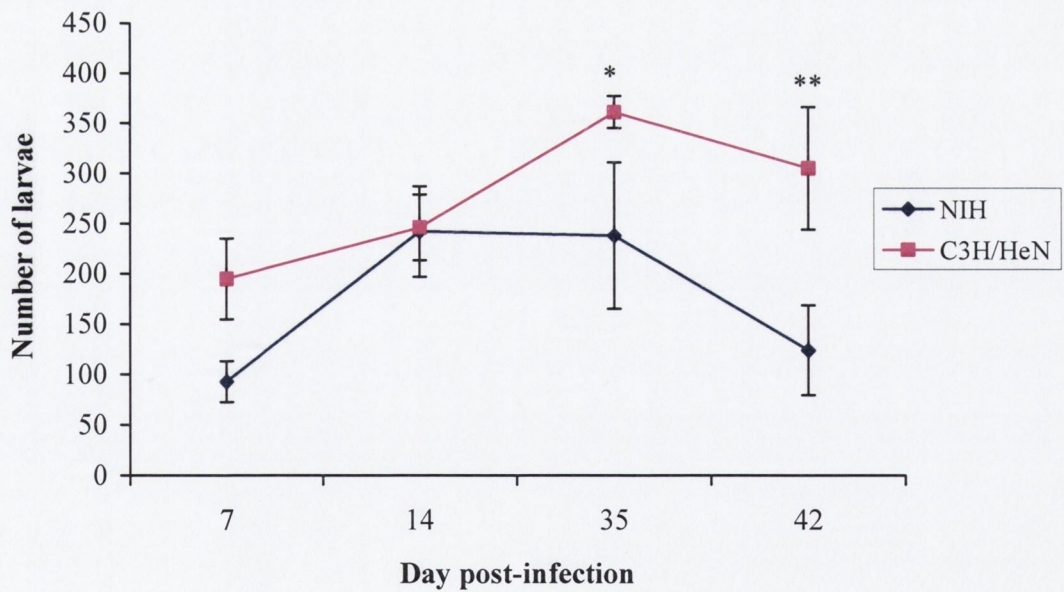
Figure 3.3.4: Mean (± SEM) number of larvae recovered from the brains of *T. canis*-infected NIH and C3H/HeN mice. (Between strain comparisons: * $P \leq 0.05$; ** $P \leq 0.01$).
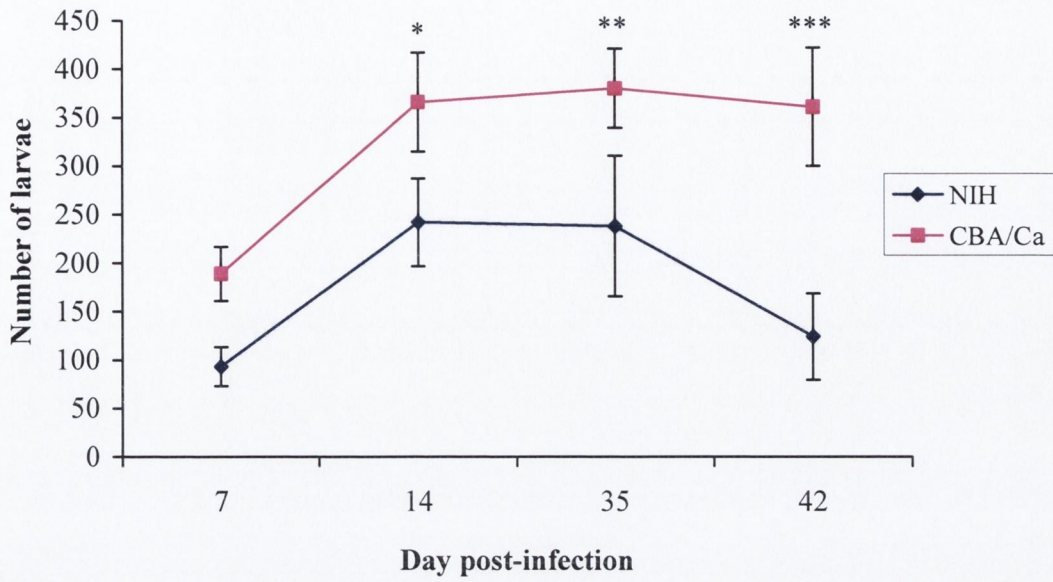
Figure 3.3.5: Mean (± SEM) number of larvae recovered from the brains of *T. canis*-infected NIH and CBA/Ca mice. (Between strain comparisons: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.0001$).
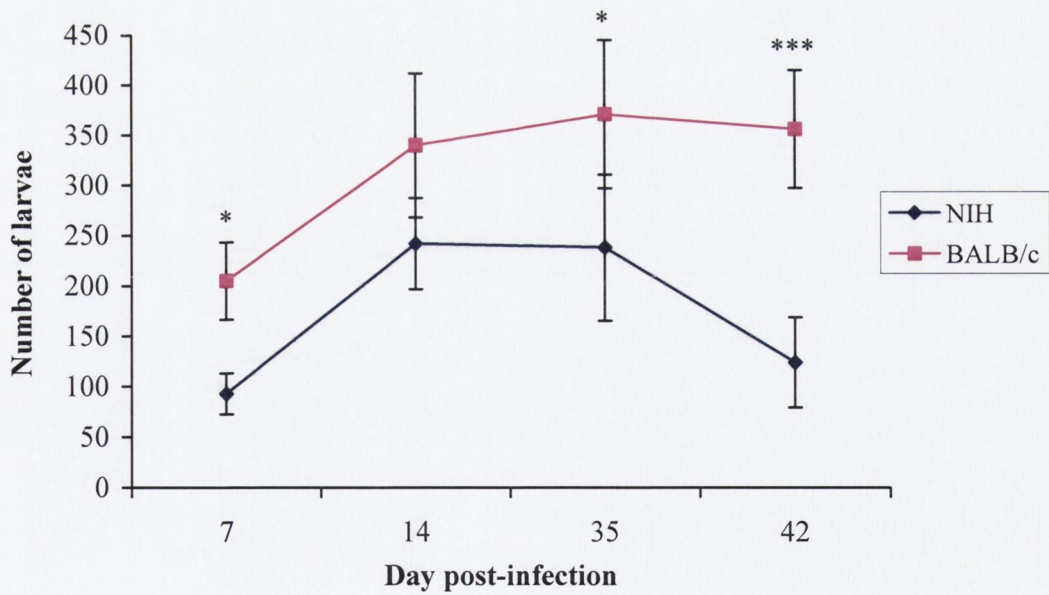
Figure 3.3.6: Mean (± SEM) number of larvae recovered from the brains of *T. canis*-infected NIH and BALB/c mice. (Between strain comparisons: * $P \leq 0.05$; *** $P \leq 0.0001$).

Since NIH mice carried the lowest larval burden overall, these mice were chosen as the resistant strain to *T. canis* infection. BALB/c mice were chosen over CBA/Ca mice as the susceptible strain, since these mice had a statistically higher larval burden in the brain than NIH mice on days 7, 35 and 42 p.i., and they had previously been reported to be more susceptible to toxocariasis. In these chosen strains, differences between the numbers of larvae recovered from the left and right cerebral hemispheres were investigated using 2-way ANOVAs, and a significant effect of day was found (BALB/c: $F_{3,32} = 3.4$, $P = 0.03$; NIH: $F_{3,32} = 5.4$, $P = 0.004$). Post-hoc tests revealed no significant differences between sides of the brain within strains ($P > 0.05$ on all days post-infection, for both strains).

3.3.1.2 Course of migration of *Toxocara canis* larvae in a resistant and susceptible strain of mice

Toxocara canis larvae were recovered from all visceral organs of BALB/c and NIH mice on all days post-infection. Results from a MANOVA demonstrated a significant effect of day and strain on all organ larval recovery (brain and visceral organs) (day: $F_{3, 93} = 2.6$, $P = 0.004$; strain: $F_{1,29} = 4.5$, $P = 0.006$). Larval recoveries from the liver did not follow the expected pattern of decline, and actually increased over the course of infection (Figure 3.3.7), with both strains carrying similar burdens on days 7, 35 and 42 p.i. (mean ± SD: BALB/c, day 7 p.i.: 18 ± 14.0; day 35 p.i.: 35 ± 20.0; day 42 p.i.: 35 ± 11.2; NIH, day 7 p.i.: 20 ± 19.7; day 35 p.i.: 37 ± 22.8; day 42 p.i.: 38 ± 14.0). A 2-way ANOVA demonstrated no significant effects of day or strain on liver larval recoveries (day: $F_{3,32} = 2.4$, $P = 0.09$; strain: $F_{1,32} = 1.0$, $P = 0.33$), and although the larval numbers increased over infection, the differences between each day (for both strains) were not significant.

Highest larval recovery from the lungs of both strains of mice occurred on day 7 p.i. (mean ± S.D: 44 ± 17.10 and 24 ± 11.40, BALB/c and NIH mice respectively) and then decreased as infection progressed, although overall numbers were slightly higher in BALB/c mice (Figure 3.3.8). A 2-way ANOVA revealed overall significant effects of day and strain on total larval numbers in the lung (day: $F_{3,32} = 11.6$, $P \leq 0.0001$; strain: $F_{1,32} = 5.5$, $P = 0.02$), and post-hoc tests demonstrated a significantly higher burden in BALB/c mice on day 7 p.i. compared with NIH mice ($P = 0.005$). Recoveries on all

other days were similar between strains (mean ± SD: BALB/c, day 14 p.i.: 20 ± 7.1; day 35 p.i.: 14 ± 7.4; day 42 p.i.: 10 ± 10.6; NIH, day 14 p.i.: 15 ± 12.2; day 35 p.i.: 11 ± 6.5; day 42 p.i.: 7 ± 5.7). There was a significant effect of day on the numbers of larvae recovered from left and right lungs of BALB/c and NIH mice (2-way ANOVA: BALB/c: $F_{3,32} = 8.1$, $P = 0.0004$; NIH: $F_{3,32} = 3.9$, $P = 0.02$). Post-hoc tests revealed no significant differences between larval recoveries from left and right lungs of NIH mice during infection (Figure 3.3.9), however there was a significantly higher larval burden in the right lung compared with the left, on day 7 p.i., in BALB/c mice ($P = 0.03$) (Figure 3.3.10).

The number of larvae recovered from the musculature was generally lower than the other organs, and varied throughout infection. These lower numbers, however, may be a reflection of the small muscle sample taken. Recoveries from both strains showed opposite patterns to each other – larval numbers decreased over infection in BALB/c mice, and then increased between days 35 and 42 p.i., whereas in NIH mice, larval numbers increased over infection, and then decreased between days 35 and 42 p.i. (Figure 3.3.11). A 2-way ANOVA revealed a significant interaction between day and strain ($F_{3,32} = 3.9$, $P = 0.02$), and post-hoc tests demonstrated that BALB/c mice had a significantly higher larval burden in their muscle than NIH mice on day 42 p.i. ($P = 0.006$).

On the whole, the total number of larvae recovered from mice over the course of infection was higher in BALB/c mice, compared with NIH (Figure 3.3.12). A 2-way ANOVA revealed significant effects of strain and day on the total numbers of larvae recovered (strain: $F_{1,32} = 16.3$, $P = 0.0003$; day: $F_{3,32} = 3.3$, $P = 0.0325$), and post-hoc tests showed that significantly more larvae were recovered from NIH mice on day 42 p.i. compared with BALB/c mice ($P = 0.002$). More larvae were recovered from BALB/c mice on days 14, 35 and 42 p.i. compared with day 7 p.i., but the differences were not significant. Significantly more larvae were recovered from NIH mice on days 14 and 35 p.i. compared with day 7 p.i. ($P = 0.04$ for both days), and less larvae were recovered on day 42 p.i. compared with day 35 p.i., although this difference was not significant ($P = 0.09$).
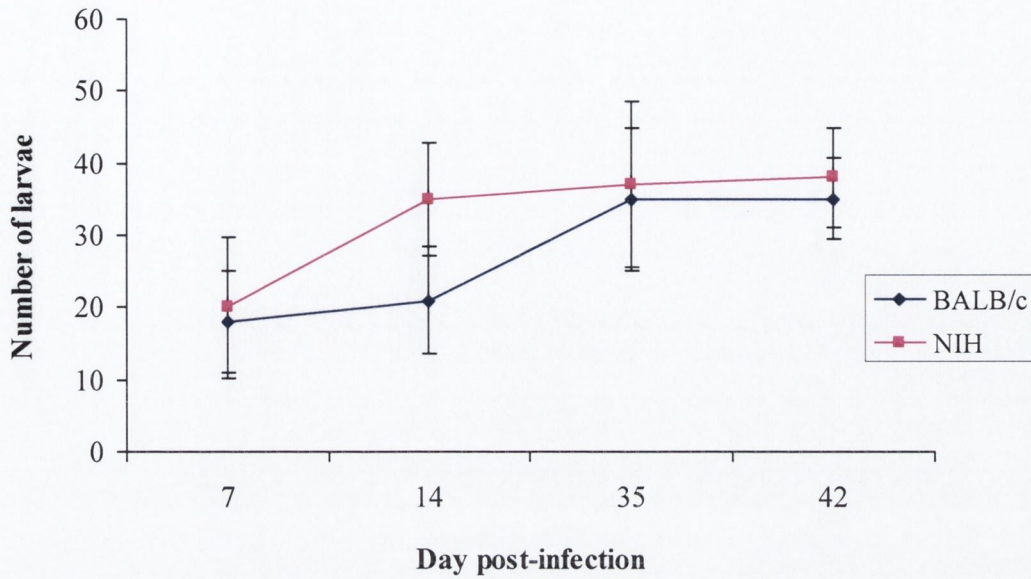
Figure 3.3.7: Mean number (± SEM) of larvae recovered from the livers of *T. canis*-infected BALB/c and NIH mice.
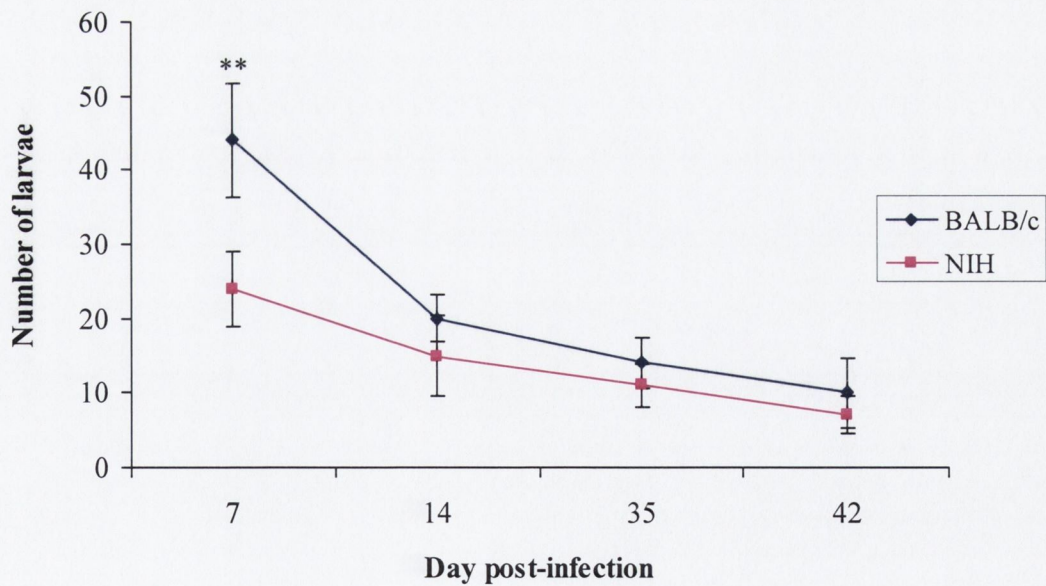
Figure 3.3.8: Mean number (± SEM) of larvae recovered from the lungs of *T. canis*-infected BALB/c and NIH mice. (Between strain comparison: ** $P \leq 0.01$).
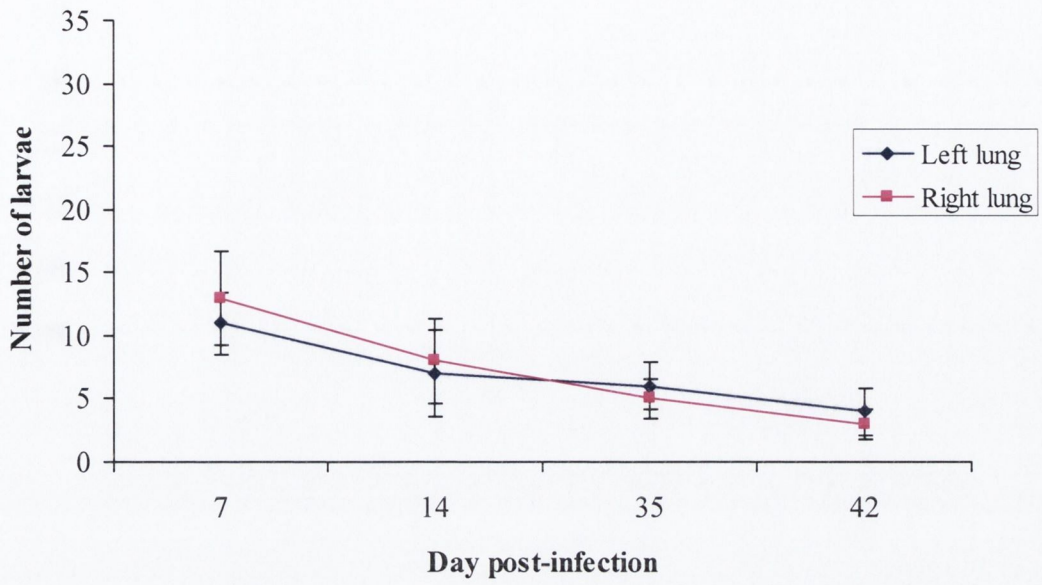
Figure 3.3.9: Mean number (± SEM) of larvae recovered from the left and right lungs of *T. canis*-infected NIH mice.
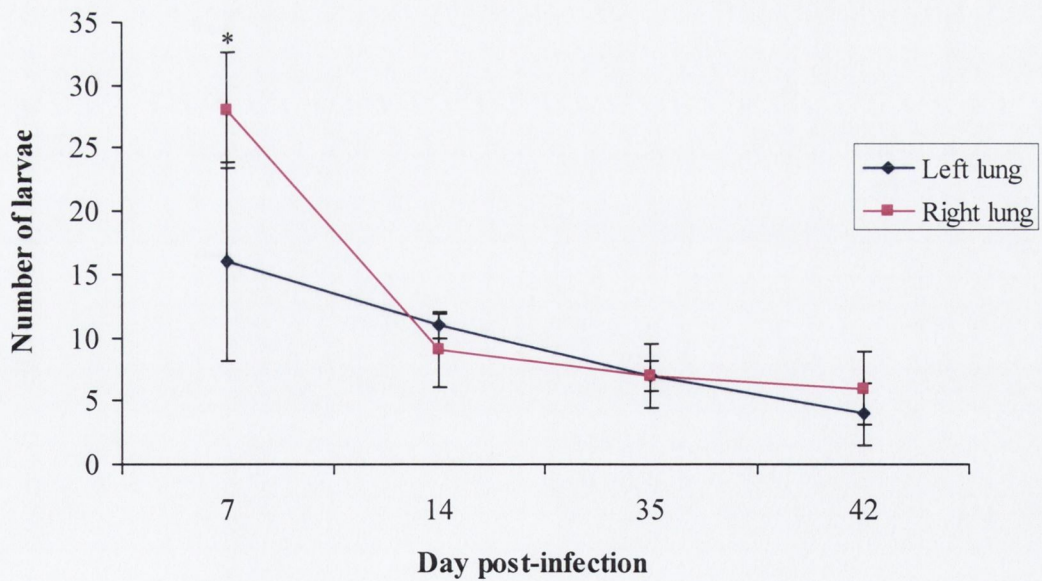
Figure 3.3.10: Mean number (± SEM) of larvae recovered from the left and right lungs of *T. canis*-infected BALB/c mice. (Between strain comparison: * $P \leq 0.05$).
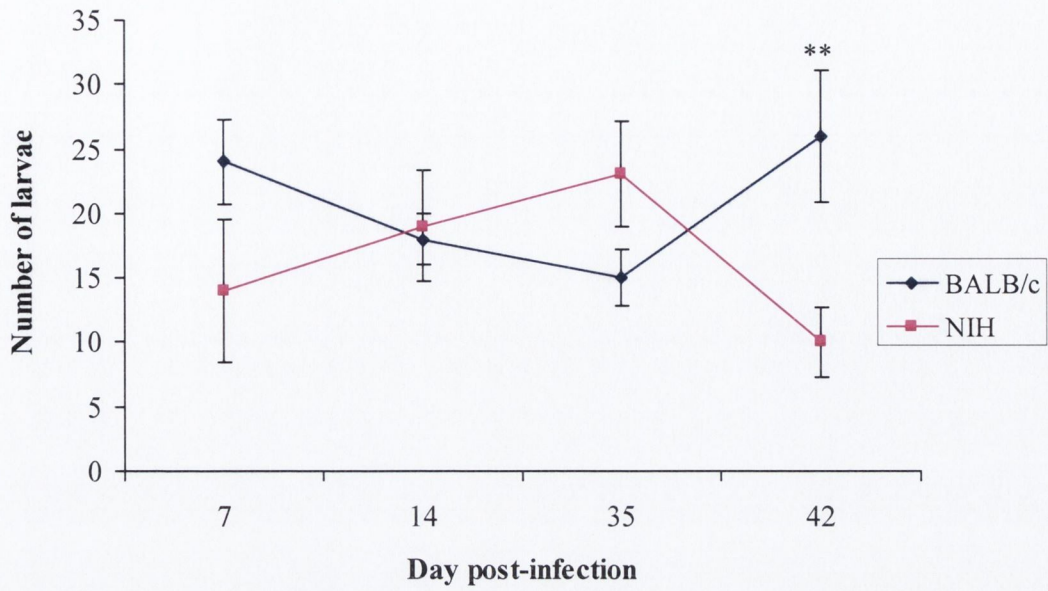
Figure 3.3.11: Mean number (± SEM) of larvae recovered from the musculature of *T. canis*-infected BALB/c and NIH mice. (Between strain comparison: ** $P \leq 0.01$).
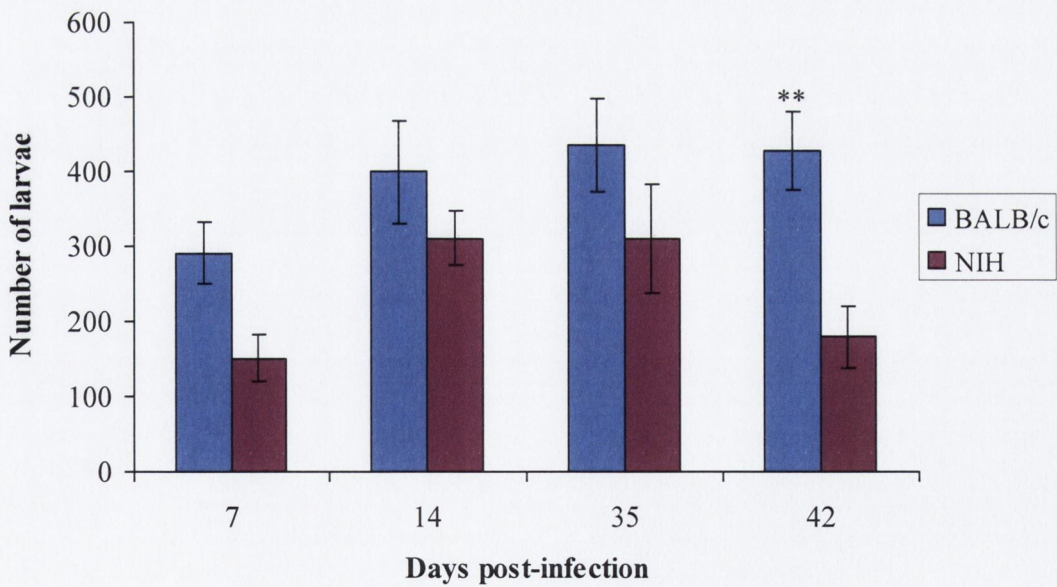
Figure 3.3.12: Mean total number (± SEM) of larvae recovered from *T. canis*-infected BALB/c and NIH mice over the course of infection. (Between strain comparison: ** $P \leq 0.01$).

Since the total number of larvae recovered during infection differed between the strains, larval recoveries from each organ were investigated as a percentage of the total recovery. Results of a MANOVA revealed significant effects of day and strain on the percentage of total larval recoveries (day: $F_{3,93} = 3.0$, $P = 0.001$; strain: $F_{1,29} = 3.2$, $P = 0.026$). Figure 3.3.13 illustrates very clearly that on all days post-infection, the vast majority of larvae were recovered from the brains of BALB/c mice compared with all other organs. A 2-way ANOVA revealed significant effects of organ, and the interaction between organ and day, on the percentage of total larvae recovered from BALB/c mice (BALB/c: organ: $F_{3,64} = 502.2$, $P \leq 0.0001$; organ x day: $F_{9,64} = 5.4$, $P \leq 0.0001$). Post-hoc tests revealed that significantly more larvae were recovered from the brain on all days post-infection compared with the other organs ($P \leq 0.0001$, for all comparison). Significantly more larvae were recovered from the brain on days 14, 35 and 42 p.i., compared with day 7 p.i. ($P \leq 0.0001$), although there were no differences between larval recoveries on the later days. There was a significant decrease in the number of larvae recovered from the lungs between days 7 and 14 p.i. ($P = 0.003$), and the numbers remained significantly lower throughout infection (day 35 p.i.: $P = 0.0001$; day 42 p.i.: $P \leq 0.0001$). The percentage of total larvae recovered from the liver and muscle was similar throughout infection.

The mean percentage of total larvae recovered from each organ in NIH mice over the course of infection is illustrated in Figure 3.3.14. As was the case for BALB/c mice, the vast majority of larvae were recovered from the brains of NIH mice compared with all other organs. A 2-way ANOVA revealed significant effects of organ, and the interaction between organ and day, on larval recoveries (organ: $F_{3,64} = 145.2$, $P \leq 0.0001$; organ x day: $F_{9,64} = 3.0$, $P = 0.0048$). Post-hoc tests revealed that a significantly higher percentage of larvae were recovered from the brain on all days post-infection compared with the other organs ($P \leq 0.0001$, for all comparisons). There was a significant increase in the percentage of larvae recovered from the brain between days 7 and 14 p.i. ($P = 0.05$), but then the percentage decreased significantly between days 14 and 42 p.i. ($P = 0.03$). A significantly higher percentage of larvae were recovered from the lungs on day 7 p.i. compared with all other days post-infection ($P = 0.006$, for all comparisons). Interestingly, a significantly higher percentage of larvae were recovered from the liver on day 42 p.i. compared with all earlier days post-

infection ($P < 0.05$ for all comparisons). The percentage of larvae recovered from the muscle was similar on all days post-infection.

Comparing percentage of total larvae recovered from each organ on each separate day post-infection revealed differences between the strains. Two-way ANOVAs for each day post-infection revealed a significant effect of organ on the percentage of total larvae recovered (day 7 p.i.: $F_{3,32} = 130.1$, $P \leq 0.0001$; day 14 p.i.: $F_{3,32} = 194.7$, $P \leq 0.0001$; day 35 p.i.: $F_{3,32} = 178.8$, $P \leq 0.0001$; day 42 p.i.: $F_{3,32} = 77.1$, $P \leq 0.0001$). There was also a significant effect of the interaction between strain and organ, on the percentage of total larvae recovered on days 35 and 42 p.i. (day 35 p.i.: $F_{3,32} = 2.8$, $P = 0.05$; day 42 p.i.: $F_{3,32} = 5.3$, $P = 0.004$). Post-hoc tests demonstrated that significantly fewer larvae were recovered from the brains of NIH mice on days 35 and 42 p.i. compared with BALB/c mice (day 35 p.i.: $P = 0.05$; day 42 p.i.: $P = 0.008$) (Figures 3.3.15 to 3.3.18). There were fewer larvae recovered on days 7 and 14 p.i. also, although the differences did not reach statistical significance ($P = 0.156$ and $P = 0.151$, respectively). The percentage of total larvae recovered from all other organs was very similar between strains, although more larvae were recovered from the livers of NIH mice compared with BALB/c – significantly so on days 14 and 42 p.i. ($P = 0.04$ and $P = 0.01$, respectively).
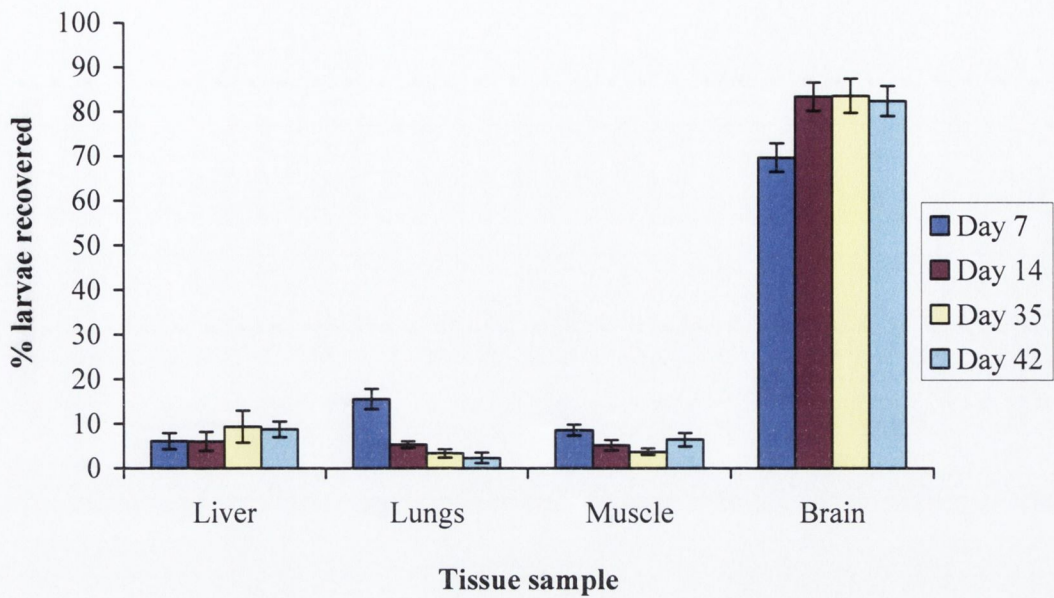
Figure 3.3.13: Mean percentage (± SEM) of total larvae recovered from each organ of *T. canis*-infected BALB/c mice, over the course of infection.
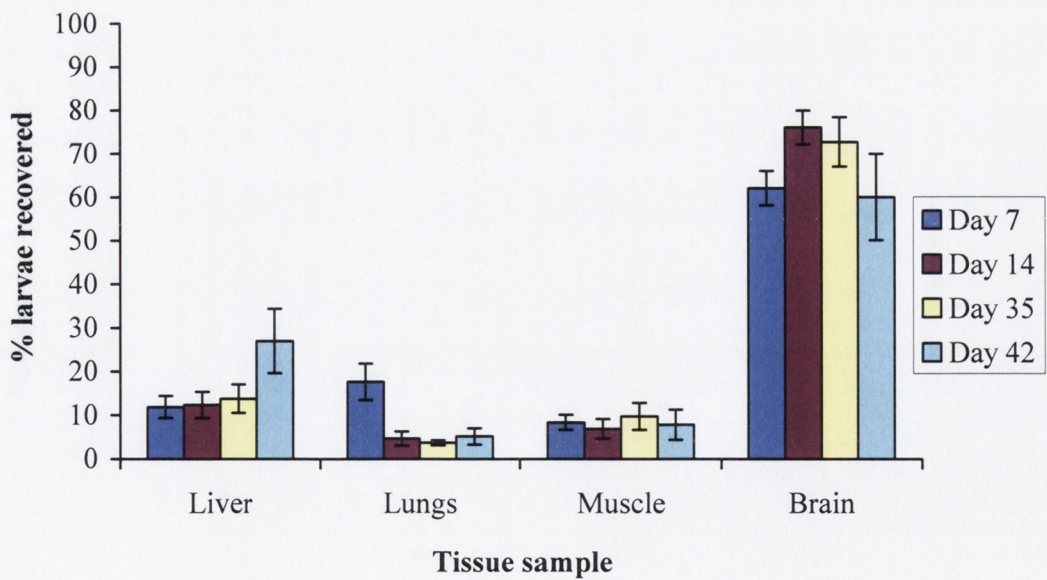
Figure 3.3.14: Mean percentage (± SEM) of total larvae recovered from each organ of *T. canis*-infected NIH mice, over the course of infection.
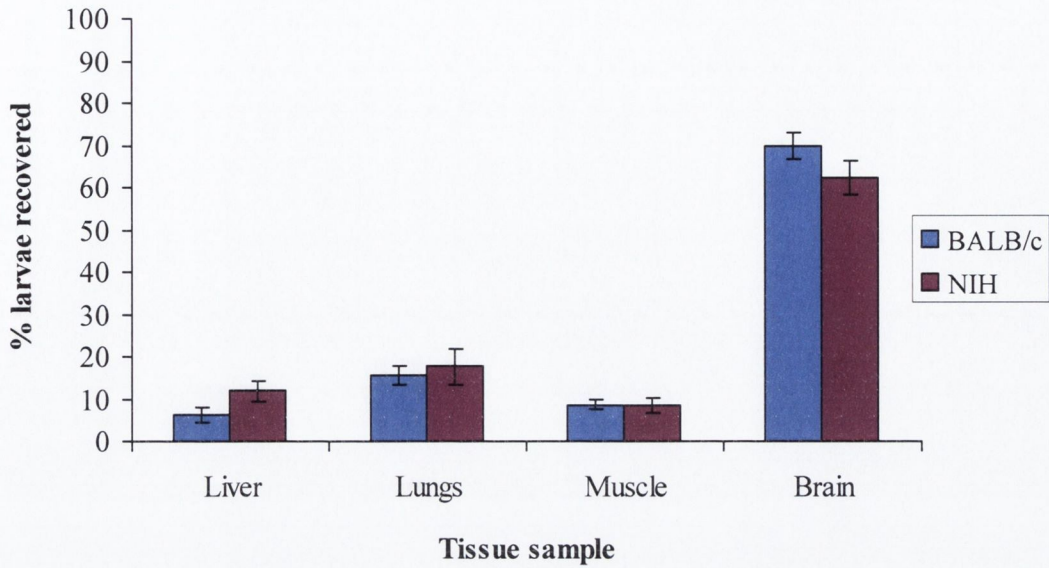
Figure 3.3.15: Mean percentage (± SEM) of total larvae recovered from different organs of *T. canis*-infected BALB/c and NIH mice on day 7 post-infection.
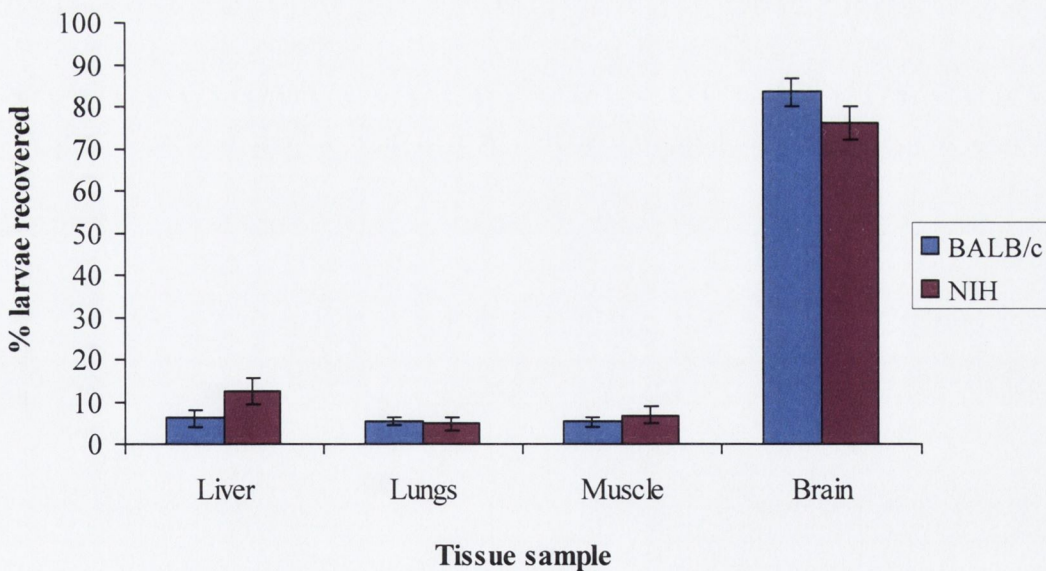
Figure 3.3.16: Mean percentage (± SEM) of total larvae recovered from different organs of *T. canis*-infected BALB/c and NIH mice on day 14 post-infection.
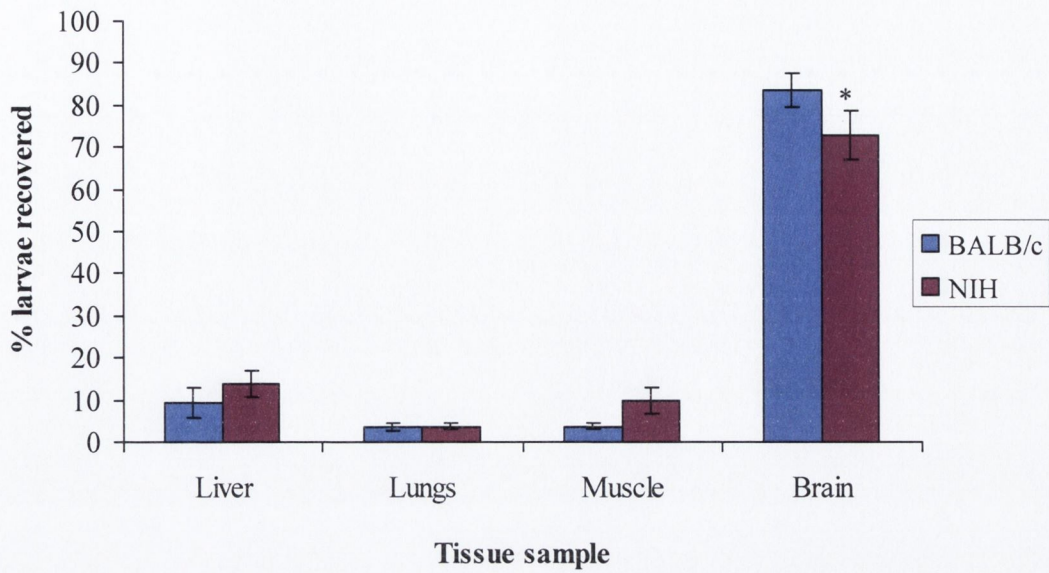
Figure 3.3.17: Mean percentage (± SEM) of total larvae recovered from different organs of *T. canis*-infected BALB/c and NIH mice on day 35 post-infection. (Between strain comparison: * $P \leq 0.05$).
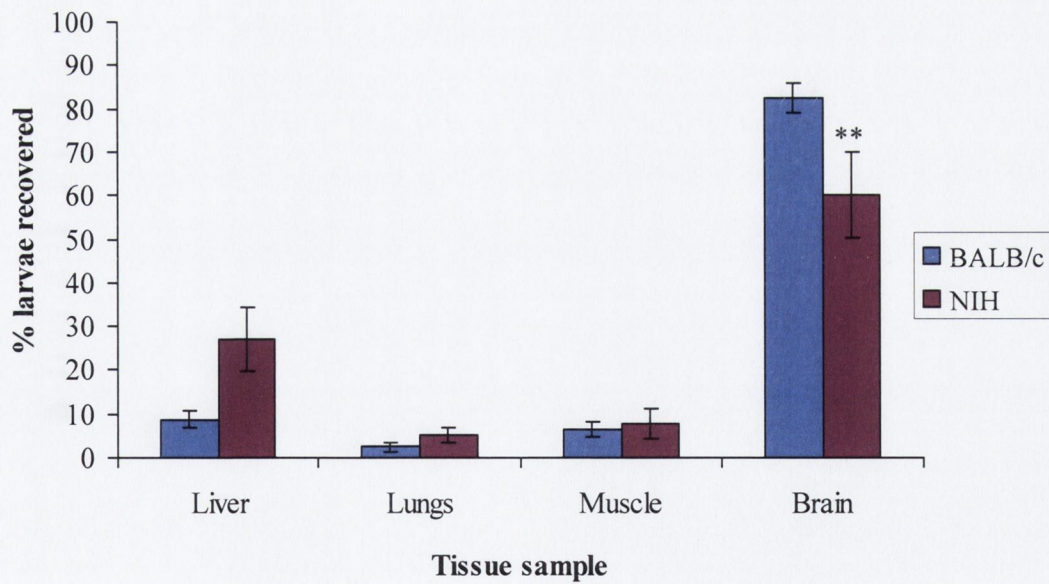
Figure 3.3.18: Mean percentage (± SEM) of total larvae recovered from different organs of *T. canis*-infected BALB/c and NIH mice on day 42 post-infection. (Between strain comparison: ** $P \leq 0.01$).

3.3.2 Experiment 2: Comparison of a susceptible and resistant strain of mice during *Toxocara canis* infection.

3.3.2.1 Larval recoveries from the brains of different strains of inbred mice

Larvae were recovered from the brains of BALB/c and NIH mice on all days post-infection, and burdens are shown in Figure 3.3.19. Although the recoveries, as a percentage of inoculum, were similar to experiment 1 (ranging from 3-18%), the individual counts for each day post-infection were very different to the first experiment, and, in fact, the strains no longer appeared to be divergent. The counts for both strains were very similar, and on day 42 p.i. there were more larvae in the brains of NIH mice compared with BALB/c mice (mean ± S.D: 350 ± 35.5 and 277 ± 64.5, respectively). A 2-way ANOVA showed there to be a significant effect of day, but not strain, on larval burdens in the brain (day: $F_{5,42} = 13.67$, $P \leq 0.0001$; strain: $F_{1,42} = 0.08$, $P = 0.77$). There were no significant differences between larval recoveries from left and right hemispheres of the brain in either strain of mice.
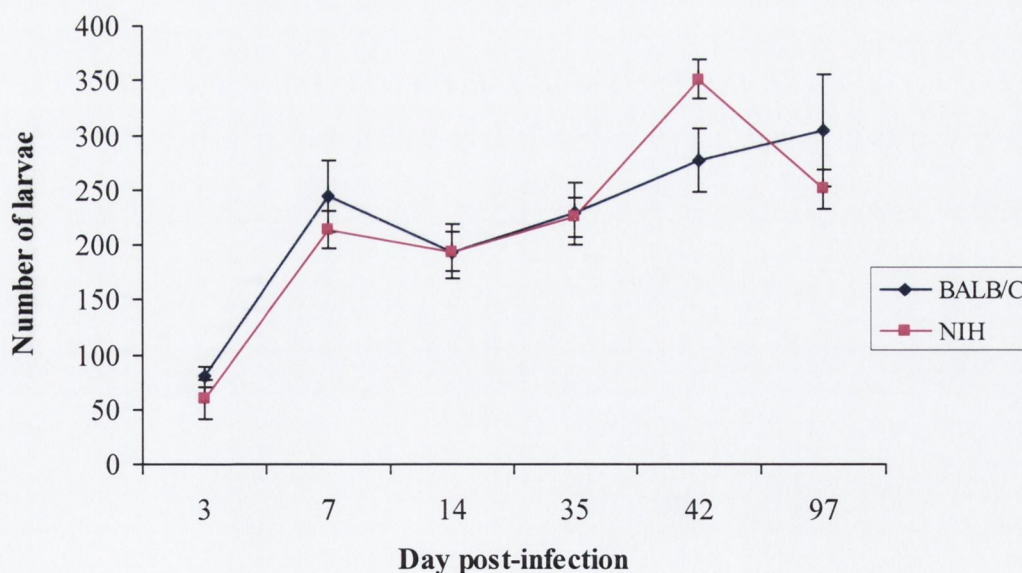
Figure 3.3.19: Mean number (± SEM) of larvae recovered from the brains of *T. canis*-infected BALB/c and NIH mice over the course of infection.

Tables 3.3.2 and 3.3.3 show the summary statistics for the larval counts from BALB/c and NIH mice, from experiments 1 and 2. It is evident in both tables that there was more variation in larval burdens obtained in experiment 1. For BALB/c mice, the maximum larval counts were much higher in experiment 1 compared with experiment 2, and for NIH mice, although the ranges of larval recoveries were similar between experiments, some of the minimum recoveries were lower in experiment 1, particularly day 42 p.i.

Table 3.3.2: Summary statistics for cerebral larval counts from BALB/c mice, from experiments 1 and 2.

Day p.i.	Experiment 1					Experiment 2			
	Mean	Min	Max	SD		Mean	Min	Max	SD
3						79.6	48	104	21.82
7	205	120	315	76.89		244.2	196	334	63.69
14	340	115	515	143.65		193.6	102	246	54.80
35	371	200	575	148.38		228.4	146	301	62.64
42	356	230	510	117.65		277	227	389	64.53
97						304.4	199	441	113.06

Table 3.3.3: Summary statistics for cerebral larval counts from NIH mice, in experiments 1 and 2.

Day p.i.	Experiment 1					Experiment 2			
	Mean	Min	Max	SD		Mean	Min	Max	SD
3						59.6	49	73	11.06
7	93	55	150	40.40		213.7	173	253	42.61
14	242	150	360	90.39		194	129	261	61.22
35	238	65	405	145.28		225.2	46	413	150.23
42	124	20	225	89.26		350.7	317	394	35.48
97						250.67	203	286	42.85

3.3.2.2 Course of migration of *Toxocara canis* larvae in a resistant and susceptible strain of mice

Toxocara canis larvae were recovered from all visceral organs of BALB/c and NIH mice on all days post-infection. Results of a MANOVA revealed a significant effect of day, but not strain, on larval recoveries from all organs of BALB/c and NIH mice (day: $F_{5,168} = 4.4$, $P \leq 0.0001$; strain: $F1,168 = 0.4$, $P = 0.829$). A 2-way ANOVA revealed a significant effect of day on the number of larvae recovered from the liver ($F_{5,42} = 17.2$, $P \leq 0.0001$). Highest recoveries were made from both strains on day 3 p.i. (Figure 3.3.20), and then decreased significantly by day 7 p.i. (post-hoc tests: BALB/c: $P \leq$ 0.0001; NIH: $P = 0.0004$). The numbers then remained low in both strains for the duration of infection, although there was a slight increase in recoveries from BALB/c mice between days 14 and 35 p.i. (mean ± SD: day 14 p.i.: 16 ± 8.94; day 35: 30 ± 21.79).

Larval recoveries from the lungs were similar between both strains throughout infection, and followed the same pattern as recoveries from the liver (Figure 3.3.21). A 2-way ANOVA revealed a significant effect of day on the number of larvae recovered ($F_{5,42} = 39.3$, $P \leq 0.0001$). Highest larval burdens in both strains were recorded on day 3 p.i., and then decreased significantly by day 7 p.i. (post-hoc tests: BALB/c: $P \leq$ 0.0001; NIH: $P \leq 0.0001$). Recoveries from both strains then remained low for the remainder of infection. Results from separate 2-way ANOVAs for each strain revealed a significant effect of day on the number of larvae recovered from the right and left lungs (BALB/c: $F5,46 = 33.4$, $P \leq 0.0001$; NIH: $F5,38 = 17.8$, $P \leq 0.0001$). Post-hoc tests revealed that there was a significantly higher larval burden in the right lung of BALB/c mice on day 3 p.i. ($P = 0.001$). There were no significant differences between right and left lung larval counts of NIH mice.

Larval recoveries from the musculature varied throughout infection, and were similar in pattern to recoveries from experiment 1 (Figure 3.3.22). A 2-way ANOVA revealed a significant effect of day, and a borderline significant effect of the interaction between day and strain, on larval recoveries from the musculature (day: $F_{5,42} = 4.4$, $P = 0.003$; day x strain: $F_{5,42} = 2.2$, $P = 0.06$). Post-hoc tests revealed that BALB/c mice carried a significantly higher burden in the musculature on day 7 p.i., compared with NIH mice

($P = 0.03$). Larval numbers peaked in BALB/c mice on day 7 p.i., with numbers increasing significantly between days 3 and 7 p.i. (post-hoc tests: $P = 0.0001$), and then decreasing significantly between days 7 and 35 ($P = 0.0004$). The numbers then remained low for the duration of infection, although there was a slight increase on day 42 p.i. Larval numbers in NIH mice peaked on day 14 p.i., with numbers increasing significantly from day 3 p.i. ($P = 0.006$), and then decreasing significantly by day 97 p.i. ($P = 0.04$).

On a whole, the number of larvae recovered from mice over the course of infection was similar in both strains, but generally lower than experiment 1. Results of a 2-way ANOVA revealed a significant effect of day on the total numbers of larvae recovered ($F_{5,42} = 5.4$, $P = 0.0006$), although post-hoc tests showed there to be no significant differences between the strains (Figure 3.3.23). However, there were differences within strains, with significantly fewer larvae being recovered from BALB/c mice on days 7, 14, 35 and 42 p.i. compared with day 3 p.i., and significantly fewer larvae being recovered from NIH mice on days 14 and 35 p.i. compared with day 3 p.i. In general, larval recoveries decreased until day 35 p.i., and then increased again, although never reaching the levels of recovery on day 3 p.i. Figures 3.3.24 and 3.3.25 illustrate the mean total number of larvae recovered from BALB/c and NIH mice in both experiments (with days 3 and 97 p.i. being excluded from experiment 2). Results from 2-way ANOVAs revealed a significant effect of experiment number on larval recoveries from BALB/c mice, and significant effects of experiment number and the interaction between experiment number and day, on larval recoveries from NIH mice (BALB/c: experiment number: $F_{1,31} = 6.2$, $P = 0.02$; NIH: experiment number: $F_{1,29} = 9.1$, $P = 0.005$; experiment number x day: $F_{3,29} = 3.9$, $P = 0.02$). Post-hoc tests revealed significantly fewer larvae were recovered from BALB/c mice in experiment 2 compared with experiment 1, on days 14 and 35 p.i. ($P = 0.027$ and 0.020, respectively). In contrast, there were significantly more larvae recovered from NIH mice in experiment 2 compared with experiment 1, on days 7 and 42 p.i. ($P = 0.01$ and 0.001, respectively).
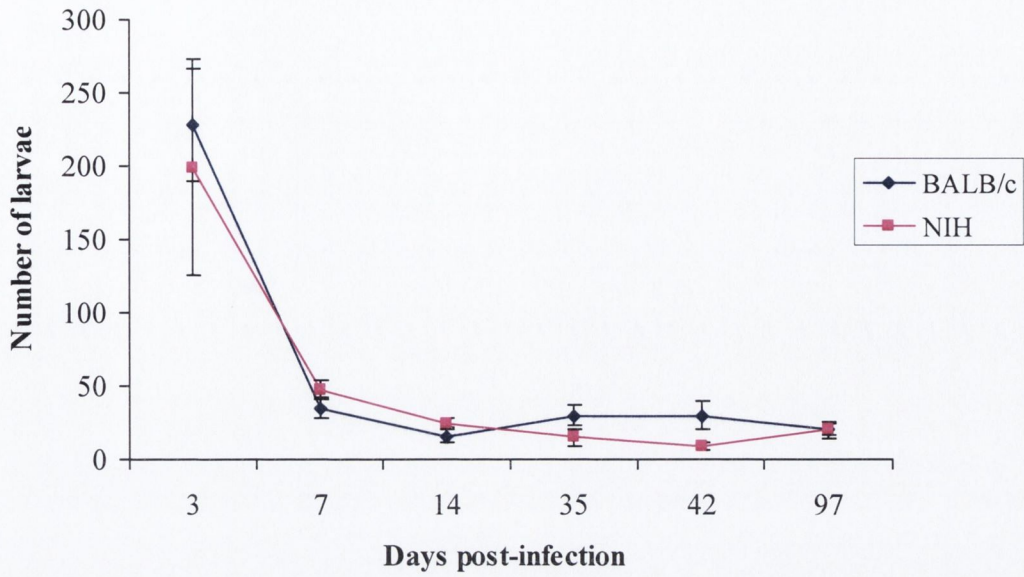
Figure 3.3.20: Mean number (± SEM) of larvae recovered from the livers of *T. canis*-infected BALB/c and NIH mice.
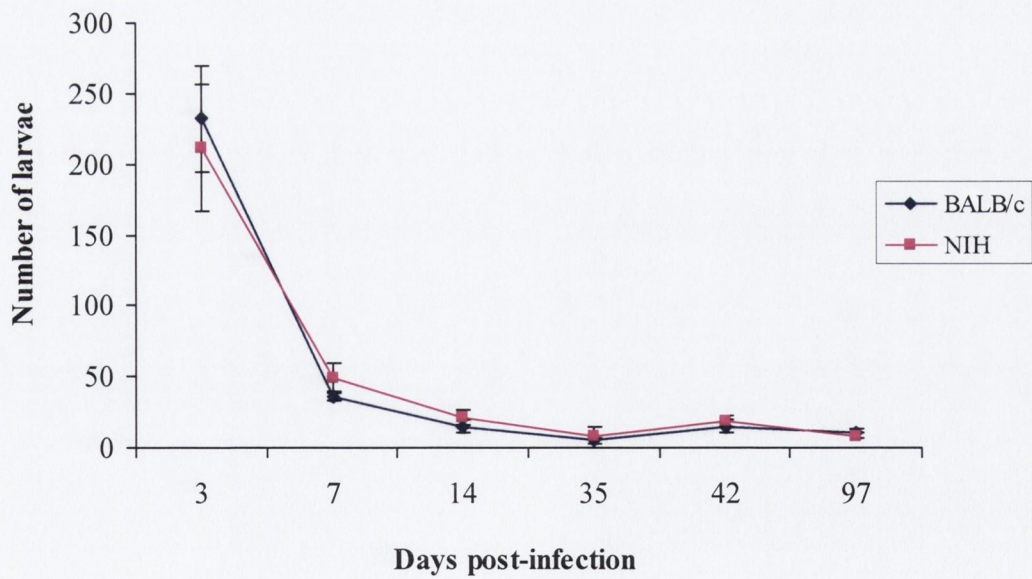
Figure 3.3.21: Mean number (± SEM) of larvae recovered from the lungs of *T. canis*-infected BALB/c and NIH mice.
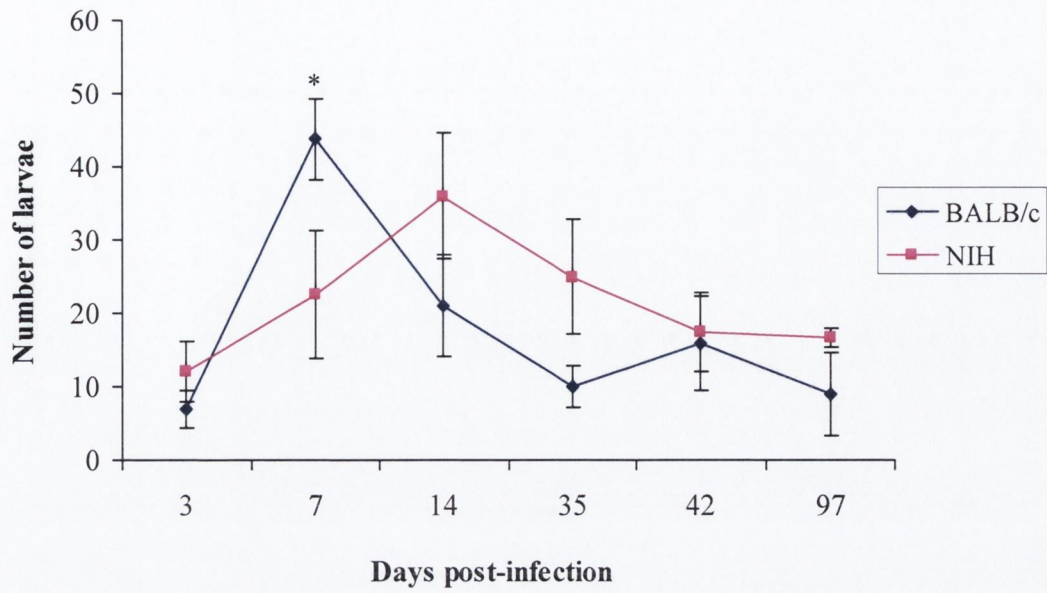
Figure 3.3.22: Mean number (± SEM) of larvae recovered from the musculature of *T. canis*-infected BALB/c and NIH mice. (Between strain comparison: * $P \leq 0.05$).
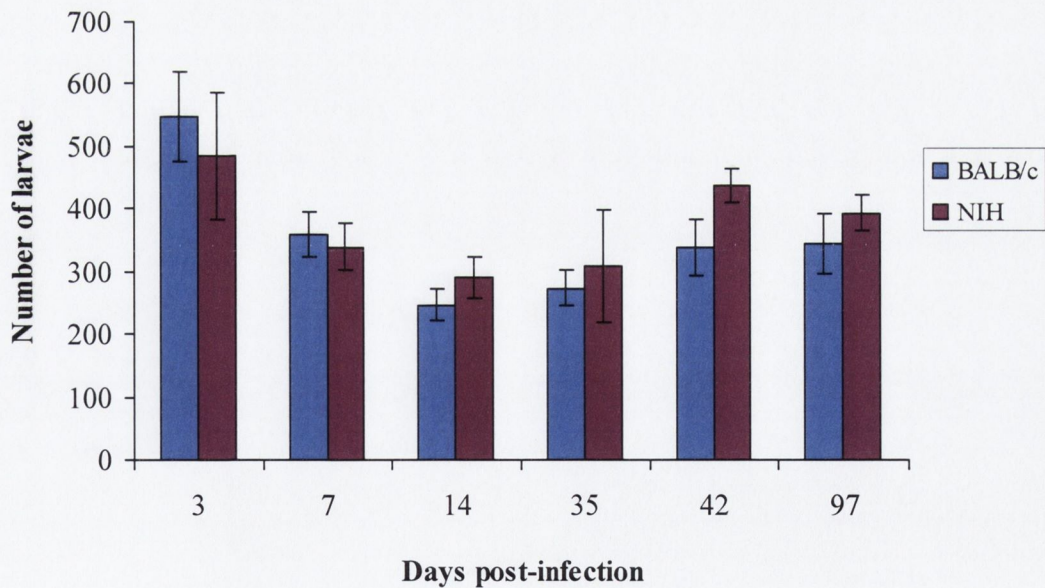
Figure 3.3.23: Mean total number (± SEM) of larvae recovered from *T. canis*-infected BALB/c and NIH mice over the course of infection.
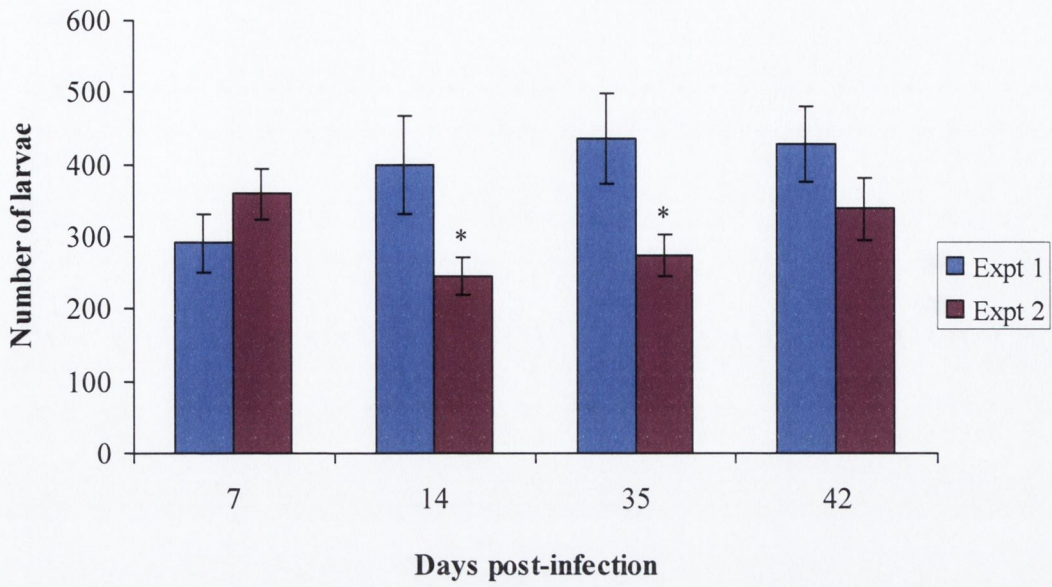
Figure 3.3.24: Mean total number (± SEM) of larvae recovered from *T. canis*-infected BALB/c mice in experiments 1 and 2. (Between experiment comparison: * $P \leq 0.05$).
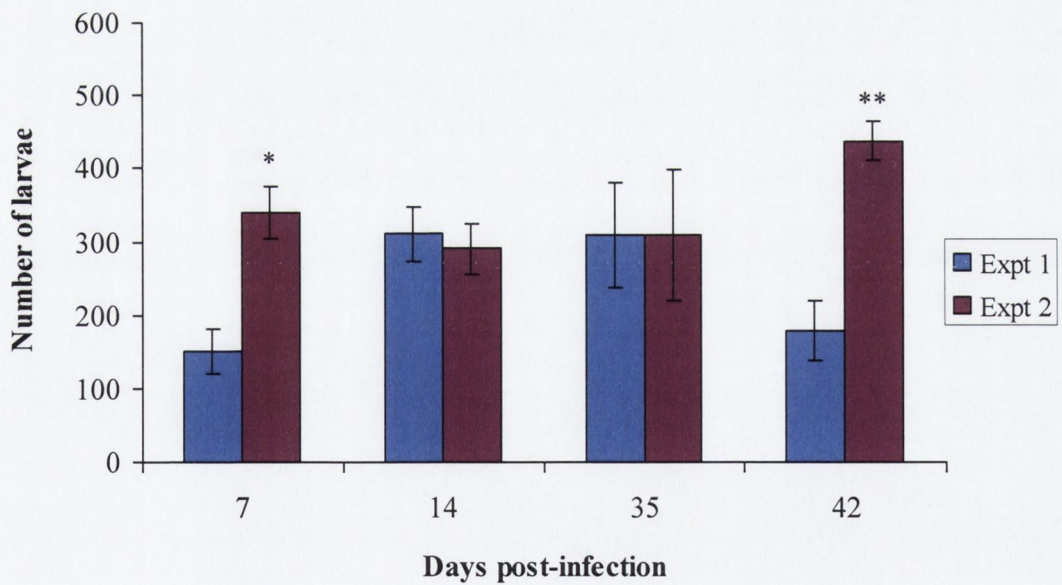
Figure 3.3.25: Mean total number (± SEM) of larvae recovered from *T. canis*-infected NIH mice in experiments 1 and 2. (Between experiment comparison: * $P \leq 0.05$; ** $P \leq 0.01$).

As for experiment 1, larval recoveries from experiment 2 were investigated as a percentage of the total number of larvae recovered over infection. Figures 3.3.26 and 3.3.27 illustrate nicely that in early infection, the majority of larvae were recovered from liver and lungs, and as infection progressed the percentage of larvae recovered from these organs decreased and the majority of larvae were recovered from the brain. Results of a MANOVA revealed significant effects of day, strain and the interaction between these factors, on the percentage of larvae recovered (day: $F_{5,168} = 6.2$, $P \leq 0.0001$; strain: $F_{1,39} = 12.2$, $P \leq 0.0001$; day x strain: $F_{5,168} = 1.9$, $P = 0.017$). Separate 2-way ANOVAs for each strain revealed significant effects of organ, and the interaction between organ and day, on the percentage of larvae recovered (BALB/c: organ: $F_{3,92} = 435.1$, $P \leq 0.0001$; organ x day: $F_{15,92} = 34.1$, $P \leq 0.0001$; NIH: organ: $F_{3,76} = 226.3$, $P \leq 0.0001$; organ x day: $F_{15,76} = 25.0$, $P \leq 0.0001$). There was a significant decrease in the percentage of larvae recovered from the liver and lungs on all days post-infection compared with day 3 p.i., for both BALB/c and NIH mice (post-hoc tests: liver: $P \leq 0.0001$, for all comparisons for both strains; lung: $P \leq 0.0001$, for all comparisons for both strains). In contrast, there was a significant increase in the number of larvae recovered from the brain on all days post-infection compared with day 3 p.i., for both strains ($P \leq 0.0001$, for all comparisons for both strains). The percentage of total larvae recovered from the musculature peaked on day 7 p.i. in BALB/c mice, with significantly more larvae being recovered on this day compared with day 3 p.i. ($P = 0.0005$). In NIH mice, the percentage of total larvae recovered from the musculature peaked on day 14 p.i., and was significantly higher compared with day 3 p.i. ($P = 0.003$).

Comparing the percentage of total larvae recovered from each organ on each day post-infection, revealed some interesting findings (Figures 3.3.28 to 3.3.33). Results from 2-way ANOVAs performed for each day post-infection revealed significant effects of organ on all days post-infection (day 3 p.i.: $F_{3,32} = 52.4$, $P \leq 0.0001$; day 7 p.i.: $F_{3,24} = 141.6$, $P \leq 0.0001$; day 14 p.i.: $F_{3,32} = 168.2$, $P \leq 0.0001$; day 35 p.i.: $F_{3,28} = 142.1$, $P \leq 0.0001$; day 42 p.i.: $F_{3,28} = 288.9$, $P \leq 0.0001$; day 97 p.i.: $F_{3,24} = 195.0$, $P \leq 0.0001$). There was a significant effect of strain on percentage larval recoveries on day 97 p.i. ($F_{3,24} = 4.6$, $P = 0.04$), and a significant effect of the interaction between strain and organ, on percentage larval recoveries on days 7, 35 and 97 p.i. (day 7 p.i.: $F_{3,24} = 3.7$, $P = 0.02$; day 35 p.i.: $F_{3,28} = 3.4$, $P = 0.03$; day 97 p.i.: $F_{3,24} = 6.0$, $P = 0.003$).

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Interestingly, post-hoc tests revealed significant differences between the percentages of total larvae recovered from the brains of BALB/c and NIH mice on days 14, 35 and 97 post-infection – with significantly fewer larvae being found in NIH mice ($P = 0.03$, $P = 0.02$ and $P = 0.0001$, respectively). Post-hoc tests also revealed a significantly lower percentage of larvae recovered from the musculature of NIH mice on day 7 p.i., compared with BALB/c mice ($P = 0.01$). The percentage of total larvae recovered from the livers of NIH mice was lower than BALB/c mice on days 35 and 42 p.i. ($P = 0.04$ and $P = 0.006$, respectively).
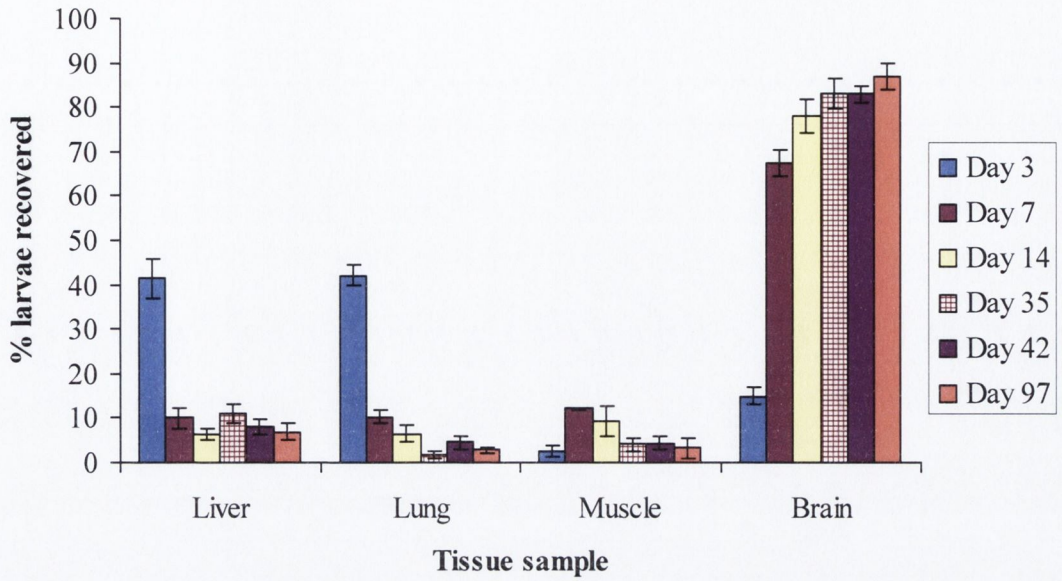
Figure 3.3.26: Mean percentage (± SEM) of total larvae recovered from each organ of *T. canis*-infected BALB/c mice, over the course of infection.
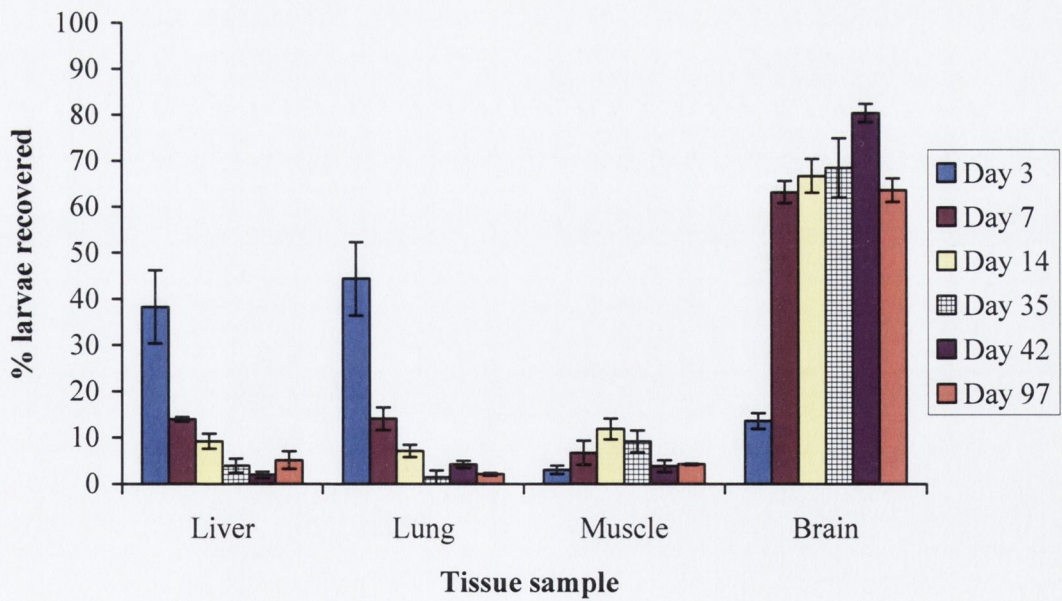
Figure 3.3.27: Mean percentage (± SEM) of total larvae recovered from each organ of *T. canis*-infected NIH mice, over the course of infection.
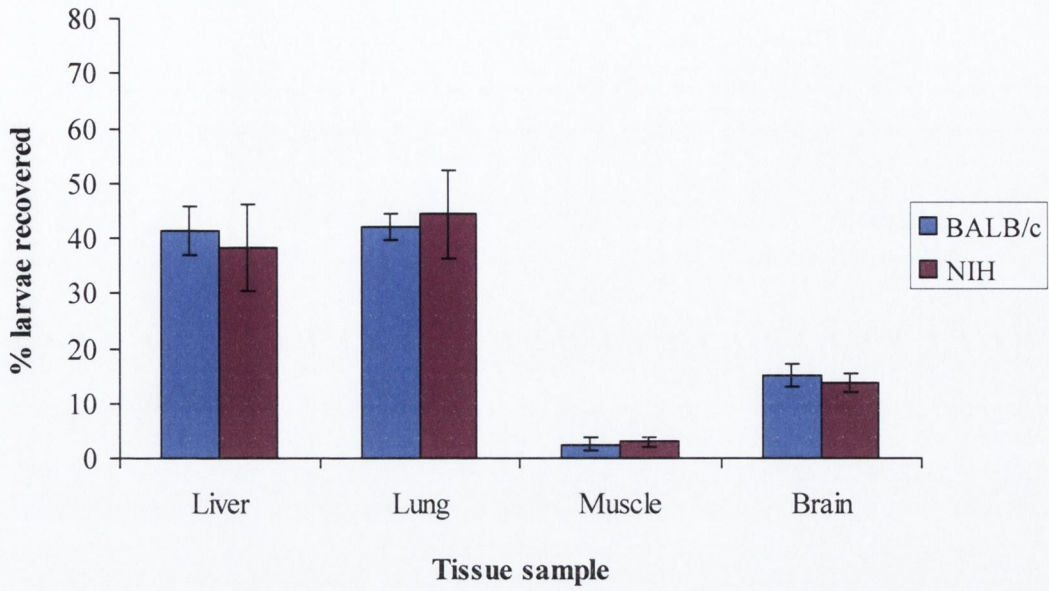
Figure 3.3.28: Mean percentage (± SEM) of total larvae recovered from different organs of *T. canis*-infected BALB/c and NIH mice on day 3 post-infection.
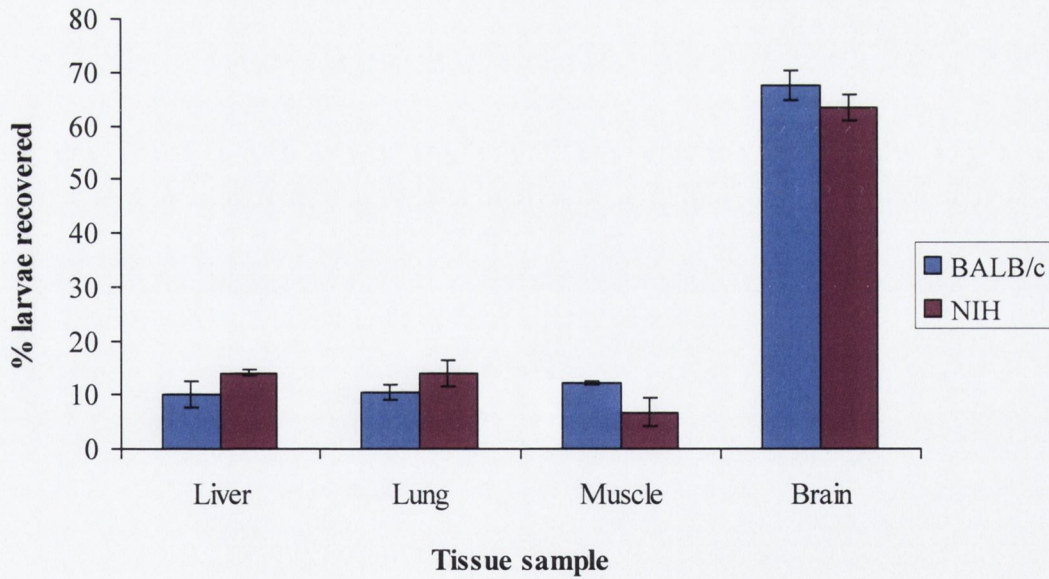
Figure 3.3.29: Mean percentage (± SEM) of total larvae recovered from different organs of *T. canis*-infected BALB/c and NIH mice on day 7 post-infection.
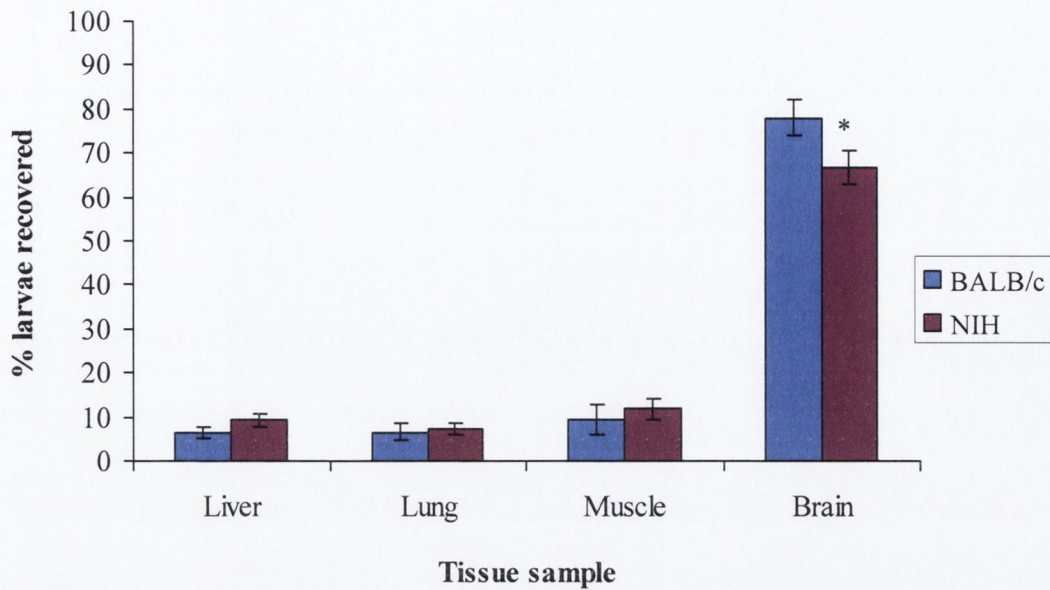
Figure 3.3.30: Mean percentage (± SEM) of total larvae recovered from different organs of *T. canis*-infected BALB/c and NIH mice on day 14 post-infection. (Between strain comparisons: * $P \leq 0.05$).
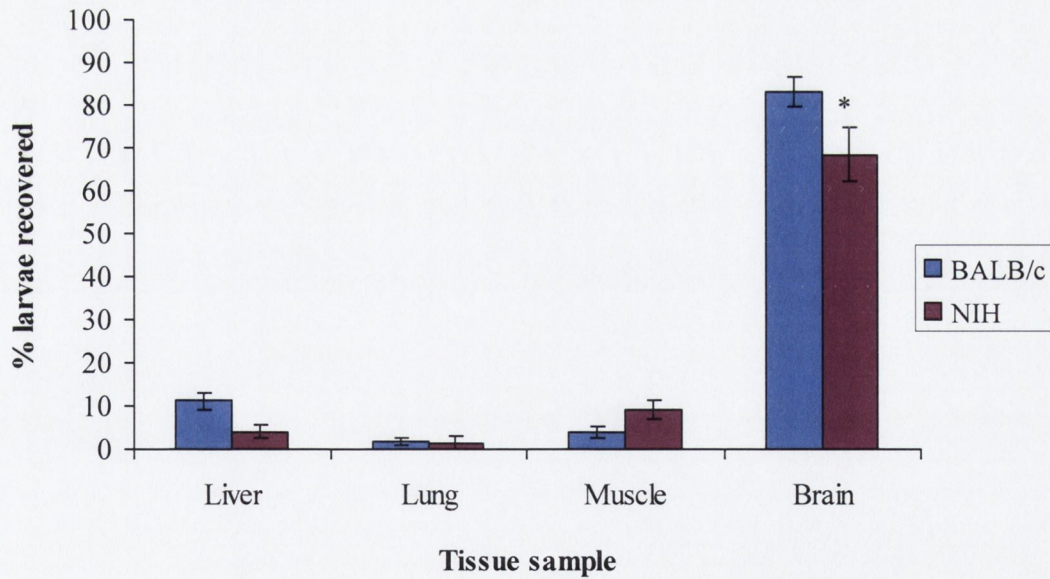
Figure 3.3.31: Mean percentage (± SEM) of total larvae recovered from different organs of *T. canis*-infected BALB/c and NIH mice on day 35 post-infection. (Between strain comparisons: * $P \leq 0.05$).
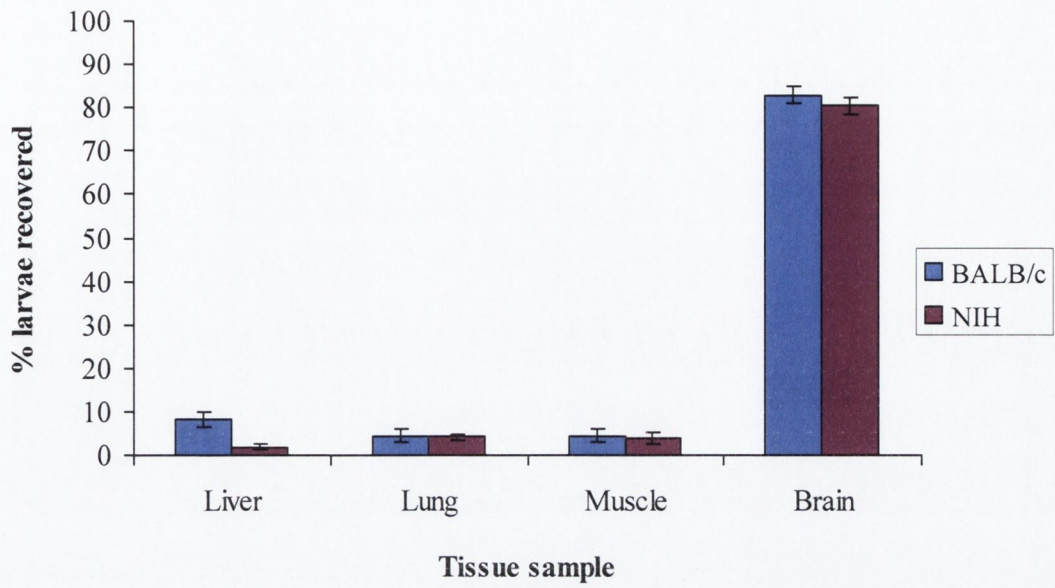
Figure 3.3.32: Mean percentage (± SEM) of total larvae recovered from different organs of *T. canis*-infected BALB/c and NIH mice on day 42 post-infection.
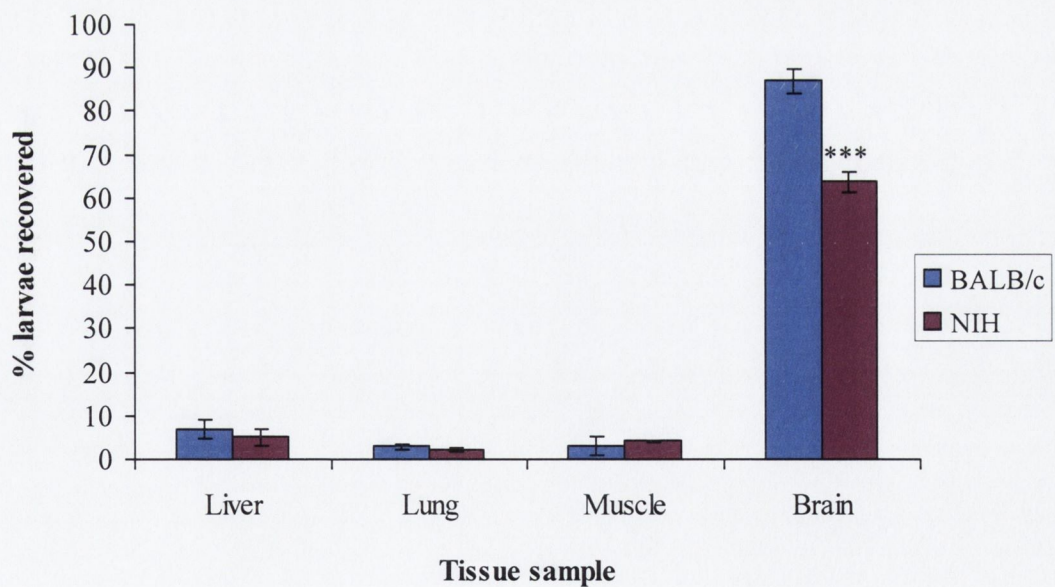
Figure 3.3.33: Mean percentage (± SEM) of total larvae recovered from different organs of *T. canis*-infected BALB/c and NIH mice on day 97 post-infection. (Between strain comparisons: *** $P \leq$ 0.0001).

3.4 DISCUSSION

The results of this chapter revealed that, firstly, *T. canis* larvae accumulate in the brains of their murine hosts – as seen by larval numbers increasing in the brain as they decrease in the visceral organs; secondly, the larvae follow the previously described migratory route encompassing the early hepato-pulmonary phase, and the later myotropic-neurotropic phase; and thirdly, the numbers of larvae in the brain vary both between and within inbred strains of mice, suggesting that cerebral larval burden may not be a reliable indicator of susceptibility or resistance to cerebral toxocariasis.

The first aim of this chapter was to identify a susceptible and resistant strain of mice to cerebral toxocariasis, with regard to cerebral larval establishment. Several different strains of inbred mice were infected with *T. canis*, and cerebral larval burden was recorded on 4 different days post-infection. *T. canis* larvae were recovered from the brains of all mice on all days post-infection, but when each strain was compared against the other, the most striking differences in larval counts occurred between NIH mice, and BALB/c and CBA/Ca mice – with BALB/c and CBA/Ca mice each carrying significantly higher larval burdens in the brain, compared with NIH mice, on 3 of the 4 days post-infection. In both BALB/c and CBA/Ca mice, the pattern of larval migration was similar to that previously described, with larval burden increasing as infection progressed. Sprent (1955) infected mice (strain unspecified) with 5000 *T. canis* eggs and recovered larvae from the brain and carcass every day for 2 weeks after infection. Although the numbers recovered from the brain were generally lower than the carcass, they increased throughout infection, peaking around day 12 post-infection – whereas recoveries from the carcass varied. Burren (1971) infected outbred mice with a dose of 1000 *T. canis* eggs, and recorded larvae from different portions of the brain over the course of 138 days. In general, the total number of larvae recovered increased over infection, although there was a slight decrease in numbers between days 14 and 19 post-infection. Kayes and Oaks (1976) reported an increase in the number of larvae recovered from the brains of outbred mice infected with varying doses of *T. canis*, over the course of a 56-day infection – although for all doses, larval numbers had declined by day 56 post-infection. Dunsmore *et al* (1983) demonstrated nicely the increase in larval numbers from the brains of inbred C57BL mice, infected with 1000 *T. canis* eggs

– with percentage of total larvae recovered ranging from 12.2% on day 4 post-infection to 87.9% on day 50 post-infection.

In this study, cerebral larval recoveries from NIH mice were generally lower than the other mice, and although they increased between days 7 and 14 post-infection, and then stabilised, they decreased significantly between days 35 and 42 post-infection. Since BALB/c mice carried a significantly higher larval burden in the brain on 3 days post-infection compared with NIH mice, they were chosen as the susceptible strain of mice to cerebral toxocariasis. BALB/c mice were chosen over CBA/Ca mice since more is known of cerebral toxocariasis in this strain, and they have previously been shown to be more susceptible to *T. canis* infection (Bardón *et al*, 1994; Epe *et al*, 1994). Since NIH mice had the lowest larval burdens overall, and differed statistically from BALB/c mice on 3 days post-infection, they were chosen as the resistant strain to cerebral toxocariasis. This choice was also supported by the literature. In a previous study, Abo-Shehada and Herbert (1989) investigated the effect of strain, sex and age of mice on resistance to *T. canis* infection, and found that NIH mice showed a relatively higher level of resistance to parasite infection than the outbred CD1 mice. Results from an earlier study, cited in the same paper, demonstrated that NIH mice produced higher serum agglutinating antibody titres against *T. canis* larval antigens than CD1 mice, making them known as 'high responders'. NIH mice have also been reported to mount a rapid, acute inflammatory response to infection with *Trichinella spiralis*, compared with the slow-responding C57BL/10 mice (Alizadeh and Wakelin, 1983). It has also been reported that NIH mice control infections with *T. spiralis* and *Trichuris muris* more rapidly than CBA mice (Lee and Wakelin, 1982). In a more recent study, Dehlawi and Goyal (2003) demonstrated that NIH mice were more resistant to infection with *T. spiralis* than CBA, C57BL/10 and B10.BR mice.

In the present study, larval counts from the visceral organs were lower than those recoveries from the brain, and differed between the strains. Highest larval burdens from the lungs were recovered on day 7 post-infection – with BALB/c mice carrying a significantly higher burden than NIH mice, and a significantly higher burden in their right lung compared with their left. As infection progressed, the larval burden decreased, although recoveries were generally higher in BALB/c mice. Interestingly, larval recoveries from the liver increased over infection (although not statistically),

with NIH mice carrying a higher burden than BALB/c. The increase in larval recoveries could possibly suggest a degree of liver trapping – a phenomenon usually only associated with repeat exposure (Sugane and Oshima, 1983). Muscle larval recoveries differed throughout infection, with the strains showing opposite patterns to each other – larval numbers decreased in BALB/c mice until day 35 post-infection and then increased, whereas numbers increased in NIH mice until day 35 post-infection and then decreased.

Overall, the total number of larvae recovered from mice over the course of infection was greater in BALB/c mice than NIH mice, with significantly more larvae being recovered from the former strain on day 42 post-infection. When larval recoveries from each organ were expressed as percentages of total burdens, the differences within both strains were striking – with a significantly higher proportion of larvae being recovered from the brain on all days post-infection compared with the other sampled organs. Interestingly, there was a lower percentage of total larvae recovered from the brains of NIH mice compared with BALB/c mice, on days 35 and 42 post-infection – compared with days 7, 35 and 42 post-infection when comparing actual larval burdens. However, the percentage of total larvae in each organ may be a better representative of the distribution of larvae within the mouse, since it takes into account any variation in the numbers of eggs which hatched successfully after inoculation. There was likely to be little variation when administering the inocula, since they were checked prior to infection to ensure correct dosage (see Appendix 1).

Based on the cerebral larval recoveries, BALB/c and NIH mice were chosen as susceptible and resistant strains, respectively. These strains were infected in a second, larger, experiment – encompassing 6 different days post-infection, including an earlier and later date so that larval migration could be investigated further. Results from this second experiment revealed that cerebral larval counts from both strains were no longer divergent – with both strains carrying similar burdens on all days post-infection. This was surprising, particularly given the statistical significance of the differences in experiment 1. The larval burdens from both strains followed the predicted pattern of migration – increasing in the brain as infection progressed – with highest larval recoveries being made on day 42 post-infection from NIH mice, and on day 97 post-infection from BALB/c mice. The results as a percentage of inocula were similar to

that of experiment 1, but the overall variation was less in experiment 2. Variation in the numbers of larvae recovered from the brains of *T. canis*-infected mice has previously been reported in outbred mice, particularly those receiving a high infective dose (Skerrett and Holland, 1997; Cox and Holland, 1998; 2001a,b). Such variation in larval numbers could be the reason the larval counts differed so greatly between the experiments in this study, although one would not expect such variation in inbred mice, particularly when the mice were the same strains, and received the same dose. Larval counts from the visceral organs were very similar between strains also - particularly from the liver and lungs.

The mean total number of larvae recovered from mice in experiment 2 was similar between the strains, although overall, fewer larvae were recovered from BALB/c mice in comparison with experiment 1, whereas more were recovered from NIH mice. When larval recoveries were investigated as a percentage of the total number of larvae recovered, the differences between organs over the course of infection were dramatic. In early infection, a statistically higher proportion of the larvae were recovered from the liver and lungs in both strains of mice, whereas by day 7 post-infection the numbers in these organs had decreased, and the majority of larvae were being recovered from the brain. This illustrates nicely the two phases of larval migration described by Abo-Shehada and Herbert (1984), where in early infection larvae are involved in a hepato-pulmonary phase, and then following day 7 post-infection, they begin the myotropic-neurotropic phase. When comparing the percentages of total larvae for each strain recovered from each organ over infection, it is apparent that fewer larvae were recovered from the brains of NIH mice compared with BALB/c mice – significantly so on days 14, 35 and 97 post-infection. This is a very interesting finding, given that the total larval counts (mentioned above) were not different between the strains.

The above results highlight the use of expressing larval burdens as a percentage of the total numbers recovered, as opposed to the actual totals in each organ. The percentage of total larval burden in each organ may be a more reliable representation of larval distribution within the tissues of the mouse, since, as mentioned earlier, it takes into account any variation in the numbers of eggs which hatched successfully in the intestine, and allows for more accurate comparisons both between and within strains. The differences in results also highlight the need for caution when assigning suitable

end-point characters to an experiment – i.e. what defines 'susceptibility' and 'resistance'. Festing and Blackwell (1988) state that when choosing suitable end-point characters, the aim should be to obtain clear-cut differences between inbred strains with minimal overlap. They mention parasite numbers in various organs at various times post-infection as an obvious choice of end-point character, but stress that care should be taken to develop a suitable scale of measurement which will not obscure strain differences. The fact that larval burdens differed so dramatically between the two experiments in this study indicate that it is not a suitable determinant of susceptibility or resistance to cerebral toxocariasis. However, perhaps a more suitable indicator would be the percentage of total larvae in each organ – since this appeared to be relatively consistent in both experiments.

A shift in susceptibility and resistance to infection has been reported in other parasitic infections, where parasite numbers have been used as the end-point characteristic. Scott (1991) investigated *Heligmosomoides polygyrus* infections in two strains of inbred mice (BALB/c and C57BL/6) which had been previously reported to be resistant and susceptible (respectively) to infection – based on parasite establishment and survival during a primary infection. In a series of experiments, the author reported that during a primary infection with 100 L_3 larvae, parasite establishment and net egg production were significantly higher in C57BL/6 mice compared with BALB/c, confirming them to be more susceptible to infection with the parasite. The degree of protection induced by an immunizing regime was also lower in C57BL/6 mice. Based on these experiments, Scott hypothesized that C57BL/6 mice would be more heavily infected than BALB/c mice, when housed together in a single population exposed to endemic infection. However, this was not the case and, in a duplication of experiments, although infection spread rapidly through the housing environment, the numbers of larvae recovered from both strains, and the net egg production through time, did not differ significantly between the strains. The author suggested that corticosteroid levels (linked to stress) or bacterial flora may have been involved in the change in level of resistance to the parasite. In a study investigating the levels of resistance and susceptibility to *Toxoplasma gondii* infection in mice, Suzuki *et al* (1993) reported that CBA/Ca mice were markedly more resistant to acute infection than BALB/c mice, but were more susceptible to chronic infection – suggesting that

resistance to chronic *Toxoplasma* infection is regulated by different mechanisms than those operative against acute infection.

Resistance or susceptibility to parasitic infection has often been linked to genes associated with the major histocompatibility complex (MHC) (Wakelin and Blackwell, 1988). In mice, this complex of genetic loci is known as the H-2 complex, and is located on chromosome 17. The main function of MHC molecules is to bind and "present" antigenic peptides on the surfaces of cells for recognition (binding) by antigen-specific T-cell receptors on the surfaces of lymphocytes. It is understandable then, that genes associated with this complex can play a part in determining how a subject responds to infection, thus determining resistance. The effects of H-2 and non-H-2 genes on the control of parasitic infections have been demonstrated over the years, with emphasis on intestinal nematodes (Wakelin, 1985).

Wakelin (1980) investigated the genetic control of immunity to *T. spiralis* infection in mice, using inbred and congenic strains. The author demonstrated that mice with the same haplotype (H-2^q) responded rapidly to infection, expelling most of the adult worms within 12 days – suggesting that H-2 genes may be involved in control of infection. However, the congenic mice (identical at all loci except the H-2 complex) responded equally as slowly as each other even though they were of different haplotypes, and one of the strains was of the H-2^q haplotype – indicating that genes of the H-2 complex are perhaps not involved in control. Else and Wakelin (1988) also reported the effects of H-2 and non-H-2 genes on the expulsion of *T. muris* from inbred and congenic mice, reporting that both types of genes play important roles in controlling the immune response which expels worms from the gut. Epe *et al* (1994) investigated the behaviour and pathogenicity of *T. canis* larvae in mice with common (BALB/c and DBA) and different (C3H/He and C57BL/6j) haplotypes, in order to obtain information about the possible role of the MHC in the control of infection. The authors reported that all strains (apart from BALB/c) with different haplotypes showed similar pathological changes with individual variation. BALB/c mice (with the same haplotype as DBA mice) behaved completely differently, and although they carried the highest larval burden in the brain, they showed no central nervous symptoms, unlike the other strains. These results would suggest that the control of *T. canis* infection is not controlled by genes on the H-2 complex.

The results from the present study would suggest that cerebral toxocariasis is perhaps controlled by both H-2 and non-H-2 genes. In the first experiment, BALB/c mice (H-2^d) were much more susceptible to cerebral infection than NIH mice (H-2^q), suggesting that resistance to infection involved genes of the H-2 complex. However, the convergent results of experiment 2 contradict this hypothesis and suggest that control of infection does not involve H-2 genes. Though, if susceptibility and resistance are based on the percentage of total larvae in the brain, then NIH mice were still more resistant than BALB/c mice in the second experiment, and it could therefore be hypothesized that H-2 genes are linked to resistance, although much more in depth studies, looking specifically at this issue and involving both inbred and congenic strains would need to be carried out in order to confirm this.

To conclude, the results of this chapter have confirmed that *T. canis* larvae accumulate in the brains of BALB/c and NIH mice as they decrease in other organs. The pattern of migration in both experiments was similar to that previously reported (Abo-Shehada and Herbert, 1984) – i.e. an early hepato-pulmonary phase followed by a later myotropic-neurotropic phase. Results also revealed that total cerebral larval burden may not be a reliable indicator of susceptibility or resistance to infection, and perhaps a more suitable end-point character is needed, such as percentage of total larval burden. It may also be worthwhile enumerating any larvae left in the remaining tissues following the Baermann procedure. This procedure works on the basis that any live larvae present in the sample will migrate out of the tissue and into the warmer solution over a 24-hour incubation period. If, however, larvae are dead, or the enzyme solution is insufficient to release them from encapsulation, they will be excluded from the counts, which may influence results. Enumerating all larvae (dead and alive) would allow for the comparison of entire counts, which may be more revealing in terms of resistance and susceptibility. Having estimations of the numbers of dead larvae in the brain may also give more insight into cerebral control of the parasite, although this will be discussed further in Chapter 6.

CHAPTER 4: Comparison of behavioural alterations in two inbred strains of mice infected with *Toxocara canis*

4.1 INTRODUCTION

The study of behavioural alterations in parasitized animals has long been an area of fascination for parasitologists. Changes in activity levels of infected murine hosts are one of the most widespread behavioural alterations observed in laboratory experimentation (see Poulin, 1994; 1995). Hutchison *et al* (1980a) observed the baseline activity of mice infected with the protozoan parasite *Toxoplasma gondii*, in comparison with control mice. They noted that both naturally and experimentally infected mice spent a significantly higher percentage of time moving around the homecage, and displayed a larger proportion of short bouts (0-2 seconds) of behaviour, compared with control mice. Infected mice also spent a significantly lower percentage of time rearing and digging, in comparison to controls. In other studies involving *T. gondii*-infected mice, it was shown that infected mice have greater activity levels when exposed to novel environments (Hay *et al*, 1983a,b; 1984); have impaired motor performance, as measured by the number of falls from a rotating cylinder (Hutchison *et al*, 1980b; Hay *et al*, 1983c); have impaired performance negotiating complex mazes (Witting, 1979); and are less likely to explore novel stimuli presented to them in a 'Y'-maze (Hay *et al*, 1984).

Rau and Putter (1984) reported decreased running ability in outbred mice infected with 400 *T. spiralis* larvae, and an increased latency of the wall-seeking response (i.e. subjects spent more time in open-field), compared with control mice or those animals inoculated with only 100 larvae. They hypothesized that such changes in behaviour could lead to a decrease in fitness of the host, and thus an increase in susceptibility to predation. In a slightly earlier study investigating the open-field behaviour of mice infected with *T. spiralis*, Rau (1983) reported a significant decrease in ambulatory and exploratory behaviour, and an increase in immobility in infected subjects. The author also demonstrated that the onset of behavioural changes coincided with the release of larvae by adult worms into the host circulation (15-20 days post-infection), and that behavioural changes were still being recorded as late as 90 days post-infection. In contrast, infection with *Trichinella pseudospiralis* (a closely related species to *T. spiralis*) has a much less pronounced effect on host behaviour. Rau (1984) reported a brief decrease in ambulatory activity in the acute phase of infection, but these levels

returned to those of pre-infection one week later. Infection did not affect the level of exploratory activity.

With reference to *T. canis*, the mouse model has been widely used to study the impact of infection on different aspects of murine behaviour. Activity levels of infected mice have been assessed in a number of studies. *T. canis*-infected mice have been shown to be more active than uninfected control mice, as measured by the number of squares crossed in an open-field, the time spent in the novel arm of a 'Y'-maze, the time spent on a running wheel, and the number of short bouts of behaviour (Hay and Aitken, 1984; Hay *et al*, 1985, 1986). Cox and Holland (2001a) reported significant differences in the baseline activities of different strains of *T. canis*-infected mice, with outbred mice spending significantly more time immobile than inbred mice.

Mice use exploratory behaviour as a means of intensively examining new surroundings and re-examining familiar ones (Mackintosh, 1981). In doing this, the mouse has an increased chance of fleeing danger successfully, and in this respect, exploratory behaviour could be viewed as adaptive. Dolinsky *et al* (1981) reported a decrease in the exploratory behaviour of *T. canis*-infected mice, compared with control mice. When given the choice of a familiar or novel environment, infected mice entered the novel environment significantly fewer times than control mice. In a similar study, Burright *et al* (1982) also reported a significant decrease in the exploratory behaviour of mice infected with varying doses of *T. canis* eggs, compared with control mice. More recently, Cox and Holland (2001b) reported that *T. canis*-infected mice were less explorative and less responsive to novelty in a 'T'-maze than uninfected control mice, although not all differences attained statistical significance.

The effect of parasitic infection on learning and memory is another area of interest for parasitologists. Spatial abilities are important in many aspects of the survival of small rodents, including successful foraging, reproduction, predator avoidance and territorial defence (Kavaliers *et al*, 1995). The effects of parasitic infection on host spatial ability have been investigated in a number of studies. Stretch *et al* (1960a,b, 1961) reported that *Schistosoma mansoni*-infected mice showed reduced ability in distinguishing between black and white in a water-maze discrimination task. Witting (1979) reported that mice infected with *T. gondii* performed less well than control mice in solving

complex mazes. Other authors have reported affects of *Heligosomoides polygyrus* and *Eimeria vermiformis* on spatial performances of infected mice (Kavaliers and Colwell, 1995; Kavaliers *et al*, 1995). Infection with *T. canis* has also been shown to affect the learning and memory of mice (Dolinksy *et al*, 1981; Cox and Holland, 2001b) and rats (Olson and Rose, 1966), with infected subjects taking longer to complete tasks and making more errors than uninfected controls, in complex mazes and water-finding tests.

A large proportion of the above studies carried out assessing murine behavioural alterations have used outbred mice as models, with inbred strains receiving less attention. Individual inbred mouse strains, produced by brother×sister mating for at least 20 generations, result in animals genetically identical at an average of 98.6% of loci. Utilised for immunological and genetic studies, due to the isogenicity within strains and the genetic heterogeneity between strains, they have transpired to be extremely useful tools for studying the interplay between genes and environment (Festing and Fisher, 2000; Beck *et al*, 2000). Undertaking behavioural studies with inbred mice is attractive since any infection-induced behavioural alterations which could be potentially masked in heterogeneous outbred mice, may appear more pronounced. Since there can be great heterogeneity between inbred strains, their use also allows for the comparison and analysis of behavioural alterations in completely divergent strains.

On this basis, the aims of this chapter were firstly to investigate and compare the baseline activity levels of two divergent strains of mice, susceptible (BALB/c) and resistant (NIH) to cerebral toxocariasis (see Chapter 3 for choice of strains), both pre- and post-infection; and secondly to investigate the effects of cerebral infection on exploratory behaviour, response to novelty, and learning and memory in both strains of mice. It was hoped that by investigating two such divergent inbred strains, any behavioural changes which occurred as a result of infection would be more pronounced in the more susceptible strain.

4.2 MATERIALS AND METHODS

4.2.1 Investigation of baseline activity in the homecage, both pre- and post-infection, in control and *T. canis*-infected BALB/c and NIH mice

Baseline activity was initially measured prior to infection, and again on day 35 p.i. Each time, the behaviour of individual mice was measured by observing their activity in the homecage. Six different categories of murine behaviour were measured, based on previous research (Hutchison *et al*, 1980).

(1) Ambulation
  - walking; running; jumping; rolling; circling; stretching.

(2) Grooming
  - biting coat; licking coat; washing face; scratching; washing genitals; chewing tail.

(3) Rearing
  - standing with paws against side of cage; straight rearing with paws held in front of body.

(4) Digging
  - digging bedding backwards; pushing bedding forwards.

(5) Immobility
  - lying on stomach; standing still.

(6) Climbing
  - hanging vertically from bars with front paws; hanging upside down.

Testing was performed in silence, with food and water restricted, on two consecutive days (testing a strain on each day) between 0900 h and 1900 h. Before testing each mouse, the other 4 mice were removed from the homecage, and the cage was placed in the testing position. A 5 min habituation period preceded testing to allow the mouse to

acclimatise to being alone in the homecage. The behaviour of the mouse was then recorded over a 20 min period on videotape using a Sony Camcorder. The camcorder was suspended above the cage, and linked to a video monitor allowing the animal's behaviour to be observed from a discrete position. Analysis of the videos was performed blind, using a True Basic© computer programme (Cox, 1996). Each 20 min period was analysed 6 times, recording an individual behaviour each time

4.2.2 Investigation of exploratory behaviour and response to novelty in control and *T. canis*-infected BALB/c and NIH mice, using a 'T'-maze

Test apparatus used was an adaptation of that previously described (Cox and Holland, 2001b). The 'T'-maze (Plate 4.1), made of varnished plywood, consisted of a start arm and two choice arms of equal length (18 x 4 x 6 inches). The start arm contained a starting box (8 inches in length), which was separated from the rest of the arm by a wooden guillotine. An end box was situated at the end of each choice arm, at an angle of 90° so the mouse had to turn to enter it. A one-day habituation period preceded testing, where each mouse was allowed free access to the entire maze for a period of 5 minutes. All recording took place in the same room as the baseline activity. During the habituation period, the following observations were recorded for each mouse:

(1) number of entries into left arm

(2) number of entries into right arm

(3) number of entries into start alley

(4) number of rears performed per mouse

Between the recordings of each mouse, the maze was thoroughly cleaned of any faecal pellets, and swabbed with 70% ethanol to remove any trace of the previous mouse. This would ensure that each individual explored freely, and did not follow the scent of the previous mouse. On the day following habituation, a running wheel was introduced to one of the choice arms in the maze as a means of investigating the level of neophobia between infected and control mice. During the testing period, each mouse was placed in the start box for 30 seconds, before raising the wooden guillotine and recording the following observations:

(1) number of entries into novel arm (i.e. arm containing running wheel)

(2) number of entries into right arm (familiar arm)

(3) number of entries into start alley

(4) number of rears performed per mouse

(5) number of times each mouse investigated the novel object (i.e. number of approaches)

(6) amount of time spent investigating novel object

(7) amount of time spent in novel arm

All behaviours were recorded on a Sony camcorder as before, and analysis was carried out blind.

4.2.3 Investigation of learning and memory in control and *T. canis*-infected BALB/c and NIH mice, by use of a water-finding task

Apparatus used in this test was similar to that described by Cox and Holland (2001b). In brief, the water-finding apparatus (Plate 4.2) consisted of an open-field enclosure measuring 30 x 50 x 15 cm. An alcove (10 x 10 x 10 cm), covered with a perspex lid, was situated on the back wall. The floor of the open field was divided into 15 identical squares to allow measurement of the subject's movement. A water bottle was suspended from the centre of the alcove ceiling, with its tip 6cm above the floor. Habituation involved recordings previously described by Cox and Holland (2001b). In brief, each mouse was placed in the left-hand corner of the apparatus and its behaviour was recorded over a 3-minute period. During this time, the following measurements were taken:

(1) number of squares crossed in the open field

(2) number of times the subject touched or sniffed the water-bottle in the alcove

(3) number of rears performed

After the habituation period, mice were returned to their homecage, and testing took place 48 h later, with the mice being deprived of water for the last 24 h. Testing followed the same criteria as the habituation, and the following measurements were taken:

(1) time taken to enter the alcove after beginning exploration

(2) time taken to touch or sniff the water-bottle after beginning exploration

(3) time taken to drink from the water-bottle after beginning exploration

In cases where the mouse did not find the water bottle, or drink, the animal was given the maximum time latency (180 s). All behaviours were recorded on a Sony camcorder as before, and analysis was carried out blind.

4.2.4 Statistical analysis

All statistical analysis was carried out at the 95% confidence interval, and data were checked for normality prior to testing. Baseline activity was initially assessed using a MANOVA, which allowed for more than one observed variable to be analysed at once – since the baseline activities are not independent of each other. The MANOVA was used to determine overall effects of strain, infection status, and the interaction between these factors, on each of the observed activities. F-ratios, with degrees of freedom, and P values are given in the text. Since the MANOVA does not allow for the analysis of between-group differences (by strain and infection status), separate 2-way ANOVAs were performed on data for each observed activity, and F-ratios, with degrees of freedom, and P values are reported. Least significant difference (LSD) post-hoc tests were carried out to investigate group differences, and P values are given in the text.

A MANOVA was used to investigate differences between the number of arm entries into each arm of the 'T'-maze, since they are not independent of each other, and F-ratios, with degrees of freedom, and P values are given where necessary. Two-way ANOVAs were used to determine effects of strain, infection status, and the interaction between these factors, on each observed activity, and F-ratios, with degrees of freedom, and P values are given where applicable. LSD post-hoc tests were carried out to further investigate group differences, and P values are reported in the text.

Water-finding task results were analysed by means of 2-way ANOVAs for each observed activity, to investigate effects of strain, infection status and the interaction between these factors. F-ratios and P values are given in text. LSD post-hoc tests were carried out to further investigate group differences, and P values are reported. A chi-

square test was used to assess the numbers of mice that drank compared with those that did not, for both strains, and the P values are given in the text.

In order to assess any effect of cage on behavioural observations, nested 2-way ANOVAs were carried out for each set of behavioural data, where cage was a random factor nested within the interaction between strain and infection status. In the case of activity, where multiple comparisons were carried out on essentially the same data set (since each activity is not independent of the others), the Bonferroni method was applied, reducing the critical P value from 0.05 to 0.008.
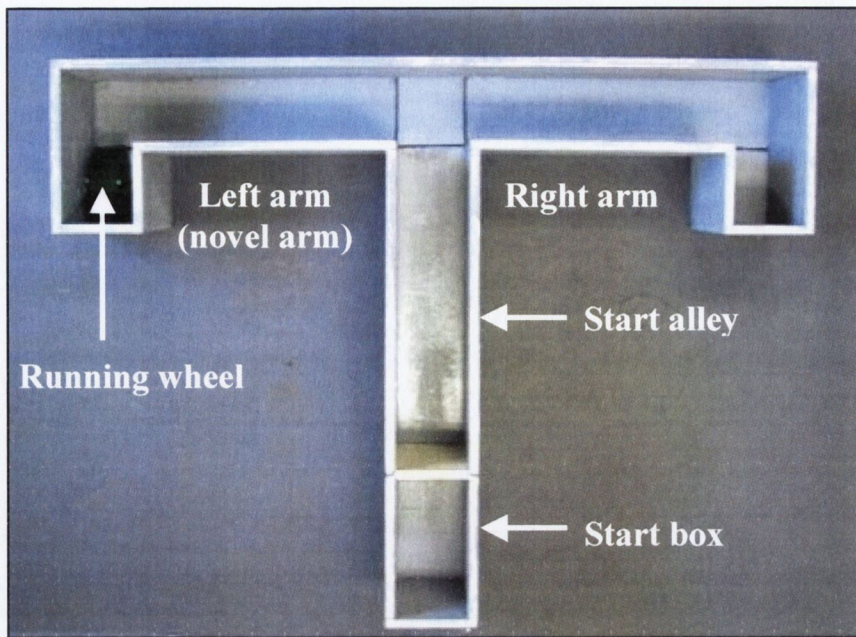
Plate 4.1: 'T'-maze used to investigate exploratory behaviour and response to novelty in *Toxocara canis*-infected and control BALB/c and NIH mice.
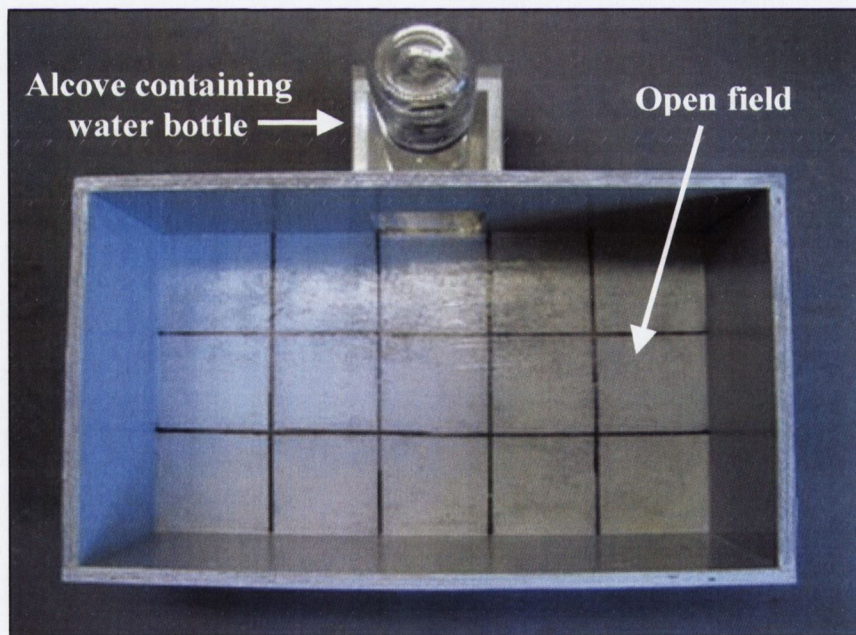
Plate 4.2: Water-finding task used to investigate learning and memory in *Toxocara canis*-infected and control BALB/c and NIH mice.

4.3 RESULTS

4.3.1 Investigation of baseline activity in the homecage, both pre- and post-infection, in control and *T. canis*-infected BALB/c and NIH mice

The baseline activity of BALB/c and NIH mice differed prior to infection (Figures 4.3.1 and 4.3.2). BALB/c mice spent the majority of their time climbing, digging, and rearing, and less time ambulating and grooming. Only a small amount of time was spent immobile. NIH mice spent most of their time rearing, and rest of the time was split similarly between ambulating, climbing, grooming and digging. NIH mice also spent very little time immobile. Post-infection behavioural observations are shown in Figures 4.3.3 and 4.3.4. BALB/c mice spent similar amounts of time ambulating, digging and rearing, much less time climbing and grooming, and were more immobile than pre-infection. NIH mice spent similar amounts of time ambulating, rearing, grooming and climbing, although in the latter activity, this was much reduced in infected mice. They spent much less time digging, and were more immobile, although this was more pronounced in the infected mice. Due to the differences in baseline activity pre-infection, both between and within strains, statistical analysis for baseline activity was performed on post- minus pre-infection data, and differences in activity are shown in Figures 4.3.5 and 4.3.6.

Figure 4.3.5 demonstrates that, overall, BALB/c mice spent more time ambulating, rearing and immobile after infection, and less time climbing and grooming. The time spent digging differs between control and infected mice – with infected mice spending less time digging. Figure 4.3.6 demonstrates that, overall, NIH mice spent slightly more time grooming, and much more time immobile after infection, but less time digging. Time spent ambulating, climbing and rearing differs between control and infected mice, with infected mice spending less time climbing and more time rearing and ambulating. Results of a MANOVA showed there to be an overall effect of strain, infection status and the interaction between these two factors on baseline activity (strain: $F_{1,30} = 2.7$, $P = 0.037$; infection status: $F_{1,30} = 10.6$, $P \leq 0.0001$; strain x infection status: $F_{1,30} = 4.9$, $P = 0.002$). The effects of strain and infection status on each activity were investigated further by means of 2-way ANOVAs. There was a

significant effect of infection status on the time spent ambulating ($F_{1,34}$ = 18.8, P = 0.0001), with both infected BALB/c and NIH mice spending significantly more time ambulating compared with control mice (post-hoc tests: BALB/c: P = 0.005; NIH: P = 0.003). However, infected NIH mice were also significantly more immobile than their control counterparts (P = 0.002), and spent significantly less time climbing (P = 0.018), whereas there were no significant differences between control and infected BALB/c mice for these activities. Infected NIH mice spent less time digging and grooming, and more time rearing compared with control mice, but the differences were not significant. Infected BALB/c mice also spent less time digging and grooming than control mice, but the differences were not significant.

Results of separate nested 2-way ANOVAs carried out for each activity, with cage as a random factor nested within the interaction between strain and infection status, revealed a significant effect of cage on the level of rearing observed (P = 0.002).
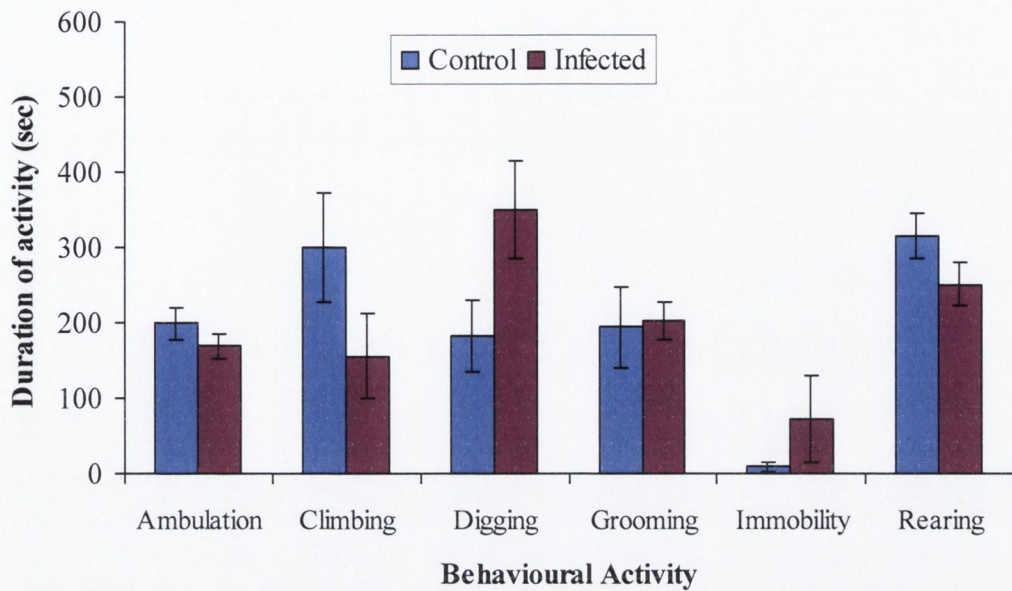
Figure 4.3.1: Mean duration (± SEM) of each baseline activity pre-infection, in assigned control and infected groups of BALB/c mice.
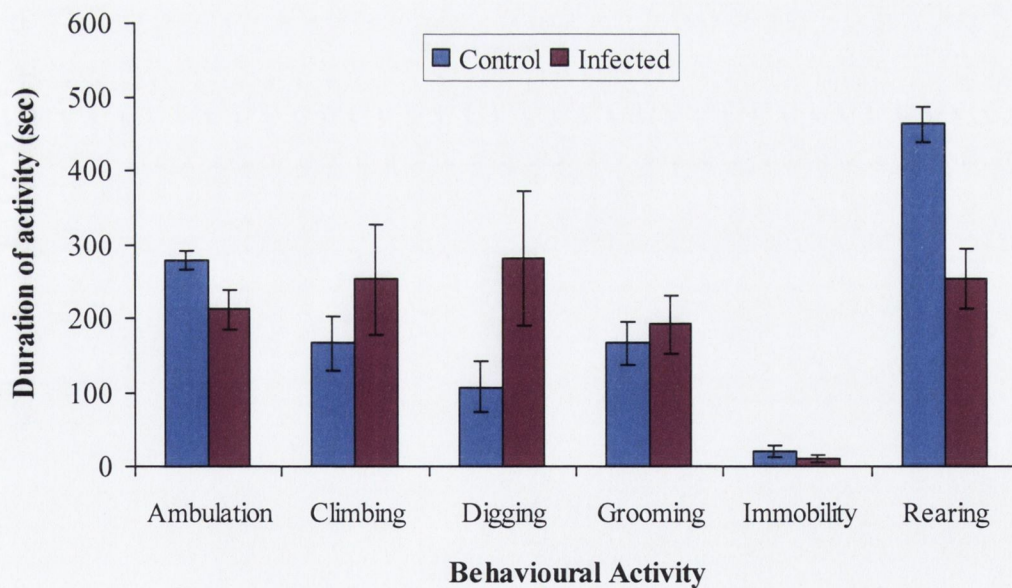
Figure 4.3.2: Mean duration (± SEM) of each baseline activity pre-infection, in assigned control and infected groups of NIH mice.
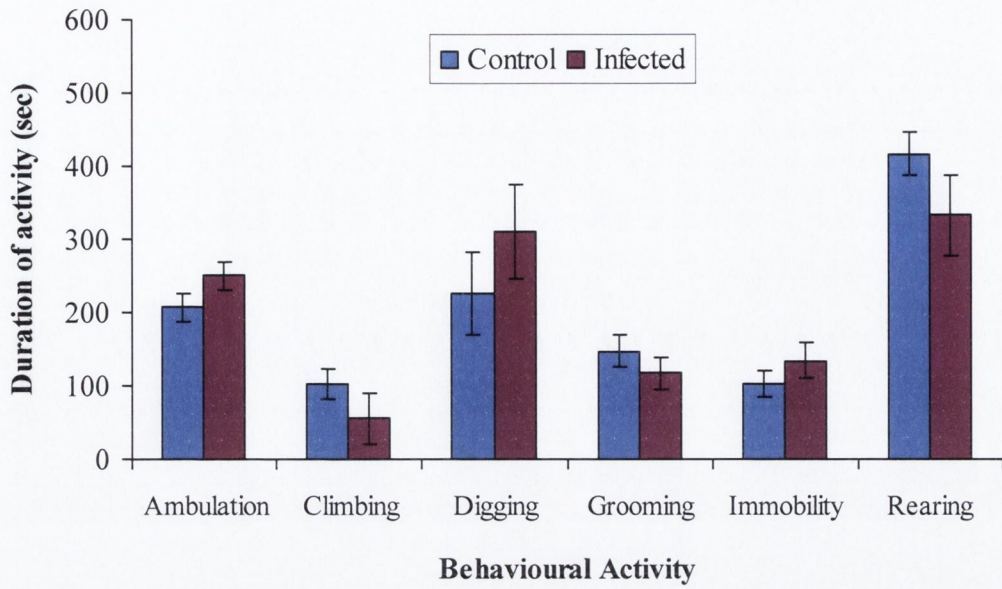
Figure 4.3.3: Mean duration (± SEM) of each baseline activity post-infection, in control and infected BALB/c mice.
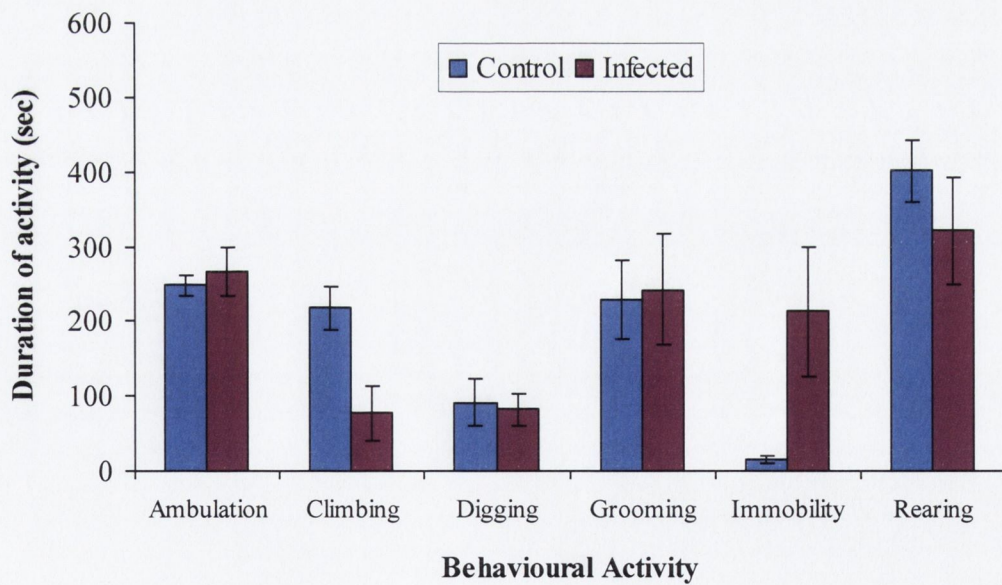
Figure 4.3.4: Mean duration (± SEM) of each baseline activity post-infection, in control and infected NIH mice.
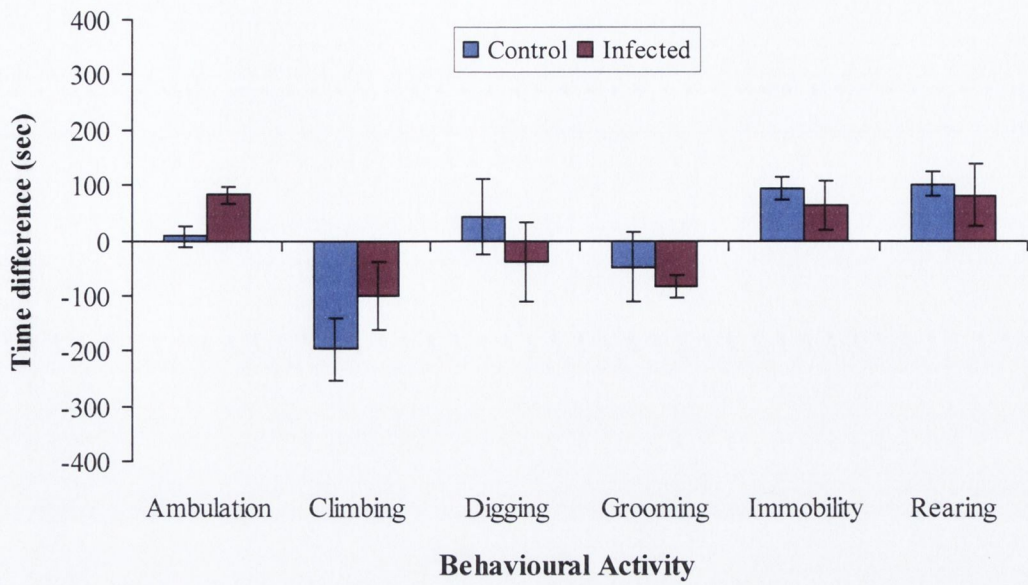
Figure 4.3.5: Mean (± SEM) time difference spent at each baseline activity between pre- and post-infection, in BALB/c mice.
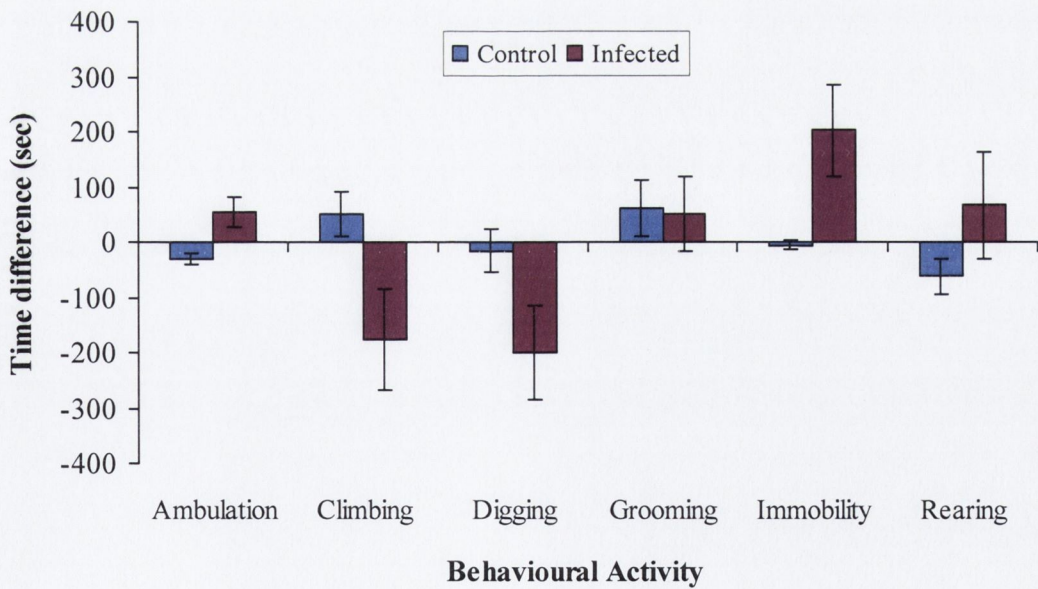
Figure 4.3.6: Mean (± SEM) time difference spent at each baseline activity between pre- and post-infection, in NIH mice.

4.3.2 Investigation of exploratory behaviour and response to novelty in control and *T. canis*-infected BALB/c and NIH mice, using a 'T'-maze

In general, infected and control mice of both strains were relatively explorative during the habituation period, entering all arms of the maze (Table 4.3.1). Results of a MANOVA demonstrated an overall significant effect of strain on the number of arms entered during habituation ($F_{1,34}$ = 8.1, $P \leq 0.0001$), with infected NIH mice entering the start alley significantly fewer times than infected BALB/c mice (mean no. entries ± S.D: BALB/c 5.4 ± 1.71, NIH 2.6 ± 1.77; $P \leq 0.0001$). There were no significant differences between control and infected mice of each strain for the number of arms entered. However, there was a significant effect of infection status on the number of rears performed, with infected NIH mice performing significantly fewer rears than their control counterparts (2-way ANOVA; infection: $F_{1,34}$ = 7.6, P = 0.009; post-hoc test: P = 0.009). Overall, the total number of arm entries during the habituation period was lower for infected mice compared with controls (Figure 4.3.7). A 2-way ANOVA revealed a significant effect of strain on the total number of arm entries made during the habituation period ($F_{1,34}$ = 9.4, P = 0.004), but there were no significant effects of infection status or the interaction between strain and infection status.

Results of separate nested 2-way ANOVAs for each activity revealed no significant effect of cage on behavioural observations (P > 0.05, for all activities).

Table 4.3.1: Mean (± S.D) number of arm entries and rears performed for control and infected BALB/c and NIH mice, during the habituation period of the 'T'-maze.

Behaviour	BALB/c		NIH	
	Control	Infected	Control	Infected
Left arm entries	4.9 ± 2.08	4.9 ± 2.08	3.1 ± 2.33	3.8 ± 2.87
Right arm entries	5.5 ± 2.51	5.4 ± 2.22	3.7 ± 2.54	4.4 ± 2.62
Start alley entries	6.9 ± 2.60	5.4 ± 1.71	3.2 ± 2.10	2.6 ± 1.77
Rears performed	34.5 ± 13.28	25.9 ± 21.80	49.8 ± 11.75	27.1 ± 21.30
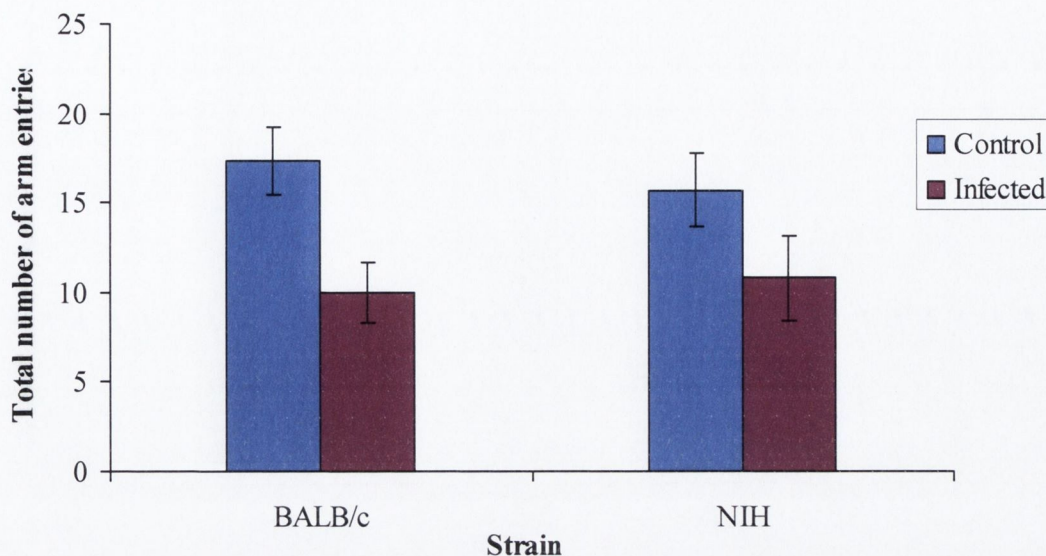
Figure 4.3.7: Mean total (± SEM) number of arms entered during the habituation period of the 'T'-maze, for control and infected BALB/c and NIH mice.

During the testing period, infected NIH mice were generally less explorative than control mice, spending less time moving between arms of the maze (Figure 4.3.8). The overall number of arms entered was less (although not significantly) for infected NIH mice compared with controls, but very similar between control and infected BALB/c mice (Figure 4.3.9). Results of a MANOVA revealed no significant effects of strain, infection status, or the interaction between these factors, on the number of entries into each arm of the maze. However, a 2-way ANOVA revealed that strain, infection status, and the interaction between these factors, had significant effects on the number of rears performed (strain: $F_{1,34} = 6.1$, $P = 0.02$; infection status: $F_{1,34} = 10.8$, $P = 0.002$; strain x infection status: $F_{1,34} = 15.1$, $P = 0.0005$), with infected NIH mice performing significantly fewer rears than control mice ($P \leq 0.0001$). Infected NIH mice did seem more responsive to novelty than control mice, spending more time in the novel arm (Figure 4.3.10; mean time (s) ± S.D: control 90.4 ± 41.47, infected 130 ± 105.78), although time spent investigating the novel object was similar between the groups (mean time (s) ± S.D: control 49.5 ± 27.33, infected 51.0 ± 67.25).
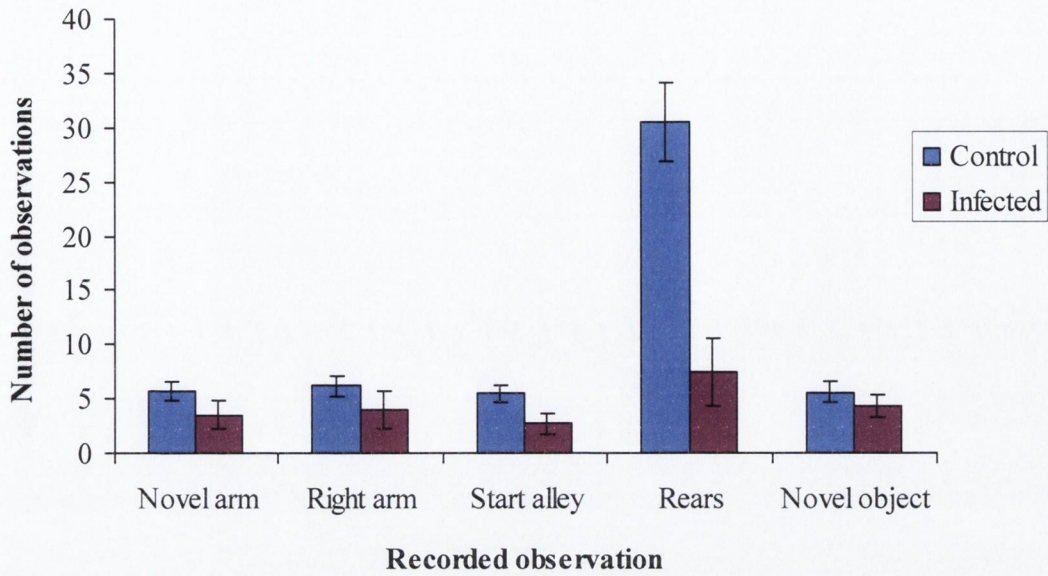
Figure 4.3.8: Mean (± SEM) number of arm entries, rears performed and novel object investigations during the testing period, by control and infected NIH mice.
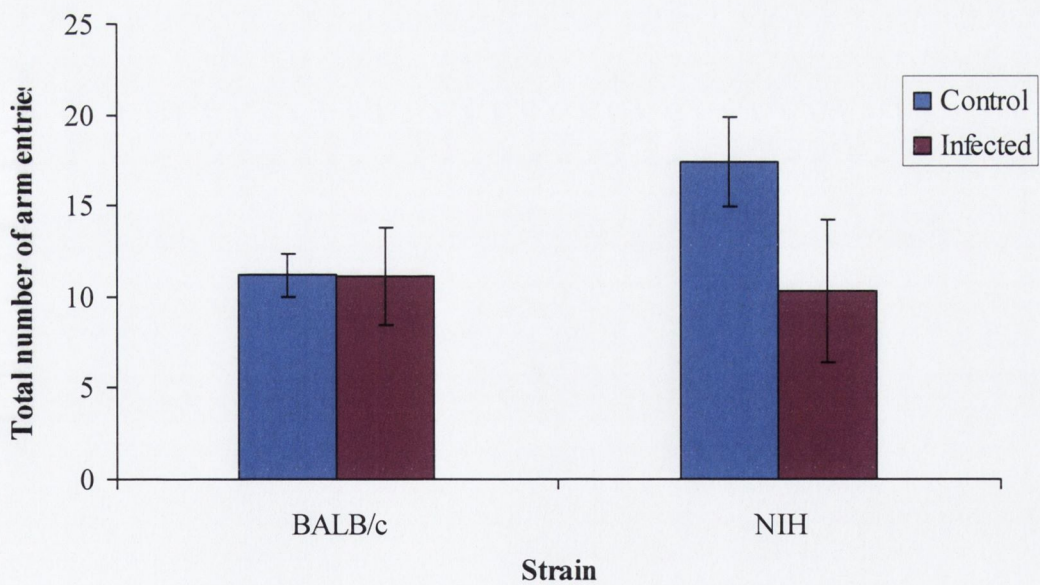
Figure 4.3.9: Mean total (± SEM) number of arms entered during the testing period of the 'T'-maze, for control and infected BALB/c and NIH mice.
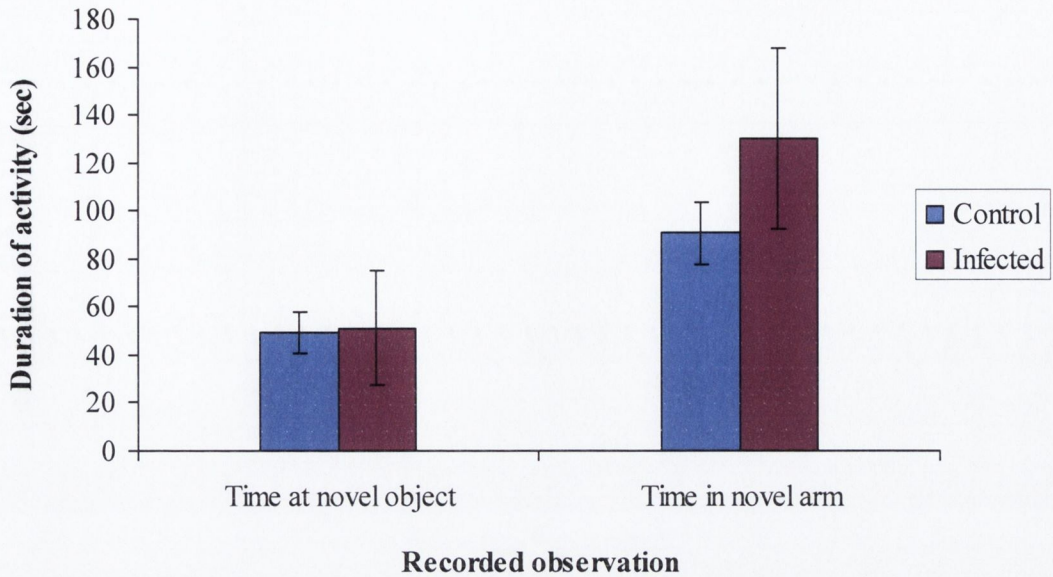
Figure 4.3.10: Mean (± SEM) amount of time spent in the novel arm, and investigating the novel object during the testing period, by control and infected NIH mice.

There were no noticeable differences in the exploratory behaviour of infected and control BALB/c mice (Figure 4.3.11). However, Figure 4.3.12 shows that infected BALB/c mice appeared less responsive to novelty than control mice, spending less time in the novel arm (mean time (s) ± S.D: control 118.3 ± 84.87, infected 86.7 ± 77.24) and less time investigating the novel object (control 58.6 ± 40.74, infected 45.3 ± 40.27), although these differences were not significant ($P > 0.05$).
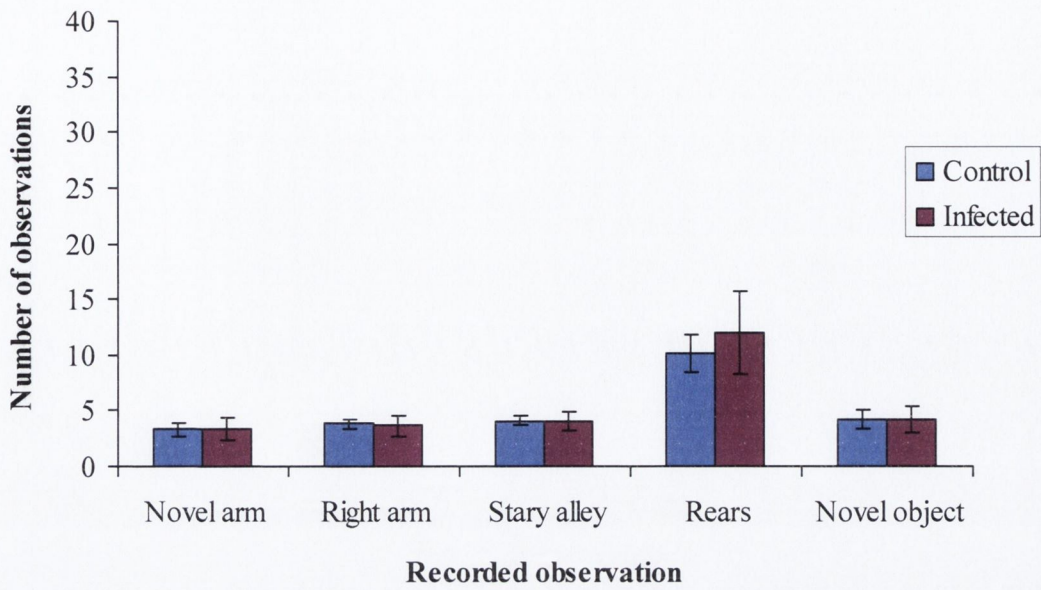
Figure 4.3.11: Mean (± SEM) number of arm entries, rears performed and novel object investigations, by control and infected BALB/c mice.
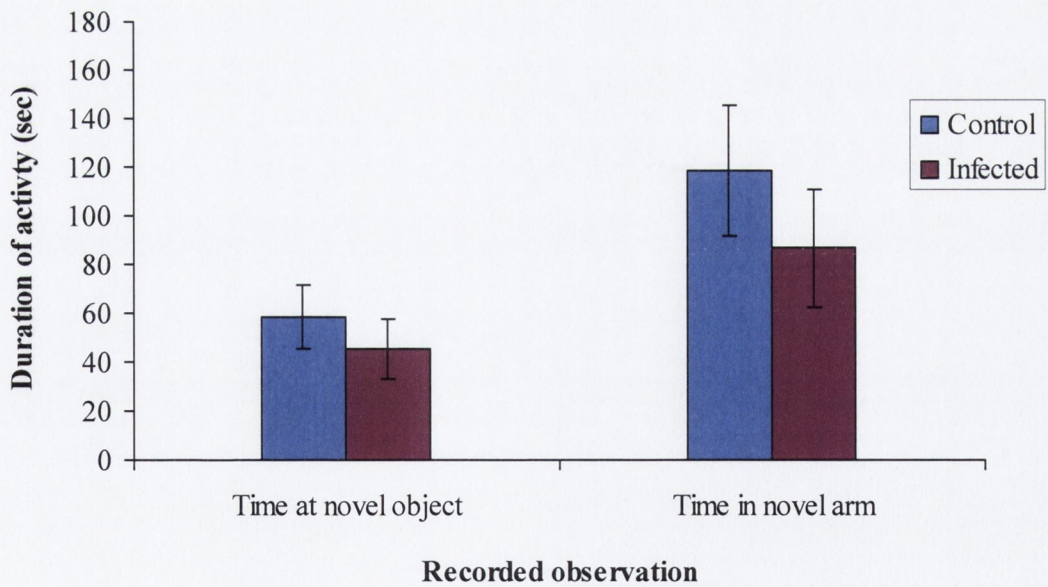
Figure 4.3.12: Mean (± S.E) amount of time spent in the novel arm, and investigating the novel object by control and infected BALB/c mice.

4.3.3 Investigation of learning and memory in control and *T. canis*-infected BALB/c and NIH mice, by use of a water-finding task

During habituation (Table 4.3.2), significant differences were noted between, and within, both strains of mice for some of the measurements recorded. Results from separate 2-way ANOVAs carried out for each observation, revealed a significant effect of infection status on the number of rears performed ($F_{1,34}$ = 25.2, $P \leq 0.0001$), and significant effects of infection status and strain on the number of times the water-bottle was touched/sniffed (infection status: $F_{1,34}$ = 6.6, P = 0.01; strain: $F_{1,34}$ = 16.5, P = 0.0003).

Post-hoc tests revealed that infected NIH mice crossed significantly fewer squares in the open field (P = 0.037), and performed significantly fewer rears ($P \leq 0.0001$), compared with control mice. They also touched/sniffed the water bottle significantly less often than infected BALB/c mice (P = 0.012). Infected BALB/c mice also performed significantly fewer rears than their control counterparts (P = 0.018), and touched/sniffed the water bottle fewer times than the control mice, although this difference did not reach significance (P = 0.06).

Table 4.3.2: Observations (± S.D) recorded for control and infected BALB/c and NIH mice, during the habituation period of the water-finding task.

Behaviour	BALB/c		NIH	
	Control	Infected	Control	Infected
No. squares crossed	52.1 ± 23.9	45.9 ± 43.7	65.6 ± 30.3	32.5 ± 25.6
No. times water bottle touched	9.7 ± 0.6	6.9 ± 4.4	5.4 ± 3.3	3.0 ± 1.9
No. rears performed	11.1 ± 7.6	2.5 ± 2.0	20.0 ± 12.2	3.4 ± 4.2

During the testing period, 9 out of 10 control BALB/c mice drank from the water-bottle, compared with only 2 out of 10 infected BALB/c mice ($\chi^2 = 9.899$, $P = 0.002$, D.F = 1), and 6 out of 10 control NIH mice drank, compared with 3 out of 8 infected NIH mice ($\chi^2 = 0.900$, $P = 0.343$, D.F = 1).

Separate 2-way ANOVAs were carried out for each recorded observation in the testing period to investigate effects of strain and infection status. These revealed significant effects of strain and infection status on the time it took for mice to enter the alcove after beginning exploration (strain: $F_{1,34} = 15.2$, $P = 0.0004$; infection status: $F_{1,34} = 5.5$, $P = 0.02$); and also significant effects of infection status, and the interaction between strain and infection status, on the time it took for mice to drink from the water bottle after beginning exploration (infection: $F_{1,34} = 16.7$, $P = 0.0002$; strain x infection status: $F_{1,34} = 6.2$, $P = 0.02$).

Post-hoc tests revealed that infected BALB/c mice took longer to enter the alcove, and locate the water bottle, after beginning exploration, compared with control mice, although these differences were not significant (Figure 4.3.13; time taken to enter alcove: $P = 0.121$; time taken to find water-bottle: $P = 0.06$). Post-hoc tests also revealed that infected BALB/c mice took a significantly longer amount of time to drink from the water bottle, compared with the control mice ($P \leq 0.0001$), suggesting some degree of memory impairment (Figure 4.3.13). Although there were no significant differences between control and infected NIH mice, infected mice generally took longer to locate the water bottle and drink from it compared with control mice (Figure 4.3.14).

Results of separate nested 2-way ANOVAs for each activity revealed no significant effect of cage on behavioural observations ($P > 0.05$, for all activities).
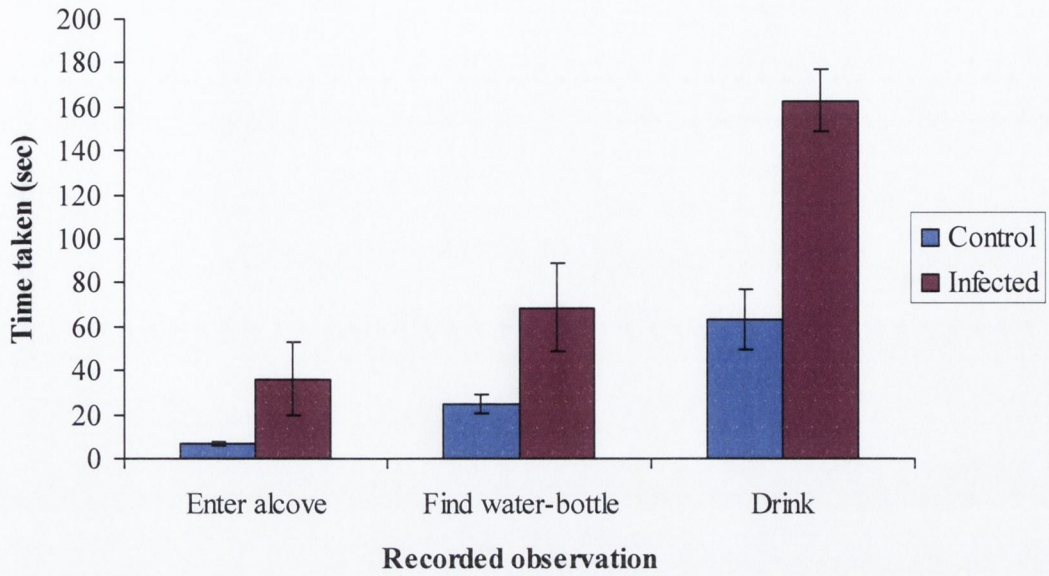
Figure 4.3.13: Mean (± S.E) time taken by control and infected BALB/c mice to carry out the various observations recorded during the water-finding task testing period.
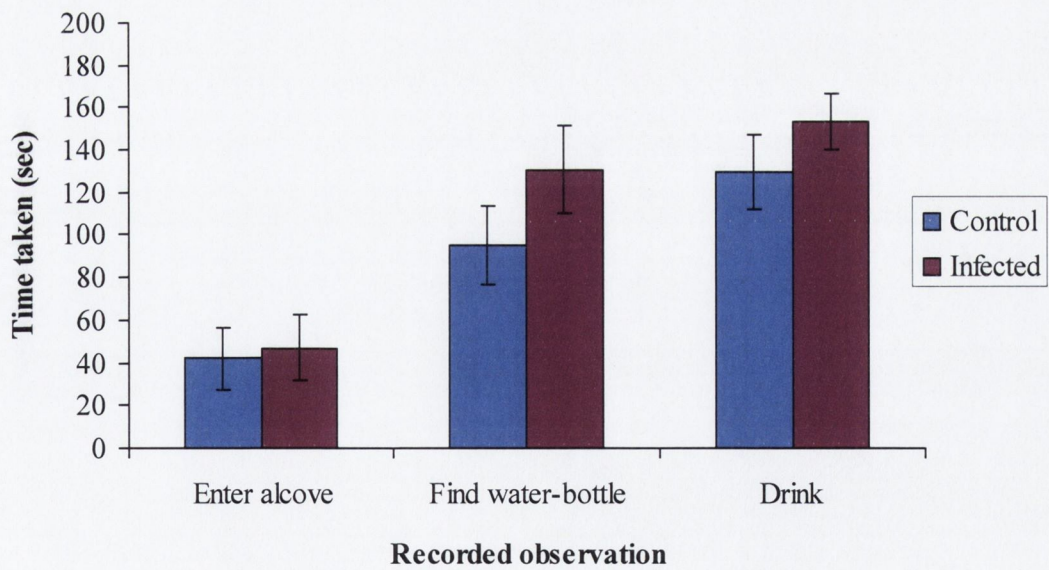
Figure 4.3.14: Mean (± S.E) time taken by control and infected NIH mice to carry out the various observations recorded during the water-finding task testing period.

4.5 DISCUSSION

The most striking result from these experiments was that *T. canis* infection led to some level of memory impairment in infected mice. Spatial awareness, and the ability to use visual cues from the surrounding environment to remember locations of specific resources, is key to the survival of small rodents. In this study, learning and memory was assessed in *T. canis*-infected mice using a water-finding task. In general, infected animals showed a reduction in exploratory behaviour and spatial awareness, taking longer to enter the alcove (after a period of water-deprivation), and locate and drink from the water bottle, compared with control animals. These findings were most pronounced in infected BALB/c mice, with animals taking significantly longer to drink from the water-bottle, after beginning exploration, compared with control mice, suggesting some level of memory impairment. Infected BALB/c mice also took longer to enter the alcove and locate the water bottle in comparison to control mice, but these differences did not reach statistical significance. Although the time taken to enter the alcove did not differ between infected and control NIH mice, infected NIH mice did take a longer amount of time to locate the water-bottle and drink from it, compared with controls, although these differences did not reach statistical significance. A possible explanation for this may be that the drive to enter the alcove was not influenced by the water source specifically but rather the need for mice to seek narrow places of shelter/safety.

A possible alternative explanation for infected BALB/c mice taking longer to enter the alcove, and drink from the water bottle, is general lethargy – since parasitic infection can render a subject less energetic than uninfected individuals. Infected BALB/c mice could have been more sluggish than uninfected mice and therefore took longer to complete any of the tasks set in the experiment. However, looking at the baseline activity of these mice (Figure 4.3.5), it is evident that although infected BALB/c mice were less active for some recorded behaviours, they were more ambulatory than their control counterparts, and less immobile - suggesting that they were not lethargic. In a follow-up experiment from this study, learning and memory was assessed in control and infected BALB/c and NIH mice under the same experimental conditions, although using modified apparatus (Plate 4.3 – Appendix 3) (Flanagan, 2005). Instead of the

open-field apparatus having only one alcove containing the water bottle, the modified apparatus utilised a further 2 separate alcoves. That way, it was possible to test whether or not the infected subject could correctly relocate the water source after a period of habituation. The results of the follow-up study were similar to those obtained in the present study, and the most interesting finding was that infected BALB/c mice took a significantly longer amount of time to drink from the water-bottle than uninfected control mice (see Appendix 3 for data). They also took longer to enter the correct alcove, and locate the water bottle within, compared with controls, although these differences were not significant. Infected NIH mice entered the alcove containing the water bottle fewer times than control mice, but the difference was not statistically significant. The idea that *T. canis*-infected mice have significantly reduced spatial awareness and exploratory skills during infection carries implications for their survival in the wild, and also implications for cognitive development in children.

Previous studies have reported the possibility of learning and memory retardation due to *T. canis* infection in outbred mice. In a study using similar apparatus to that used in this study, Cox and Holland (2001b) demonstrated that outbred LACA mice in moderate and high larval dose groups showed a greater latency to enter the alcove, locate the water-bottle and drink from it, compared with controls, reflecting a degree of memory loss, although the differences were not statistically significant. Dolinksy *et al* (1981) used a passive avoidance test and 'T'-maze to measure the effect of *T. canis* infection on outbred mice. The authors noted that infected mice took longer to avoid the shock than control mice, and took more trials to acquire and retain spatial discrimination, indicating impairment of learning, although differences were not significant. Olson and Rose (1966) reported that the ability of rats to solve maze problems was impaired as a result of *T. canis* infection, and this effect appears to be dose dependent. Rats infected with 20,000 ova made significantly more errors in the maze than control rats. However, at the lower doses of 5000 and 7500 ova, there was no statistical difference between control and infected rats.

Effects on spatial learning have been reported in infections with other parasites. Piekarski (1981) investigated the effects of a latent *Toxoplasma gondii* infection on the learning and memory capacity of mice and rats, and reported that learning capacity was impaired in both species of animal, but particularly so in mice. The author also

reported that the memory and overall activity of infected mice was impaired in comparison to controls. Kavaliers and Colwell (1995) and Kavaliers *et al* (1995) examined the effects of acute *Heligmosomoides polygyrus* and *Eimeria vermiformis* on the acquisition and retention of spatial information in outbred mice, using a water-maze task. In both studies, they found that infected mice were significantly poorer at acquiring the location of the hidden platform, and retaining the information, compared with control mice, and speculated that the cause may be a side-effect of the immunological and neuromodulatory responses of the host. However, in a similar experiment, using *Strongyloides ratti*-infected rats, Braithwaite *et al* (1998) found no evidence of learning or memory impairment in infected animals - even in those receiving a high infective dose. In an earlier study, Kershaw *et al* (1959) assessed the effect of *Schistosoma mansoni* infection on the learning capabilities of rats by counting the number of errors made in solving a 'T'-maze. They noted that infected rats made significantly more errors when solving the maze than controls, therefore displaying a retardation of ability to learn. Witting (1979) assessed learning and memory in *T. gondii*-infected mice using a series of maze experiments, and reported severe memory impairment in infected animals compared with controls. The level of impairment was also significantly correlated with the number of tissue cysts present in the brain.

Of those studies cited above using mice, behavioural testing was carried out using outbred strains, which could potentially explain the absence of significance in some of the results. Outbred mice are derived from stocks of unrelated individuals, so are not uniformly identical like their inbred counterparts. The heterogeneity of outbred mice could be a possible reason behind wider variation in results within strains, and hence non-significant results. The use of inbred mice in the present study perhaps reduced within-strain variation, hence making *T. canis*-induced behavioural alterations more pronounced.

Behavioural differences in the present study were also evident in the baseline activity testing. This behaviour was assessed on post- minus pre-infection data, to take into account variation between the strains prior to infection. Based on this, we found that, in general, baseline activity of infected BALB/c mice seemed less affected by infection compared with infected NIH mice. Infected BALB/c mice spent significantly more time ambulating compared with control mice, perhaps indicating hyperactivity,

although the amount of time spent digging and grooming decreased in comparison to control mice, but the differences were not statistically significant. The amount of time spent climbing decreased compared with pre-infection observations, and the amount of time spent rearing and immobile increased, but the differences did not exceed those of the control mice. Infected NIH mice spent a significantly longer amount of time ambulating compared with control mice, which is interesting considering they also spent a significantly longer amount of time immobile. The increase in ambulation, however, could be due to the fact that there was no obvious increase in any other activity and, in fact, climbing and digging decreased significantly compared with control mice. Climbing and digging could be considered to be explorative and adaptive to survival, so a significant decrease in these behaviours could potentially impact the animal's survival in the wild, and hence exposure to predation.

Exploration is a potentially risky but essential behaviour employed by rodents to source food and shelter in their natural habitat. One of the adaptive values of successful exploration, however, lies in the ability to escape from predators (Montgomery and Gurnell, 1985). Results from the 'T'-maze test in this study show that exploration was decreased in infected NIH mice, compared with control mice. In general, the infected mice entered fewer arms of the maze, and performed significantly fewer rears than controls. This decrease in exploration may reflect the level of immobility seen in the earlier assessment of baseline activity, or is perhaps an indication of familiarity between the animals and their environment. Infected BALB/c mice, on the other hand, demonstrated no change in exploratory behaviour compared with control mice – displaying similar numbers of arm entries and rears performed. This may be a reflection of the increase in ambulatory behaviour observed in the assessment of baseline activity, or it perhaps indicates a lack of familiarity with the environment, further suggesting a degree of memory impairment in these mice. Although infected NIH mice were less explorative than control mice, they did spend more time in the novel arm, although this could be due to their immobility rather than an interest in the novel object itself. Infected NIH mice also spent more time investigating the novel object than the controls, although the overall number of times the object was investigated was lower. Infected BALB/c mice spent less time investigating the novel object, and less time in the novel arm than control mice, although these differences were not significant.

Previous studies have reported the affects of *T. canis* infection on exploratory behaviour in mice. Cox and Holland (2001b) divided infected outbred LACA mice into different larval intensity groups, and assessed each group in terms of exploration and response to novelty using a 'T'-maze. In general, infected mice were less explorative and less responsive to novelty than the control mice, and this was particularly so in the higher larval intensity group (66 to 557 larvae in the brain), although differences did not reach statistical significance. Dolinsky *et al* (1981) infected outbred mice with 1000 *T. canis* eggs, and reported that the number of times infected mice changed levels of their cage to explore an upper level was significantly lower than the other groups. Furthermore, the mean latency to initial ascent was considerably longer in infected mice compared with controls. Burright *et al* (1982) also reported a decrease in exploration in *T. canis*-infected outbred mice, with the most pronounced affect being observed in the highest dose group. Hay *et al* (1983d), however, investigated the exploratory behaviour of *T. canis*-infected mice using a 'Y'-maze, and found infected mice to be just as active and explorative as control mice. Infected mice spent significantly more time in the novel arm, and significantly less time in the familiar arm, compared with control mice. A number of studies into the effects of *Toxoplasma gondii* infection on exploratory behaviour and response to novelty have also reported that infected mice are less responsive to novelty, yet more active than control mice (Hutchison *et al*, 1980c; Hay *et al*, 1983a).

The evidence of memory impairment in *T. canis*-infected mice in this study is interesting because it was evident in BALB/c mice – the chosen susceptible strain. Experiment one identified BALB/c and NIH as susceptible and resistant strains of mice (respectively) to cerebral *T. canis* infection (see Chapter 3). BALB/c mice carried a significantly higher percentage of larvae in their brains than NIH mice, on days 35 and 42 post-infection in experiment 1, and on days 14, 35 and 97 post-infection in experiment 2. The significantly higher percentage of larvae in the brains of BALB/c mice around the time of behavioural testing in experiment 2, correlates with the apparent impairment of memory evident in these mice, suggesting that heavier cerebral larval burdens lead to significantly more pronounced behavioural alterations.

The ability of a parasite to alter its host's behaviour has been an area of increasing interest for researchers over the years (Moore, 2002), particularly whether or not changes in behaviour are an adaptive manipulation by the parasite or a mere side-effect of pathology. Although changes in behaviour can often appear adaptive, it is difficult to prove such a finding. Poulin (1995) outlined a set of criteria that a host-parasite alteration must satisfy before it can be called a parasitic adaptation – these included complexity of the trait, purposiveness of design, convergence and fitness effects. Of a number of studies assessed, where host behavioural changes were believed to facilitate parasite transmission, very few met with all criteria. The facilitation of parasite transmission is one of the most popular motivations thought to be behind behavioural changes (Hay *et al*, 1983a,d; Rau, 1983; Rau and Putter, 1984;). Predisposing its host to predation may be beneficial for some parasites (e.g. *T. gondii, T. canis, T. spiralis*), but for others, with direct life-cycles involving no intermediate hosts, the changes would seem futile (e.g. *H. polygyrus*). It is more likely that changes in behaviour result from the host's response to parasitic infection. With respect to *T. canis*, and this study, the fact that such a range of behaviours were affected (although not all statistically) implies that there was no specific alteration induced by the parasite. Although memory appeared to be significantly altered in infected subjects, the fact that *T. canis* can be maintained in such a wide range of paratenic hosts (including earthworms, chickens, and man), means it is unlikely that this behavioural alteration would have the same consequences for all hosts. The reasons behind the behavioural differences in this study are more likely to involve more complex and interacting factors. It is possible that the immune response produced in the brain is leading to pathology, and hence behavioural changes, but this will be discussed in detail in Chapter 6.

In conclusion, the key findings of this chapter suggest that cerebral toxocariasis leads to memory impairment in the murine host. Although actual larval counts were similar between the strains, those mice exhibiting evidence of memory impairment had statistically higher percentages of larvae in the brain. These results are interesting, not only because of the implications they carry for the animal's survival in the wild (and hence exposure to predation), but also because of the wider implications with respect to human infection. Evidence for cerebral toxocariasis in humans is patchy but there are some indications that larval involvement in the human brain may have some subtle public health implications (Magnaval *et al*, 1997). In a study involving over 4000

children aged 1-15 years, Marmor et al (1987) compared 155 case-control pairs, in terms of a range of parameters including neuropsychological examinations. They found that more parents of cases reported hyperactivity in their children than did parents of controls, and furthermore, cases performed less well on several measures of neuropsychological tests. In a later study, Hill *et al* (1985) reported the presence of *T. canis* larvae in the brain of a child at autopsy. The child had died from 'non-accidental injury', and was said to have cried excessively. The clinical significance of cerebral toxocariasis in humans remains unclear since there are usually other factors involved when the patient presents – such as epilepsy, dementia, and mental retardation (Kaplan *et al*, 2004; Magnaval *et al*, 1997; Glickman *et al*, 1979). However, given the results of this study, and the many others cited, it is evident that cerebral *T. canis* infection leads to behavioural alterations of some extent, and although these results are in rodents, they still carry implications for human health, especially given the high seroprevalence of *T. canis* in some countries (Worley *et al*, 1984; Thomson *et al*, 1986; Holland *et al*, 1995), and the close partnership between dog and man. Furthermore, this model system may also provide insights into the impact of chronic geohelminth infection on cognitive development, something that has proved to be very difficult to investigate in human subjects (Holland and Hamilton, 2005).

CHAPTER 5: Humoral immune response to *Toxocara canis* infection in two inbred strains of mice

5.1 INTRODUCTION

Parasites are known to induce a variety of immunological responses in the infected host, depending on which arm of the immune response has been triggered. Extracellular parasites, such as helminths, are known to predominantly activate the Th2 arm of the immune system, inducing cytokines such as interleukin (IL)-4, IL-5, IL-6 and IL-10 (among others). Production of these cytokines evokes eosinophilia, mastocytosis, and the production of antibodies - mainly IgE and IgG1 (Mosmann *et al*, 1986). Intracellular parasites tend to activate the Th1 arm of the immune system, inducing production of the cytokines IL-2, IFN-γ and TNF-β, provoking a more cell-mediated immunity, although there is production of the antibody IgG2a (Mosmann *et al*, 1986; Scott *et al*, 1989). The immune response evoked, however, is never strictly polarized to either one arm or the other, and in some cases there can be a mixed Th1/Th2 response to infection (Abraham *et al*, 2002; Cortes *et al*, 2003).

The production of antibodies during helminth infection is well documented (Wakelin, 1984; see review Else and Finkelman, 1998). Elevated IgE levels is one of the classic indicators, and this antibody has been implicated as a mediator of antibody-dependent cell-mediated cytotoxicity against a number of helminth infections, including *Schistosoma mansoni* (Olds and Mahmoud, 1980; Capron *et al*, 1982), *Onchocerca volvulus* (Wakelin, 1984) and *Trichinella spiralis* (Callahan *et al*, 1988). The production of IgG1 has also been associated with the control of helminth infections, including *T. spiralis* (Appleton and McGregor, 1987) and *Heligmosomoides polygyrus* (Pritchard *et al*, 1983).

Infection with *T. canis* has been shown to induce a Th2 systemic immune response. Kayes *et al* (1985) investigated the immune responses of inbred CBA/J mice to graded infections with *T. canis*, and noted that the levels of combined IgG and IgM increased in a dose-dependent manner. Eosinophil counts were also observed to increase significantly with infective dose. Havasiová-Reiterová *et al* (1995) examined the humoral immune response in C57BL/6J mice to various doses of *T. canis*, and reported a positive correlation between the level of total immunoglobulin and the number of eggs ingested, with a dose of 50 eggs eliciting a maximal immune response. Cuéllar *et*

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al (2001) reported isotype specific immune responses to *T. canis* infection in mice, with BALB/c mice showing higher levels of IgG1 compared with C3H and C57BL/10 mice. Buijs *et al* (1994) reported peak levels of IgE in *T. canis*-infected mice on day 14 post-infection, and Pinelli *et al* (2001) reported more pronounced total serum IgE levels in BALB/c mice infected with 1000 *T. canis* eggs, compared with C57BL/6J mice.

Although the systemic immune response to *T. canis* infection has been documented, there has been little attention paid to the role (if any) that antibodies may play in controlling larval migration. It has been hypothesised in the past that the pronounced humoral immune response elicited to higher infective doses of *T. canis* may be the reason larvae accumulate in the brain, where there is believed to be little immunoreactivity (Kayes *et al*, 1985). The aim of this chapter, therefore, was to characterise and compare the humoral immune response in two strains of inbred mice, susceptible and resistant to cerebral *T. canis* infection, to ascertain any association between the type of immune response produced and control of the parasite.

5.2 MATERIALS AND METHODS

5.2.1 Collection of sera at post mortem

In experiment 2, 15 mice per strain were sacrificed on days 3, 7, 14, 35, 42 and 97 post-infection (see Chapter 3). On each post mortem day, blood was taken from 5 infected and 5 control mice of each strain, by cardiac puncture using a 25-gauge needle attached to a 1ml syringe. The blood was then transferred to 1.5ml microcentrifuge tubes, and incubated overnight at 4°C. Following incubation, samples were centrifuged at 1200 G for 8 minutes, and serum was transferred to clean microcentrifuge tubes and stored at -20°C until required for immunological analysis.

5.2.2 Preparation of *Toxocara canis* excretory/secretory antigen

Following the collection and embryonation of *T. canis* eggs (see Chapter 2), excretory/secretory antigen was prepared by colleagues at the RIVM (The Netherlands) as follows:

A total of 10^5 embryonated eggs were washed with phosphate buffered saline (PBS, pH 7.2), and incubated in 0.5% natrium hypochloride for 20 minutes to soften the egg shells. Following extensive washing with PBS, eggs were suspended in 5 ml Modified Eagle Medium (GIBCO, N.Y., USA) and larvae were freed from the shells after careful homogenisation. Living larvae were separated from eggs shells and other debris by allowing larvae to migrate through cotton wool contained in tubes filled with medium, at 37°C overnight. The migrating larvae were collected and counted. A suspension of 150 larvae per ml medium was incubated at 37°C for 6 days, after which, the medium was harvested and used as the E/S antigen.

5.2.3 Detection of *Toxocara*-specific IgG1 and IgG2a in sera of BALB/c and NIH mice, using an indirect enzyme-linked immunosorbent assay (Figure 5.3.1)

1. A 1:10 dilution of E/S antigen (80µg/ml) was made with 0.1M sodium carbonate (pH 9.6), and 100µl was added to each well of a 96-well medium binding microtitre plate (Greiner, Germany).

2. Plates were incubated overnight at 37°C, and washed twice in PBS containing 0.05% v/v Tween-20 (PBS/Tween).

3. Serum samples were diluted in two-fold dilutions, starting at 1:40, in 2% bovine serum albumin (BSA) in PBS (Boehringer Mannheim, Germany), and 100µl was added to appropriate wells. Different dilutions were included so the optimum for all samples could be chosen.

4. Plates were incubated for 1 hour at 37°C, and washed twice in 0.05% PBS/Tween.

5. 100µl of rabbit anti-mouse IgG1 (at a dilution of 1:10,000) or IgG2a (at a dilution of 1:250), conjugated to horse-radish peroxidase (HRP) (Zymed Laboratories, San Francisco, USA), and diluted in 4% BSA/PBS/Tween was added to wells.

6. Plates were incubated for 1 hour at 37°C, and washed twice in 0.05% PBS/Tween.

7. 100µl of substrate solution (3,3'5,5'-tetramethylbenzidine (TMB) with hydrogen peroxide (H_2O_2)) was added to each well (giving a blue colour change), and the reaction was stopped after 5 minutes (for IgG1) or 10 minutes (for IgG2a), by the addition of 100µl 2M H_2SO_4 (giving a yellow colour change).

8. The absorbance of each plate was read at 450nm, and was directly proportional to the amount of antibody present in the test sera.

Positive, negative and reagent controls were run on each plate to ensure accuracy and consistency of results. Results for samples in each group (i.e. BALB/c control, BALB/c infected, NIH control, NIH infected) were corrected to the plate with the highest positive control optical density value, to allow for between-plate comparisons.

Toxocara-specific IgG1 and IgG2a levels are expressed as the optical density at 450nm of a 1:40 dilution.

(a)

(b)

(c)

(d)
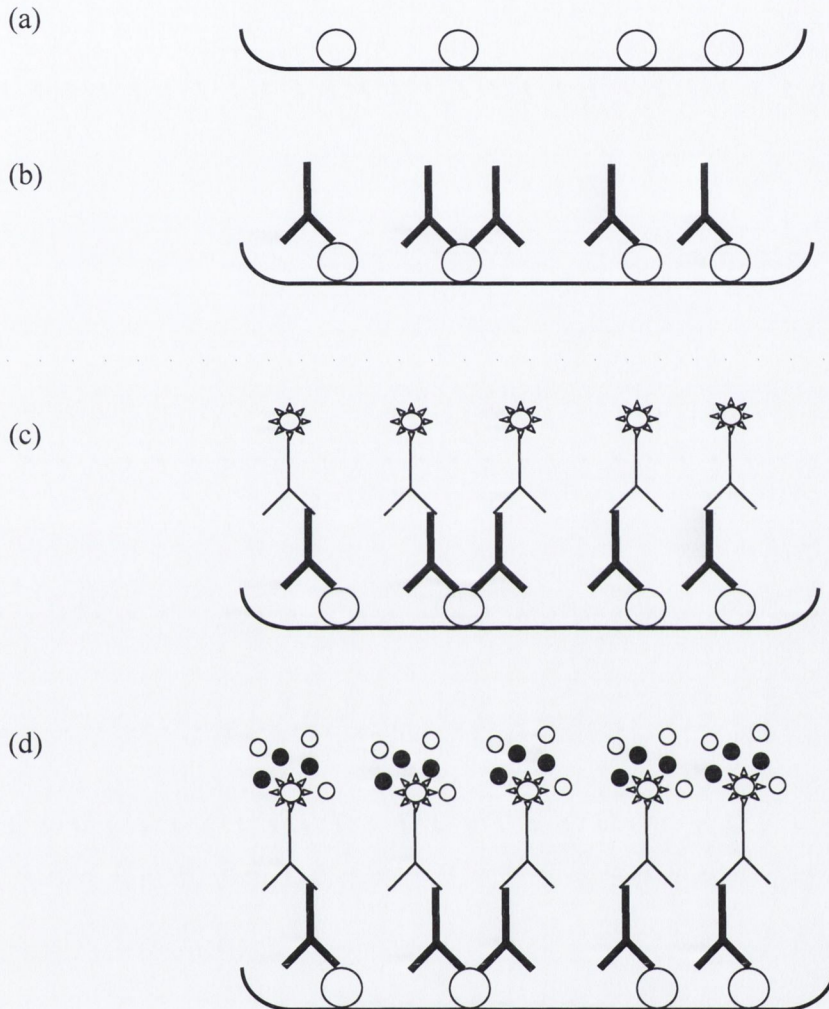
Figure 5.3.1: Indirect ELISA. (a) E/S antigen is attached to solid phase; (b) IgG1/2a present in test sera binds to antigen; (c) HRP-labelled anti-mouse IgG1/2a detects any bound antibodies; (d) substrate is added, and colour change is measured.

5.2.4 Detection of total IgE in sera of *Toxocara canis*-infected BALB/c and NIH mice, using a capture ELISA (Figure 5.3.2)

Total IgE antibody levels were measured in sera using a capture ELISA (BD OptEIA™, BD Biosciences Pharmingen, San Diego, USA) according to the manufacturer's instructions. In brief:

1. A 1:250 dilution of anti-mouse IgE monoclonal antibody (capture antibody) was made in coating buffer (0.1M sodium carbonate, pH 9.6), and 100µl was added to each well of 96-well microtitre plates (Nunc, Denmark).

2. Plates were sealed, and incubated overnight at 4°C.

3. Following incubation, plates were washed 3 times in 0.05% PBS/Tween.

4. 200µl 10% foetal bovine serum (FBS) (Greiner, Germany) was added to each well as a blocking agent, and plates were incubated for 1 hour at room temperature.

5. Plates were washed 3 times in 0.05% PBS/Tween.

6. Doubling dilutions of recombinant mouse IgE standards were made up in 10% FBS, with concentrations from 100ng/ml to 1.6ng/ml. FBS acted as the zero standard (0ng/ml).

7. 100µl of each standard solution was added in duplicate to wells.

8. Serum samples were diluted in two-fold dilutions, starting at 1:40, in 10% FBS, and 100µl was added to appropriate wells.

9. Plates were sealed, and incubated at room temperature for 2 hours.

10. Plates were then washed 3 times in 0.05% PBS/Tween.

11. 100µl of detection antibody (biotinylated anti-mouse IgE monoclonal antibody with avidin-HRP conjugate), at a dilution of 1:250 in 10% FBS, was added to each well.

12. Plates were sealed, and incubated at room temperature for 1 hour.

13. 100µl of substrate solution (TMB with H_2O_2) was added to each well, and the reaction was stopped after 10 minutes by the addition of 100µl 2M H_2SO_4.

14. The absorbance of each plate was read at 450nm.

The mean absorbance of each set of duplicate standards was plotted against the log concentration of IgE, to create a standard curve from which all the results of test sera were calculated. Once IgE concentrations of test sera were calculated from the graph, results were corrected for the dilution factor of the sera to obtain final concentrations, and these are expressed in ng/ml.
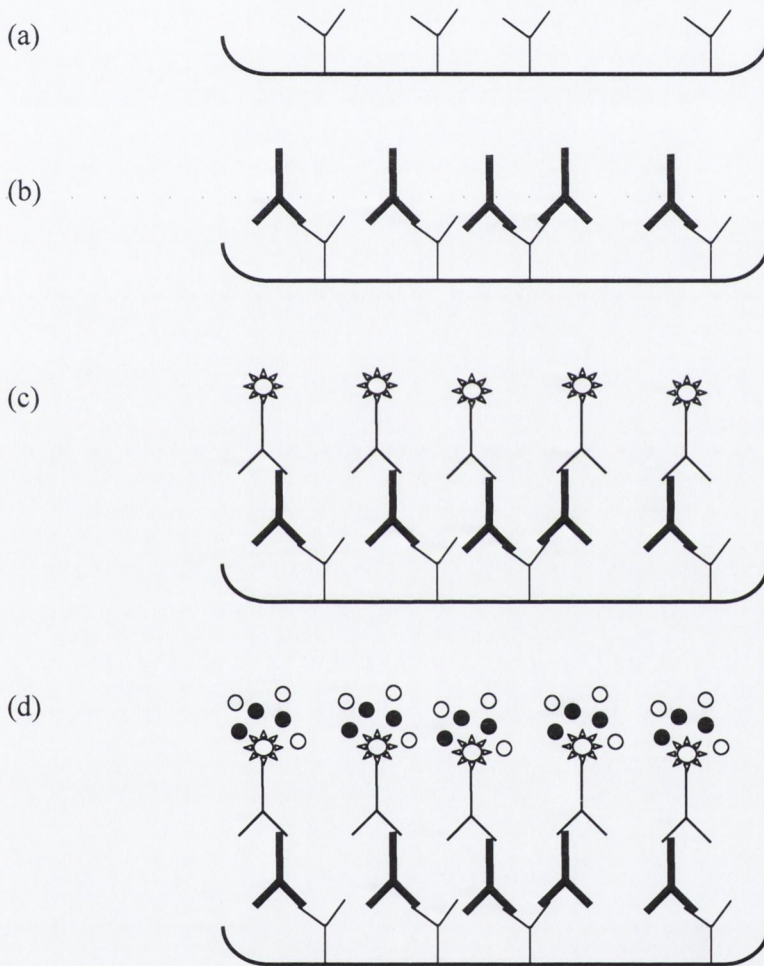
Figure 5.3.2: Capture ELISA. (a) anti-mouse IgE attached to solid phase; (b) IgE present in test sera binds to capture antibody; (c) biotinylated anti-mouse IgE + avidin-HRP detects any bound antibodies; (d) substrate is added, and colour change is measured.

5.2.5 Statistical analysis

All statistical analysis was carried out at the 95% confidence limit, and data were square-root-transformed and checked for normality prior to testing. Effects of day and strain on the levels of IgG1, IgG2a and IgE in the sera of infected and control mice were investigated by means of 2-way ANOVAs, and F-ratios, degrees of freedom and P values are reported in the text. Between-group differences on each day post-infection were investigated using least significant difference (LSD) post-hoc tests, and P values are reported. The same analysis was applied when comparing the levels of antibodies between infected BALB/c and infected NIH mice, and appropriate statistics are reported.

5.3 RESULTS

5.3.1 Detection of *Toxocara*-specific IgG1 and IgG2a in sera of BALB/c and NIH mice, using an indirect enzyme-linked immunosorbent assay

5.3.1.1 *Toxocara*-specific IgG1

The level of *Toxocara*-specific IgG1 increased in both strains of infected mice over the course of infection, compared with control mice (Figures 5.3.3 and 5.3.4). Two-way ANOVAs revealed significant effects of day, infection status, and the interaction between these factors, on the levels of *Toxocara*-specific IgG1 detected in the sera of control and infected BALB/c and NIH mice (Table 5.3.1). Post-hoc tests demonstrated significantly higher levels of the antibody in infected mice compared with control mice, for both strains, on days 14, 35, 42 and 97 post-infection (post-hoc tests: BALB/c: day 14 p.i.: $P = 0.0007$; days 35, 42 and 97 p.i.: $P \leq 0.0001$; NIH: day 14 p.i.: $P = 0.001$; days 35, 42 and 97 p.i.: $P \leq 0.0001$). After day 14 p.i., the levels of IgG1 in infected mice of both strains, increased significantly over the course of infection (BALB/c: day 14 p.i. – day 35 p.i.: $P \leq 0.0001$; day 35 p.i. – day 42 p.i.: $P \leq 0.0001$; day 42 p.i. – day 97 p.i.: $P = 0.02$; NIH: day 14 p.i. – day 35 p.i.: $P \leq 0.0001$; day 42 p.i. – day 97 p.i.: $P \leq 0.0001$).

A 2-way ANOVA revealed significant effects of day, strain, and the interaction between these factors on the levels of IgG1 in the sera of infected BALB/c and NIH mice (day: $F_{5,41} = 275.1$, $P \leq 0.0001$; strain: $F_{1,41} = 149.9$, $P \leq 0.0001$; day x strain: $F_{5,41} = 25.8$, $P \leq 0.0001$). Post-hoc tests demonstrated that infected BALB/c mice had significantly higher levels of IgG1 on days 35, 42 and 97 post-infection, compared with NIH mice ($P \leq 0.0001$ for all comparisons) (Figure 5.3.5).

Table 5.3.1: Effects of day, infection status, and the interaction between these factors, on the levels of *Toxocara*-specific IgG1 in the sera of control and infected BALB/c and NIH mice.

Strain	Effect	*F*-ratio	D.F	*P*-value
BALB/c	Day	190.5	5,46	≤ 0.0001
	Infection status	1150.7	1,46	≤ 0.0001
	Day x infection status	212.0	5,46	≤ 0.0001
NIH	Day	103.7	5,38	≤ 0.0001
	Infection status	621.1	1,38	≤ 0.0001
	Day x infection status	102.6	5,38	≤ 0.0001

Figure 5.3.3: Mean level (± SEM) of *Toxocara*-specific IgG1 detected in the sera of control and infected BALB/c mice. (Between-group comparisons: ** $P \leq 0.05$; *** $P \leq 0.0001$).

Figure 5.3.4: Mean level (± SEM) of *Toxocara*-specific IgG1 detected in the sera of control and infected NIH mice. (Between-group comparisons: ** $P \leq 0.05$; *** $P \leq 0.0001$).
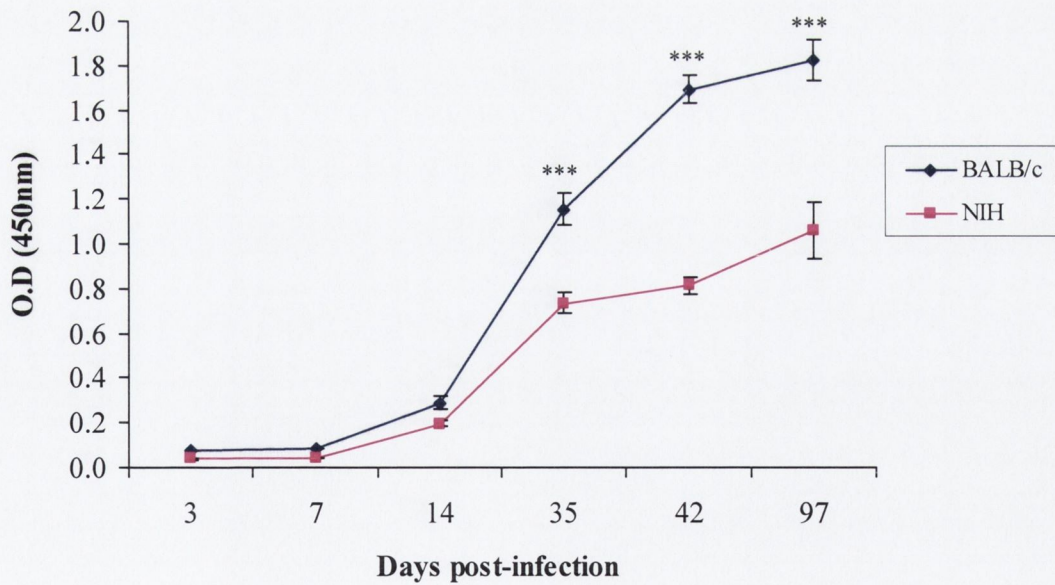
Figure 5.3.5: Mean level (± SEM) of *Toxocara*-specific IgG1 detected in the sera of infected BALB/c and NIH mice. (Between-group comparisons: *** $P \leq 0.0001$).

5.3.1.2 *Toxocara*-specific IgG2a

The levels of *Toxocara*-specific IgG2a in both strains of infected mice were generally lower than those of IgG1. Levels in infected BALB/c mice fluctuated over the course of infection, and were higher than levels detected in control mice (Figure 5.3.6). In infected NIH mice, the levels of IgG2a increased over the course of infection, and were higher than in control mice (Figure 5.3.7). Two-way ANOVAs revealed significant effects of day, infection status, and the interaction between these factors, on the levels of *Toxocara*-specific IgG2a detected in the sera of control and infected BALB/c and NIH mice (Table 5.3.2). Post-hoc tests demonstrated significantly higher levels of the antibody in infected BALB/c mice compared with control mice, on days 14, 35, 42 and 97 post-infection (post-hoc tests: day 14 p.i.: $P \leq 0.0001$; day 35 p.i.: $P = 0.05$; day 42 p.i.: $P \leq 0.0001$; day 97 p.i.: $P = 0.006$). Post-hoc tests also revealed significantly higher levels of the antibody in infected NIH mice, compared with control mice, on days 7, 14, 35, 42 and 97 post-infection (day 7 p.i.: $P = 0.01$; days 14, 35 and 42 p.i.: $P \leq 0.0001$; day 97 p.i.: $P = 0.0005$).

A 2-way ANOVA revealed significant effects of day, and the interaction between day and strain, on the levels of IgG2a in the sera of infected BALB/c and NIH mice (day: $F_{5,39} = 17.4$, $P \leq 0.0001$; day x strain: $F_{5,39} = 3.8$, $P = 0.0068$). Post-hoc tests demonstrated that infected BALB/c mice had significantly higher levels of IgG2a on days 14 and 42 post-infection, compared with NIH mice (day 14 p.i.: $P = 0.009$; day 42 p.i.: $P = 0.002$) (Figure 5.3.8[*]).

[*]Note: Due to high background levels of IgG2a in BALB/c control mice, the mean control value was taken away from each data point of the infected BALB/c mice, and these points were plotted. The same principle was also applied to NIH mice, so that the two strains could be compared.

Table 5.3.2: Effects of day, infection status, and the interaction between these factors, on the levels of *Toxocara*-specific IgG2a in the sera of control and infected BALB/c and NIH mice.

Strain	Effect	*F*-ratio	D.F	*P*-value
BALB/c	Day	6.0	5,38	0.0003
	Infection status	25.8	1,38	≤ 0.0001
	Day x infection status	4.7	5,38	0.0019
NIH	Day	3.9	5,39	0.005
	Infection status	116.7	1,39	≤ 0.0001
	Day x infection status	8.4	5,39	≤ 0.0001
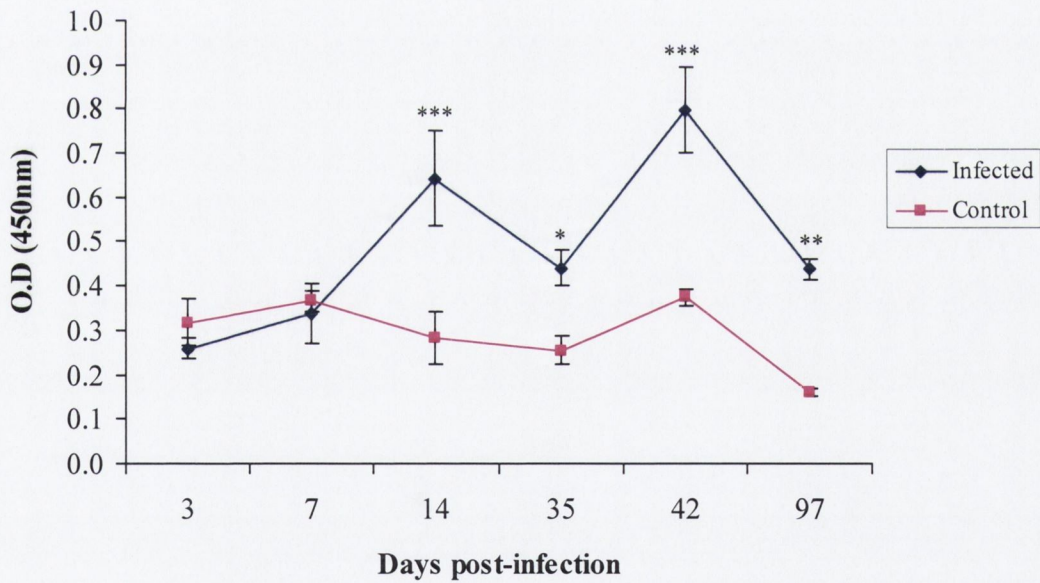
Figure 5.3.6: Mean level (± SEM) of *Toxocara*-specific IgG2a detected in the sera of control and infected BALB/c mice. (Between-group comparisons: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.0001$).
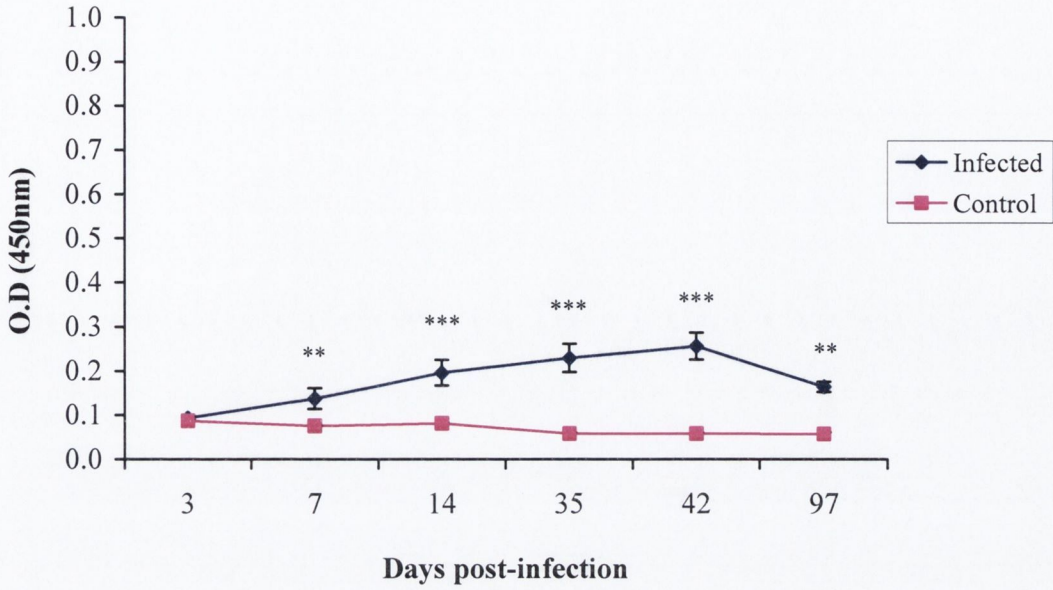
Figure 5.3.7: Mean level (± SEM) of *Toxocara*-specific IgG2a detected in the sera of control and infected NIH mice. (Between-group comparisons: ** $P \leq 0.01$; *** $P \leq 0.0001$).
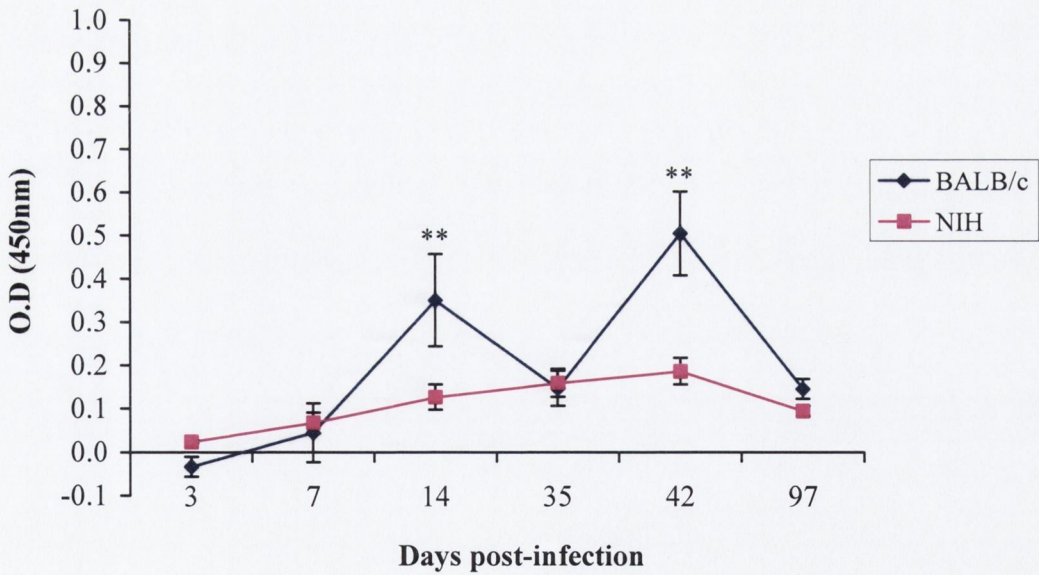
Figure 5.3.8: Mean level (± SEM) of *Toxocara*-specific IgG2a detected in the sera of infected BALB/c and NIH mice. (Between-group comparisons: ** $P \leq 0.01$).

5.3.2 Detection of total IgE in sera of *Toxocara canis*-infected BALB/c and NIH mice, using a capture ELISA.

The levels of total serum IgE in both strains of infected mice increased over the course of infection (Figures 5.3.9 and 5.3.10). Two-way ANOVAs revealed significant effects of day, infection status, and the interaction between these factors, on the levels of total IgE detected in the sera of control and infected BALB/c and NIH mice (Table 5.3.3). Post-hoc tests demonstrated significantly higher levels of the antibody in infected mice compared with control mice, for both strains, on days 14, 35, 42 and 97 post-infection (post-hoc tests: $P \leq 0.0001$ for all comparisons). Following day 7 p.i., levels of IgE increased significantly in infected mice of both strains, over the course of infection ($P \leq 0.0001$, for all consecutive between-day comparisons). However, between days 35 and 42 p.i., the levels in infected BALB/c mice did not differ significantly, and between days 42 and 97 p.i., the levels in NIH mice had reached a plateaux.

A 2-way ANOVA revealed significant effects of day, strain, and the interaction between these factors on the levels of IgE in the sera of infected BALB/c and NIH mice (day: $F_{5,45} = 199.7$, $P \leq 0.0001$; strain: $F_{1,45} = 41.0$, $P \leq 0.0001$; day x strain: $F_{5,45} = 9.1$, $P \leq 0.0001$). Post-hoc tests demonstrated that infected NIH mice had significantly higher levels of IgE on days 14, 35 and 42 post-infection, compared with BALB/c mice (day 14 p.i.: $P = 0.0002$; day 35 p.i.: $P = 0.0003$; day 42 p.i.: $P \leq 0.0001$) (Figure 5.3.11).

Table 5.3.3: Effects of day, infection status, and the interaction between these factors, on the levels of total serum IgE in the sera of control and infected BALB/c and NIH mice.

Strain	Effect	*F*-ratio	D.F	*P*-value
BALB/c	Day	66.5	5,47	≤ 0.0001
	Infection status	390.7	1,47	≤ 0.0001
	Day x infection status	49.3	5,47	≤ 0.0001
NIH	Day	257.3	5,46	≤ 0.0001
	Infection status	1804.4	1,46	≤ 0.0001
	Day x infection status	214.5	5,46	≤ 0.0001
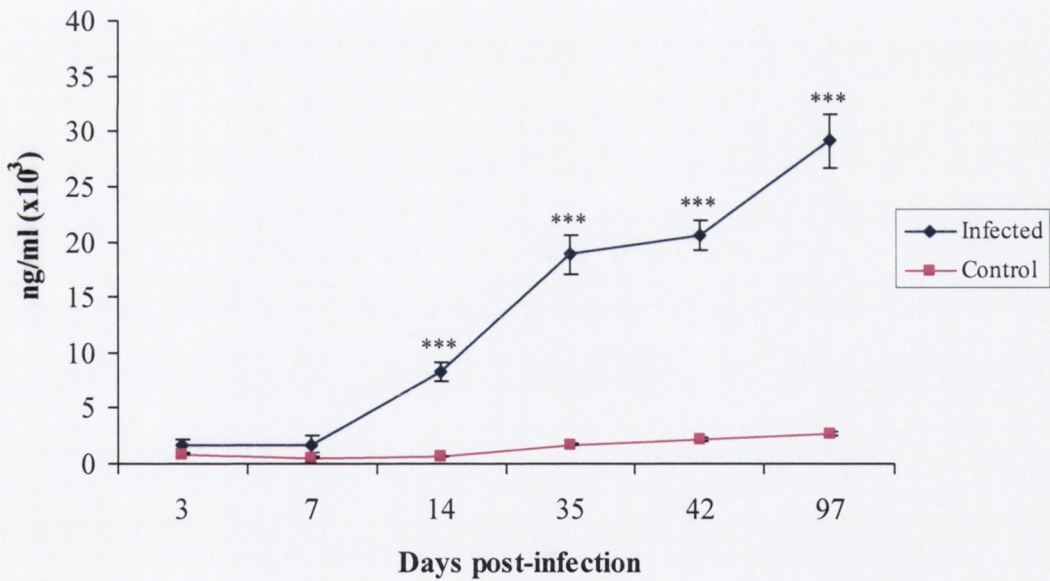
Figure 5.3.9: Mean level (± SEM) of total serum IgE detected in the sera of control and infected BALB/c mice. (Between-group comparisons: *** $P \leq 0.0001$).

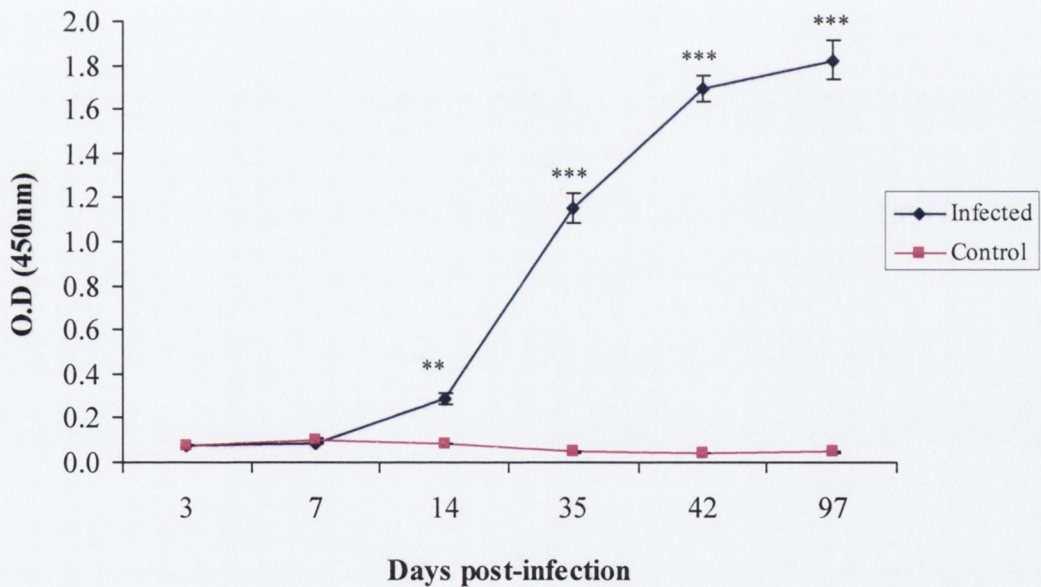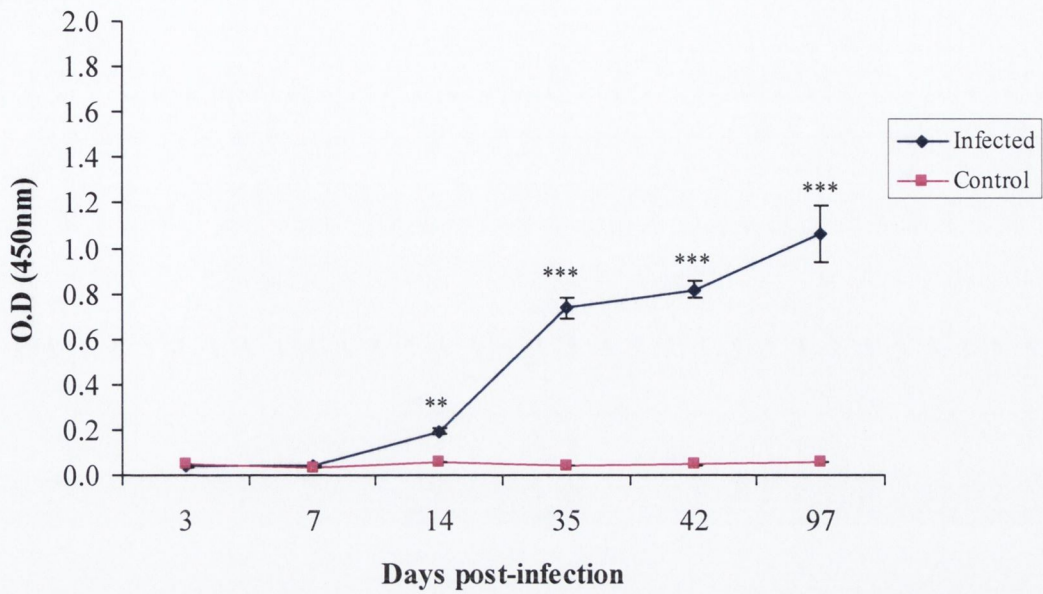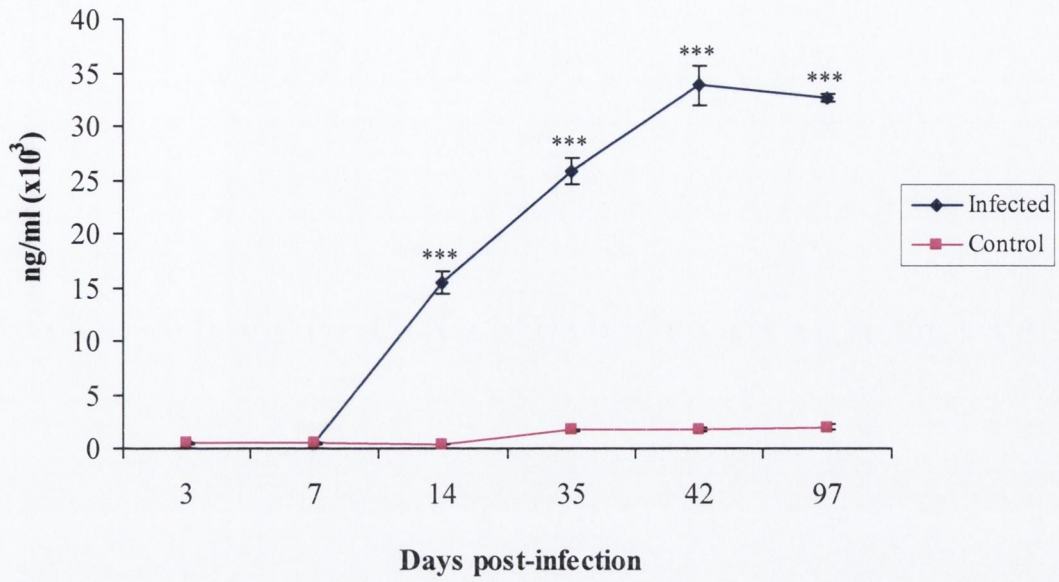**Figure 5.3.10:** Mean level (± SEM) of total serum IgE detected in the sera of control and infected NIH mice. (Between-group comparisons: *** $P \leq 0.0001$).
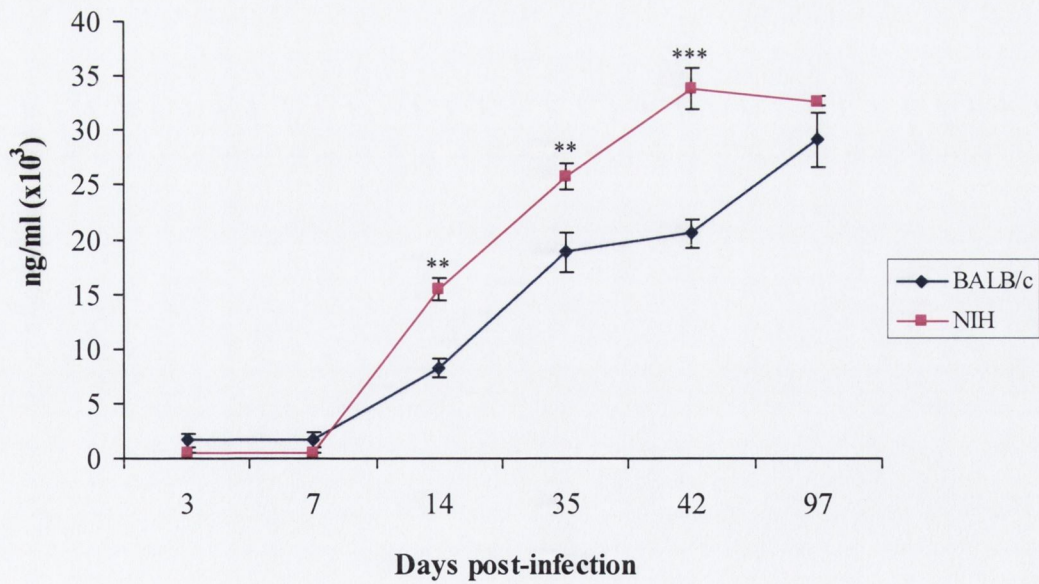
Figure 5.3.11: Mean level (± SEM) of total serum IgE detected in the sera of infected BALB/c and NIH mice. (Between-group comparisons: ** $P \leq 0.01$; *** $P \leq 0.0001$).

5.4 DISCUSSION

The results of this chapter revealed that *T. canis*-infected mice elicited a more pronounced Th2-type of humoral immune response, with the production of *Toxocara*-specific IgG1 and total serum IgE. This was particularly so in BALB/c mice, but there appeared to be no definite correlation between antibodies produced and larval burden in the brain. There was a significantly pronounced IgE response in infected NIH mice, perhaps indicating a role for this antibody isotype in cerebral larval establishment.

The levels of *Toxocara*-specific IgG1 and IgG2a, and total serum IgE, were measured in infected BALB/c and NIH mice over the course of a 97-day infection. Significantly higher levels of all antibodies were detected in infected mice of both strains, compared to their control counterparts, from day 14 p.i. onwards. The kinetics of IgG1 production in infected mice of both strains was similar, with antibody levels being detected on day 14 p.i. and then increasing significantly throughout infection, with the peak level being detected on day 97 p.i. When infected mice of both strains were compared, it was observed that BALB/c mice had significantly higher levels of IgG1 in their serum on days 35, 42 and 97 p.i., indicating them to be 'higher responders' than NIH mice. The levels of IgG2a were lower than IgG1 in both strains of infected mice, and remained low throughout infection – although levels appeared to fluctuate in infected BALB/c mice. Both strains of infected mice exhibited significantly higher levels of IgG2a compared with control mice from day 14 p.i. onwards – with peak levels being detected in infected BALB/c mice on days 14 and 42 p.i., and in infected NIH mice on day 42 p.i. When infected mice of both strains were compared, it was evident that BALB/c mice had significantly higher levels of IgG2a on days 14 and 42 post-infection, although this may have been due to a technical error rather than BALB/c mice actually being higher responders than NIH mice. The high background levels of BALB/c control mice (shown in Figure 5.3.6) would also suggest that levels in infected mice are lower than they appear.

The predominant induction of IgG1 observed in *T. canis*-infected mice in this study is similar to previous studies. Cuéllar *et al* (2001) reported isotype specific immune responses to *T. canis* infection in BALB/c, C57BL/10 and C3H mice inoculated with

4000 eggs. Levels of IgG1 were highest in all strains, being detected from day 14 p.i. and remaining high throughout infection – and this was particularly so in BALB/c mice. The IgG2a response was much lower in all strains, although significantly higher in BALB/c mice compared with all other strains. The authors suggested that both Th1 and Th2 cells were active during infection, although with a Th2-dominant response. This is similar to the results of the present study where overall lower levels of IgG2a compared with IgG1 were observed, but with BALB/c mice demonstrating significantly higher levels of IgG2a compared with control mice. Carter (1992) assessed the antibody production on day 14 p.i., in mice infected with 35S-labelled *T. canis* larvae, and noted the production of parasite-specific IgG1 and IgM. Fan *et al* (2003) investigated the levels of IgG1 and IgG3 in ICR mice infected with 250-260 *T. canis* eggs, and observed a predominant IgG1 response from week 4 to week 67 p.i. Levels fluctuated over the course of infection, with a peak on week 28 p.i., and were significantly higher than control mice throughout. Levels of increased IgG1 have also been reported in the sera of human patients seropositive for *Toxocara*-specific IgG (Obwaller *et al*, 1998).

The levels of total serum IgE measured in *T. canis*-infected BALB/c and NIH mice in the present study were detectable from day 14 p.i., and increased significantly throughout infection. Infected mice of both strains had significantly higher levels of the antibody than their control counterparts from day 14 p.i. onwards, with a peak in infected BALB/c mice on day 97 p.i., and in NIH mice on day 42 p.i. When infected mice of both strains were compared, there was a significantly more pronounced response in NIH mice, compared with BALB/c mice, on days 14, 35 and 42 p.i., although by day 97 p.i. levels were similar between the strains. This is an interesting result, given the more pronounced IgG1 response in BALB/c mice.

Raised levels of IgE have been reported in previous studies with *T. canis*. Buijs *et al* (1994) reported peak levels of IgE on day 14 p.i., in sera of BALB/c mice infected with various doses of *T. canis* eggs. Pinelli *et al* (2001) reported significantly higher levels of total serum IgE as early as day 8 p.i., in both *T. canis*-infected BALB/c and C57BL/6 mice, compared with control mice. In a more recent paper, Pinelli *et al* (2005) reported significantly higher levels of IgE in *T. canis*-infected BALB/c mice,

compared with controls - with antibody levels being detected on day 14 p.i. and increasing throughout infection.

The role of T helper subsets (particularly Th2), and the cytokines they release has been well researched in response to helminth infections (Sher and Coffman, 1992). The hallmarks of infection are most notably elevated serum IgE and IgG1 levels, eosinophilia and mastocytosis (Okano *et al*, 1999; Bueno *et al*, 2001; Cortes *et al*, 2003) – all generally being induced by the Th2 cytokines IL-4 and IL-5 (Mosmann and Coffman, 1987; Pene *et al*, 1988; Sanderson, 1990). The production of IgG1 and IgE has been reported in many cases of helminth infection and, in some, has been associated with parasite control and conferred resistance. Lebrun and Spiegelberg (1987) reported the concomitant production of IgG1 and IgE in *Nippostrongylus brasiliensis*-infected mice, with decreased levels of IgG2a. Similarly, Zakroff *et al* (1989) reported an increase in the IgG1 and IgE serum levels of mice infected with four helminth parasites (*N. brasiliensis*, *Mesocestoides corti*, *Taenia crassiceps* and *Trichinella spiralis*). Prichard *et al* (1983) demonstrated that purified IgG1 reacted with antigenic components common to both *Heligmosomoides polygyrus* adult homogenate and adult excretory-secretory antigen. In addition, IgG1 was the only purified immunoglobulin isotype to cause significant reduction in worm burdens in the gastrointestinal tract of infected mice, and was also observed to cause severe stunting of worms. Ahmad *et al* (1991) demonstrated that the transfer of purified IgE antibody to *T. spiralis*-infected rats resulted in the rapid expulsion of worms. They also observed that muscle larval burdens were enhanced in mice with suppressed IgE production.

IgE is one of the main antibodies produced in response to helminth infection, but overall it makes up a very small percentage of total immunoglobulin. Despite its low levels, however, it is known to bind avidly to high affinity receptors (FcεRI) on mast cells found just beneath the skin and mucosa (Roitt, 1993). Activation of mast cells occurs when the bound IgE is cross-linked by multivalent antigen, which signals the cell to release the contents of its granules (primarily histamine) into the surrounding tissue, initiating a local inflammatory response, with accumulation of blood fluid and proteins, including antibodies (Roitt, 1993). This activation also leads to the synthesis of lipid mediators such as prostaglandin D2 and leukotriene C4, which recruit and

activate neutrophils, basophils and eosinophils, contributing further to the IgE-mediated response. The activation of mast cells and eosinophils has been implicated in the control of some intestinal nematodes. Mice deficient in mast cells (either through treatment with anti-stem-cell factor, or anti-*c*-kit antibodies) show impaired clearance of *T. spiralis* infection compared with control mice (Alizadeh and Wakelin, 1984; Donaldson *et al*, 1996). Primary infection with *H. polygyrus* is chronic in most strains of mice and tends to lack a mast cell response. However, where worm expulsion is observed, a mast cell response begins to develop (Else and Finkelman, 1998). Eosinophils have been associated with control of *Schistosoma mansoni* – with depletion of these cells, using polyclonal anti-eosinophil antisera, leading to increased severity of infection in mice (Butterworth *et al*, 1977). Degranulated eosinophils adhering to the surface of *S. mansoni* larvae *in vitro* have also been observed – suggesting a role in parasite killing, in the presence of IgE (Butterworth *et al*, 1977). An eosinophil-mediated protective immune response has also been suggested against the migratory larval stages of *Strongyloides venezuelensis* (Korenaga *et al*, 1994) and *Angiostrongylus cantonensis* (Sasaki *et al*, 1993).

In many other cases, the presence of eosinophils and mast cells play no part in resistance to parasitic infection – with the ablation of these cells having no effect on parasite burdens, in the cases of *N. brasiliensis*, *H. polygyrus* and *T. spiralis* (Madden *et al*, 1991; Urban *et al*, 1991; Herndon and Kayes, 1992). We can merely speculate about the roles elevated levels of *Toxocara*-specific IgG1 and total serum IgE play in the control of *T. canis* in this study. Given the larval burdens and pattern of migration reported in Chapter 3 (with larval numbers decreasing in liver and lungs after day 7 p.i., whilst increasing in the brain), it would appear that the antibodies produced in response to infection are doing little to control the migrating larvae. This may be because the humoral response is produced late in infection (i.e. day 14 p.i.), by which time the majority of larvae are already resident in the brain (70-80% of the total larvae recovered). The significantly higher levels of IgE recorded in NIH mice is interesting, given these mice carried a significantly lower larval burden (as a percentage of the total number of larvae recovered) in the brain on 3 of the days post-infection, compared with BALB/c mice. This may indicate a potential role of IgE-mediated control against larvae migrating in the visceral organs, reducing the overall numbers free to migrate to the brain. However, a recent study by Jones *et al* (1994) reported the lack of IgE

receptors (FcεRII) on bronchoalveolar lavage eosinophils recovered from *T. canis* infected mice, suggesting that murine eosinophils do not kill helminth larvae by an IgE-dependent mechanism. The authors did, however, detect the IgG1 receptor (FcγRII) on eosinophils, indicating that perhaps IgG1 is involved in antibody-dependent cell-mediated cytotoxicity reactions or antigen-specific degranulation. In a much earlier paper, Fattah *et al* (1986) investigated whether *T. canis* larvae could be killed in the presence of human blood eosinophils taken from a child recovering from toxocariasis. Using both electron and light microscopy, the authors noted the adherence of eosinophils to the surface of the larvae after as little as 10 mins, and after 40 mins, the cells had flattened against the cuticle and degranulated. However by 3 hours, the cells had begun to detach, and the larvae remained alive for at least one week afterward. The further addition of patient serum, or a pure solution of eosinophils, failed to kill the larvae, suggesting that eosinophil-dependent killing mechanisms may be less important than other components of the immune system in the control of *T. canis* larvae. If IgE does play a role in the control of cerebral larval establishment, further research would be required to elucidate the mechanisms involved.

The apparent lack of effect of the Th2 humoral immune response on migrating *T. canis* larvae observed in this study may be because it is ineffective against that particular stage of the parasite. In a recent paper, Mulcahy *et al* (2005) reviewed the literature on Th2-mediated protection against helminth infection, and concluded that the response has been characterised principally for helminths at mucosal sites. When migration is a feature in the life cycle of a helminth, a dominant Th2 immune response is often much more effective against mucosal-dwelling stages of the parasite than against migratory stages. *T. canis* in its definitive host is an example of this – adult worms are expelled from the small intestine of dogs by a combination of innate and adaptive immunity, whereas larval stages can persist in the tissues indefinitely (Lloyd *et al*, 1981). Similarly, *T. spiralis* adults are susceptible to expulsion from the murine gut by classic Th2 responses, whereas migratory larval stages can persist indefinitely in skeletal-muscle cells (Helmby and Grencis, 2003). It is more often reported that the immune response effective at eliminating tissue-dwelling stages is orchestrated by the Th1 arm of the immune response (Toledo *et al*, 2001; Abraham *et al*, 2002; Helmby *et al*, 2003). Mulcahy *et al* (2005) suggest, therefore, that the migratory phase of some parasite life cycles could be a useful mechanism for avoiding the effector consequences of the Th2

response, suited to protecting against parasites at mucosal surfaces. Immune evasion could subsequently afford the parasite more time in its host, thus increasing the chances of it completing its life cycle.

In conclusion, the results of this chapter have revealed a dominant Th2-type of humoral immune response during *T. canis* infection, although there was evidence of Th1-type of immune response also, with the production of IgG2a in infected BALB/c mice. Bearing in mind the larval recoveries reported in Chapter 3, and given the evidence of other host-parasite systems, where the Th2 immune response is ineffective against tissue-dwelling stages, it seems likely that the predominant Th2 humoral response observed in this study does little to control migrating larvae. It is possible that the elevated antibody levels may play a more effective role in protection against secondary infection rather than primary.

CHAPTER 6: Relative quantification of the cerebral immune response in two inbred strains of mice infected with *Toxocara canis*

Figure   **Page**

Table   **Page**

6.1 INTRODUCTION

Toxocara canis infection in the murine host has been shown to result in parasite accumulation in the brain (Sprent, 1955; Lee, 1960, Dunsmore *et al*, 1983; Medved'ová *et al*, 1994; Skerrett and Holland, 1997). As described in Chapter 3, *T. canis* larvae generally display two stages of migration – an early hepato-pulmonary phase, with migration predominantly through the liver and lungs, and a later myotropic-neurotropic phase, with migration to the muscle and brain (Abo-Shehada and Herbert, 1984). Dunsmore *et al* (1983) were the first to clearly illustrate the concept of larval accumulation during *T. canis* infection, demonstrating that as larval numbers increased in the brains of infected mice, they decreased in other tissues. The results from the present study, reported in Chapter 3, have also demonstrated larval accumulation in the brains of two strains of inbred mice – with percentages of total larvae recovered from the brain increasing from 10% in early infection to 90% by late infection. Variation in the numbers of larvae recovered from the brains of *T. canis*-infected mice has been reported in both outbred and inbred strains (Epe *et al*, 1994), even in individual mice of the same strain, receiving the same infective dose (Skerrett and Holland, 1997; Cox and Holland, 2001), suggesting a role of immunity in the establishment of cerebral infection.

As reported in previous studies, and in Chapter 5 of this study, infection with *T. canis* induces a Th2 humoral immune response, with the production of IgE and IgG1 (Buijs *et al*, 1994; Cuéllar *et al*, 2001). However, given the larval burdens in the different organs of both strains of mice in this study, it is apparent that the humoral immune response does little to control migrating larvae. The immune response in the brain during *T. canis* infection has received very little attention. Previous studies have reported the lack of cellular response to larvae resident in the brain, and the presence of viable larvae, as verified by observing their movement in tissue preparations (Sprent, 1955; Burren, 1971; Dunsmore *et al*, 1983; Summers *et al*, 1983). The presence of the blood-brain-barrier, and the idea that immune reactivity is rarely observed in the brain, has commonly led to the concept that the brain is an immune privileged organ (Owens *et al*, 1994). This in turn has led to the suggestion that *T. canis* larvae accumulate here as a means of avoiding the host's immune response, thus prolonging their survival in

the host, and increasing the chances of transmission to an appropriate definitive host upon the ingestion of murine cerebral tissue.

Cytokines are small, secreted proteins which mediate and regulate many aspects of the immune response and inflammatory reactions. Th1 and Th2 cells are defined by their production of cytokines – with Th1 cells producing IFN-γ, IL-2 and TNF-β, and Th2 cells producing IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Mosmann and Coffman, 1987). The various cytokines produced during a Th1- or Th2-type response are the driving force behind many mechanisms activated during infection, such as strong IgG1 and IgE production, and eosinophilia, in a Th2-type response, and macrophage activation (with the release of nitric oxide, other reactive nitrogen and oxygen intermediates, and cytokines), and IgG2a production during a Th1-type response. There is abundant evidence of the production of cytokines in response to various CNS-dwelling parasites, including *Taenia solium* (neurocysticercosis), *Plasmodium falciparum* and *P. berghei* (cerebral malaria), and *Toxoplasma gondii* (cerebral toxoplasmosis), diminishing any idea that the brain is an organ of immune privilege (Restrepo *et al*, 1998; Brown *et al*, 1999; Gazzinelli *et al*, 1993). In fact, in these studies, the quality and quantity of immune response produced has often been responsible for the induction of pathology.

Characterisation of the immune response produced in parasitic invasion of the CNS is important not only for understanding the host-parasite relationship in the brain, but also the associated pathology. Elucidating the cerebral immune response in *T. canis* infection will provide important insights into the disease process and the consequences of such infection – particularly with respect to human infection when, often, patients present with other CNS disorders such as epilepsy, dementia and mental retardation (Kaplan *et al*, 2004; Magnaval *et al*, 1997; Glickman *et al*, 1979).

The main aim of this chapter, therefore, was to characterise the cerebral immune response produced during *T. canis* infection in two strains of inbred mice, susceptible (BALB/c) and resistant (NIH) to infection, by the detection of different Th1 and Th2 cytokines in brain tissue using real-time RT-PCR.

Quantitative RT-PCR is a sensitive and accurate technique that measures PCR-product accumulation during the exponential phase of the reaction, thus enabling quantification of the PCR product in "real time".

6.2 MATERIALS AND METHODS

6.2.1 Collection of tissues at post-mortem

In experiment 2, 15 mice per strain were killed on days 3, 7, 14, 35, 42 and 97 post-infection (see Chapter 3). The brains from 5 control and 5 infected mice of each strain, were carefully removed and sagitally bisected into right and left cerebral hemispheres, and transferred to clean cryovials (Sarstedt). Each sample was snap frozen immediately in liquid nitrogen, and transferred to the -80°C freezer for storage until required for analysis.

6.2.2 Total RNA isolation from the brains of *T. canis*-infected BALB/c and NIH mice

Due to time constraints, total RNA was extracted from the right cerebral hemisphere only. The decision to choose the right hemisphere was based on previous work by Good *et al* (2001), where more *T. canis* larvae were found in the telencephalon and diencephalons of the right-hand side of the brain, compared with the left. Extraction of total RNA was carried out according to manufacturer's instructions. In brief:

Tissue homogenisation
  1. 2ml, sterile, screw-cap tubes (Greiner Bio-one) were filled 1/3 full with 1mm zirconia homogenisation beads (BioSpec Products Inc.), and 1ml TRIzol® reagent (Invitrogen) was added.
  2. Brain samples (whole, right cerebral hemisphere) were then added, and the screw-caps closed and covered with parafilm to ensure no leakage.
  3. Samples were homogenised in a Ribolyser for 2 x 20 secs, at speed setting 6.
  4. Homogenates (approx. 1ml) were transferred to clean 1.5ml screw-cap tubes (Sarstedt), and filled to 1.5ml with TRIzol.

Phase separation
  1. Samples were incubated at room temperature for 5 mins.

2. 1ml solution was transferred to clean 1.5ml tubes, and 200µl chloroform was added. (The remaining samples were stored at -80°C in case of future extraction).

3. Samples were vortexed for 15 secs, and incubated at room temperature for 3 mins.

4. Samples were then centrifuged for 15 mins at 12,000 G, at 4°C, separating the solution into a lower red phenol-chloroform phase, an interphase, and a colourless aqueous phase. RNA remains exclusively in the aqueous phase.

5. The aqueous layers were carefully transferred to clean 1.5ml tubes, and TRIzol was added at a ratio of 1:1.

6. Samples were vortexed, and 200µl chloroform was added.

7. Samples were vortexed again for 15 secs, incubated at room temperature for 3 mins, and centrifuged for 15 mins at 12,000 G, at 4°C.

8. If layers of solution were distinct, aqueous layers were transferred to clean 1.5ml tubes, and RNA precipitation was performed. If layers were not distinct, aqueous layers were transferred to clean 2ml tubes, and steps 5-7 were repeated.

RNA precipitation

1. 500µl isopropyl alcohol was added to each aqueous layer, and tubes were inverted gently to mix the solution, and incubated at room temperature for 10 mins.

2. Samples were centrifuged for 10 mins at 12,000 G, at 4°C, and supernatants were carefully aspirated and discarded, leaving a faint pellet on the side and bottom of tube.

RNA wash

1. 1ml 75% ethanol was added to each sample, mixed gently, and centrifuged for 5 mins at 7500 G, at 4°C.

2. As much ethanol as possible was aspirated, and samples were left to air-dry for 10-15 mins. Samples were never allowed to dry completely.

Redissolving the RNA

1. Each sample was diluted in 140μl RNase-free water, and incubated for 10 mins at 55-60°C on a heat block.

2. Samples were vortexed, and 50μl transferred to clean 1.5ml microcentrifuge tubes for treatment with a DNA-*free*™ kit. (The remaining samples were treated with RNAguard (Amersham), and stored at -80°C).

Treatment of samples with DNA-*free* kit

To remove any contaminating DNA from the prepared RNA samples, each sample was treated using a DNA-*free*™ kit (Ambion), according to manufacturer's instructions. In brief:

1. 5.6μl 10X DNase I Buffer (100mM Tris-HCl pH 7.5, 25mM $MgCl_2$, 5mM $CaCl_2$) was mixed with 1μl rDNase (2 Units/μl).

2. 6μl of this solution was added to each 50μl RNA sample, and incubated at 37°C for 45 mins, on a shaker platform set at 100 rpm.

3. DNase inactivation reagent was resuspended by flicking the tube, and 5.6μl was added to each sample.

4. Samples were incubated at room temperature for 2 mins, mixing occasionally, before being centrifuged at 10,000 G for 1 min, to pellet the inactivation reagent.

5. The supernatants (i.e. DNA-free RNA samples) were transferred to clean microcentrifuge tubes, and stored at -80°C until required for reverse transcription.

6.2.3 Reverse transcription (RT) of RNA samples

Isolation of mRNA from total RNA

1. 9µl of RNA sample was added to 1µl of oligo $(dT)_{12-18}$ primer (0.5µg/µl), and centrifuged gently for a few seconds to ensure the sample was at the bottom of the tube.

2. Samples were incubated on a heat block at 95^oC for 5 mins, followed by 5 mins on ice.

Reverse transcription of mRNA to cDNA

1. Mastermix was prepared as follows:

Reagent	Volume for one sample (µl)
10X PCR Buffer	2.5
100mM DTT[a]	2.5
10mM dNTP	2.5
25mM $MgCl_2$	5.0
40U/µl RNAsine	0.5
10U/µl AMV RT[b]	1.0
MiliQ	1.0
Total mastermix volume	**15**

[a] Dithiothreitol

[b] Avian myeloblastosis virus reverse transcriptase

2. 15µl of RT master mix was added to each mRNA sample, giving a total reaction volume of 25µl, and samples were centrifuged gently for a few seconds.

3. Samples were incubated for: 1 hour at 42^oC

   5 mins at 95^oC

   5 mins on ice

4. Samples were stored at -20^oC until required for polymerase chain reaction (PCR).

6.2.4 Amplification of the house-keeping gene, β-actin, by polymerase chain reaction (PCR), to validate success of RNA extraction method

In order to determine whether or not the method of RNA extraction was successful, a standard PCR was performed, targeting the house-keeping gene, β-actin, using experimental samples, and known positive and negative control samples.

1. Mastermix was prepared as follows:

Reagent	Volume for one sample (μl)	Final concentration
10X PCR Buffer	2.5	
10mM dNTP	0.5	0.2mM
25mM MgCl₂	1.5	1.5mM
50pmol Forward primer[a]	0.16	0.32μM
50pmol Reverse primer[b]	0.16	0.32μM
5U/ μl Taq polymerase	0.25	1.25U
MiliQ	18.43	
Total mastermix volume	**23.5**	

β- actin primer sequences (5'→ 3'):
[a] GTG GGC CGC TCT AGG CAC CAA
[b] CTC TTT GAT GTC ACG CAC GAT TTC
Product size: 540bp

2. 1.5μl cDNA template of appropriate samples was added to each mastermix, bringing the final reaction volume to 25μl.

3. The following PCR reaction was performed, with a total of 33 cycles:

PCR step	Temperature	Duration	
Denaturation	95°C	2 mins	
Denaturation	95°C	30 secs	
Annealing	60°C	1 min	1 cycle
Elongation	72°C	1 min	
Last elongation	72°C	7 minutes	
End	4°C	hold	

4. Following PCR, 10μl of each sample, mixed with 2.5μl loading buffer, were loaded onto a 1.5% agarose gel.

5. The gel was run at 125V for approximately 40-50 minutes, and bands were visualised on a UV transilluminator.

6.2.5 Relative quantification of cytokine mRNA in the brains of *T. canis*-infected BALB/c and NIH mice, using real-time PCR

Interleukin (IL)-5, IL-10, interferon (IFN)-γ and inducible nitric oxide synthase (iNOS) mRNA transcripts were quantified, relative to the house-keeping gene, β-actin, using the Roche LightCycler®. FastStart DNA Master^{PLUS} SYBR Green *I* kits (Roche) were used.

SYBR Green *I* is a DNA-binding dye that incorporates into double stranded DNA (dsDNA), and in doing so, emits fluorescence. During the PCR reaction, the increase in SYBR Green I fluorescence is directly proportional to the amount of dsDNA generated. The cycle number at which the level of fluorescence exceeds background levels is called the crossing point (CP), and this is used as a quantitative measurement of the input target. CP values decrease linearly with increasing input target quantity.

Since SYBR Green *I* will bind to any dsDNA, it can detect not only the specific target, but also non-specific PCR products and primer dimer. To overcome this problem, a melting curve analysis is performed at the end of each PCR program, where the reaction mixture is slowly heated to 95°C, causing dsDNA to melt and thus a decrease in SYBR Green I fluorescence. The LightCycler instrument continuously monitors this fluorescent decrease and displays it as melting peaks – each peak representing the characteristic melting temperature of a particular DNA product (where the DNA is 50% double-stranded and 50% single-stranded).

FastStart DNA Master^PLUS SYBR Green *I* kits were used following manufacturer's instructions. In brief:

1. The PCR mastermix was prepared as follows (details of primer sequences, concentrations, annealing temperatures, and product sizes are given in Table 6.2.1):

Reagent	Volume for one sample (μl)
PCR grade water	11
Forward primer	1.5
Reverse primer	1.5
Reagent mix	4
Total mastermix volume	**18**

2. The required number of 20μl LightCycler capillaries were placed in the pre-cooled LightCycler centrifuge adapters, and 18μl of mastermix was added to each.

3. 2μl of cDNA template was added to appropriate capillaries, and the stoppers placed on after each (to prevent any cross-contamination). 2μl PCR grade water was used as the negative control, and 2μl plasmid was used as the positive control (two plasmids were used during this work - one containing gene sequences for all cytokines studied, and one for iNOS).

4. The sealed capillaries were then placed into the LightCycler carousel, and centrifuged at 3000 rpm for 15 secs.

5. Prior to loading the carousel into the LightCycler 2.0 Instrument, a self-test was performed to check the instrument functionality. The self-test was only performed if the run was the first one of the day.

6. Following the self-test, the carousel was placed into the LightCycler, and the appropriate program was selected and run. The same PCR program (Table 6.2.2) was run for all cytokines, with only the annealing temperature changing each time (see Table 6.2.1 for temperatures).

7. Amplification curves were generated (Figure 6.2.1), and a melting curve analysis was performed following the last PCR cycle as a means of confirming amplification of the correct product (Figure 6.2.2).

Table 6.2.1: Primers used in real-time PCR

mRNA target	Primer sequence[1] (5'→ 3')	$T_A{}^2$	$T_M{}^3$	Conc.[4]	Product size (bp)
β-actin	F: GTGGGCCGCTCTAGGCACCAA	60	90	0.75	540
	R: CTCTTTGATGTCACGCACGATTTC				
IL-5	F: TCACCGAGCTCTGTTGACAA	60	83	1.87	201
	R: CCACACTTCTCTTTTTGGCG				
IL-10	F: ATGCAGGACTTTAAGGGTTACTTG	60	86	1.87	254
	R: TAGACACCTTGGTCTTGGAGCTTA				
IFN-γ	F: GCTCTGAGACAATGAACGCT	55	85	1.87	227
	R: AAAGAGATAATCTGGCTCTGC				
iNOS	F: GCCTCATGCCATTGAGTTCATCAACC	69	88	3.75	372
	R: GAGCTGTGAATTCCAGAGCCTGAAG				

[1]F and R indicate forward and reverse primers, respectively
[2]Optimum annealing temperature of primers (^{o}C)
[3]Melting temperature of product (^{o}C)
[4]Final concentration of primers in reaction mix (μM); Stock solution concentrations were:
β-actin = 10pmol/μl; IL-5, IL-10 and IFN-γ = 25pmol/μl; iNOS = 50pmol/μl

Table 6.2.2: Real-time PCR program, used for amplifying cytokine and iNOS cDNA from brain tissue. A total of 45 cycles were performed.

PCR step	Temperature	Slope (^{o}C/s)	Duration
Initial Denaturation	95°C	20	10 mins
Denaturation	95°C	20	10 secs
Annealing	see Table 6.2.1	20	10 secs
Elongation	72°C	20	5 secs
	95°C	20	0 secs
Melting curve analysis	65°C	20	15 secs
	95°C	0.1	1 sec
Cooling	40°C	20	30 secs
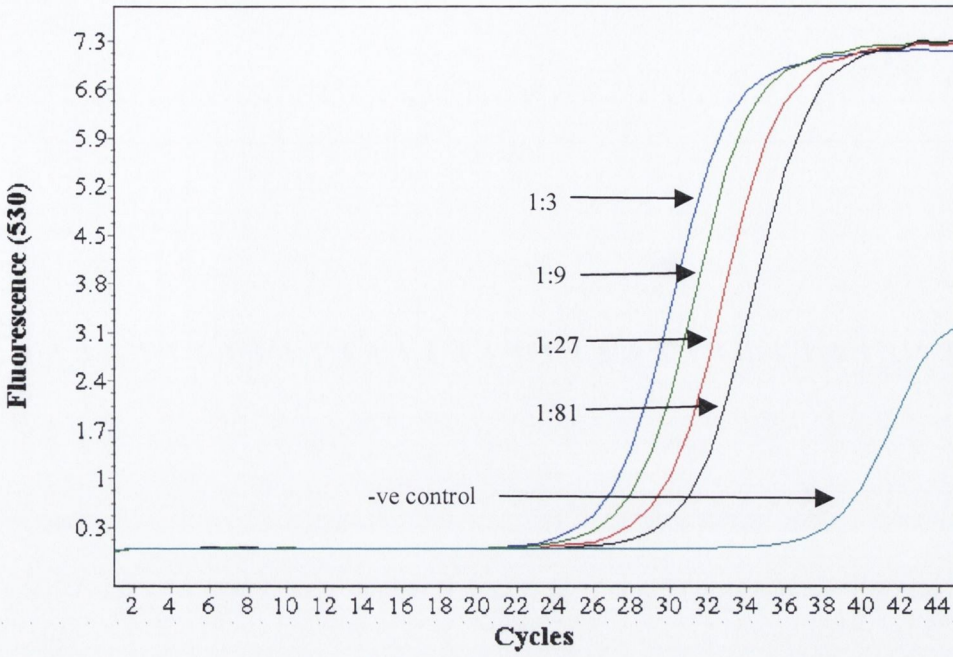
Figure 6.2.1: Amplification curves from real-time PCR reaction. The graph illustrates curves from a 3-fold serial dilution of β-actin (1:3 dilution to 1:81 dilution). The negative (-ve) control is also shown.
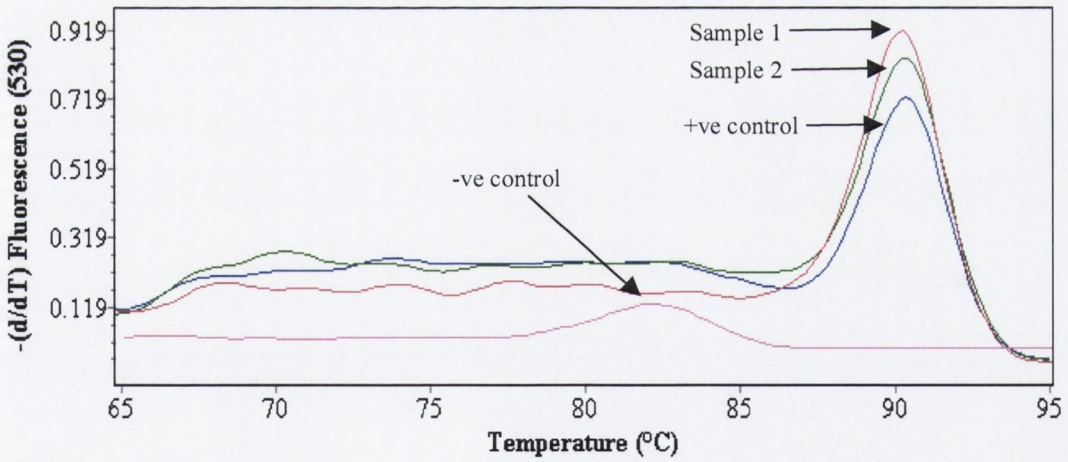
Figure 6.2.2: β-actin melting curve analysis. The graph illustrates 2 experimental samples (1 and 2) with melting peaks at 90°C. The plasmid positive (+ve) control also has a melting peak at 90 °C, and the negative (-ve) control has a peak at 82 °C.

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6.2.6 Relative quantification analysis

Relative quantification compares the ratio of a target DNA sequence to a reference (housekeeping) gene, measured in an unknown sample, to the same ratio measured in a "calibrator", or known, sample. In this study, the unknown samples were the infected mice, the reference gene was β-actin, and the "calibrators" were the uninfected control mice.

Standard curves were created for IL-5, IL-10, IFN-γ, iNOS and β-actin, by serially diluting (3-fold) samples of cDNA, and plotting the obtained crossing point (CP) values against an arbitrary log concentration. The concentration of each experimental sample was calculated from the appropriate standard curve, and relative quantities of each target were calculated as follows:

$$\frac{\text{Conc. of target (infected mice)} / \text{Conc. of } \beta\text{-actin (infected mice)}}{\text{Conc. of target (control mice)} / \text{Conc. of } \beta\text{-actin (control mice)}}$$

"Normalising" the results against the housekeeping gene corrected for any inefficiencies that may have occurred during the reverse transcription or PCR steps. Results are reported as the mean percentage increase of mRNA levels over control mice.

6.2.7 Statistical analysis

All statistical analysis was carried out at the 95% confidence limit, and data were checked for normality prior to testing. The overall effects of day, strain, and the interaction between these factors, on the levels of cytokines in the brain were investigated using a MANOVA (only on those data where values for all cytokines were obtained), and appropriate statistics are quoted in the text. The effect of day on the levels of IL-5, IL-10, IFN-γ and iNOS in infected mice, relative to their controls, was investigated for each strain by means of a one-way ANOVA, and F-ratios, degrees of freedom and P values are given in the text. The effects of day and strain on the levels of each cytokine in infected mice of both strains, relative to their controls, were investigated using a 2-way ANOVA, and the statistics are given. Between-group differences on each day post-infection were investigated using least significant difference (LSD) post-hoc tests, and P values are reported.

6.3 RESULTS

The levels of each cytokine and iNOS differed both within and between strains over the course of infection, with generally a more pronounced response being observed in infected BALB/c mice. Results of a MANOVA revealed significant effects of day, strain, and the interaction between these factors, on the level of cytokines and iNOS detected in the brains of both BALB/c and NIH infected mice (day: $F_{5,160} = 7.2$, $P \leq 0.0001$; strain: $F_{1,37} = 17.1$, $P \leq 0.0001$; day x strain: $F_{5,160} = 4.4$, $P \leq 0.0001$). The analysis of each cytokine is described below.

6.3.1 Relative quantification of IL-5 levels in the brains of *T. canis*-infected BALB/c and NIH mice, using real time PCR

The levels of IL-5 mRNA varied throughout infection in both strains of mice, relative to their controls (Figures 6.3.1 and 6.3.2). By day 3 p.i., there was a 28% increase in levels of IL-5 in the brains of infected BALB/c mice, relative to their controls, and this level remained similar over day 7 p.i. (32%) and day 14 p.i. (23%). By day 35 p.i., the levels in infected mice had peaked and were 54% greater than levels in control mice. By day 42 p.i., however, levels had decreased, and continued to decrease over the remainder of infection - being lower than those of control mice by day 97 p.i.

A one-way ANOVA revealed a significant effect of day on the levels of IL-5 in the brains of infected BALB/c mice, relative to their controls ($F_{5,23} = 21.6$, $P \leq 0.0001$). IL-5 levels did not differ significantly between days 3, 7 and 14 p.i., but there was a significant increase between days 14 and 35 p.i. (LSD post-hoc tests: $P \leq 0.0001$). This level then decreased significantly between days 35 and 42 p.i., and days 42 and 97 p.i. ($P \leq 0.0001$, for both comparisons).

Overall, the levels of IL-5 in infected NIH mice, relative to their controls, were lower than BALB/c mice, and fluctuated over the course of infection. A 23% increase over control mice was detected on day 3 p.i., but this had decreased to 13% by day 7 p.i. Levels of mRNA increased again on day 14 p.i., with a 27% increase over controls being detected, but they fell over days 35 and 42 p.i. By day 97 p.i., levels of mRNA

detected were similar to those seen on day 14 p.i., with a 27% increase over control mice.

Results of a one-way ANOVA revealed a significant effect of day on the levels of IL-5 mRNA in the brains of infected NIH mice, relative to their controls ($F_{5,21}$ = 4.7, P = 0.005). Post-hoc tests showed that levels did not differ significantly between days 3 and 7 p.i., but there was a significant increase between days 7 and 14 p.i. (P = 0.01), and then a significant decrease in levels between days 14 and 35 p.i. (P = 0.002). There was a slight increase in the levels of mRNA between days 35 and 42 p.i. (although not significantly), followed by a significant increase between days 42 and 97 p.i. (P = 0.02).

Figure 6.3.1: Mean (± SEM) percentage increase of IL-5 levels in the brains of infected BALB/c mice, relative to their controls.

Figure 6.3.2: Mean (± SEM) percentage increase of IL-5 levels in the brains of infected NIH mice, relative to their controls.

When infected mice of both strains were compared against each other, differences throughout infection in the levels of IL-5 were evident (Figure 6.3.3). Results of a 2-way ANOVA revealed significant effects of day, strain, and the interaction between these factors, on the levels of IL-5 in the brains of infected BALB/c and NIH mice (day: $F_{5,44}$ = 6.8, $P \leq 0.0001$; strain: $F_{1,44}$ = 10.5, $P = 0.0023$; day x strain: $F_{5,44}$ = 21.7, $P \leq 0.0001$). Post-hoc tests revealed that infected BALB/c mice had significantly higher levels of IL-5 in their brains, relative to controls, compared with infected NIH mice, on days 7, 35 and 42 p.i. ($P = 0.03$, ≤ 0.0001 and 0.02, respectively). Interestingly, by day 97 p.i., levels of IL-5 were significantly higher in infected NIH mice compared with infected BALB/c mice, relative to their controls ($P \leq 0.0001$).

Figure 6.3.3: Mean (± SEM) percentage increase of IL-5 levels in the brains of infected BALB/c and NIH mice, relative to their controls. (Between group comparisons: * $P \leq 0.05$; *** $P \leq 0.0001$).

6.3.2 Relative quantification of IL-10 levels in the brains of *T. canis*-infected BALB/c and NIH mice, using real time PCR

The levels of IL-10 mRNA in infected BALB/c mice increased, relative to control mice, early in infection, and remained at a similar level before decreasing in the later stages of infection (Figure 6.3.4). By day 3 p.i., there was a 34% increase in levels of IL-10 relative to control mice, and this had increased to 139% by day 7 p.i. The peak increase was detected on day 35 p.i., with levels in infected mice being 161% greater than controls. By day 97 p.i., levels of the cytokine were similar to those seen early in infection, with infected mice showing a 38% increase over control mice.

Results of a one-way ANOVA revealed a significant effect of day on the levels of IL-10 detected in the brains of infected BALB/c mice, relative to their controls ($F_{5,21}$ = 9.7, $P \leq 0.0001$). Post-hoc tests demonstrated that mRNA levels increased significantly between days 3 and 7 p.i. ($P \leq 0.0001$). There were no significant differences between the levels of IL-10 on days 7, 14, 35 and 42 p.i., however, levels decreased significantly between days 42 and 97 p.i. ($P = 0.001$).

The levels of IL-10 in the brains of infected NIH mice, relative to their controls, were generally lower than those of infected BALB/c mice (Figure 6.3.5). Levels of mRNA increased as infection progressed, with the peak increase being detected on day 14 p.i. – where levels of IL-10 were 90% higher than control mice. By day 35 p.i., levels had decreased to a 61% increase over control mice, and they continued to decrease throughout the remainder of infection.

Results of a one-way ANOVA revealed a significant effect of day on the levels of IL-10 in the brains of infected NIH mice, relative to their controls ($F_{5,22}$ = 6.7, $P = 0.001$). Post-hoc tests demonstrated that levels of IL-10 increased significantly between days 3 and 7 p.i., and between days 7 and 14 p.i. ($P = 0.02$ and 0.01, respectively). Levels of the cytokine then decreased significantly between days 14 and 35 p.i. ($P = 0.005$), and continued to decrease as infection progressed, although the differences were not significant.

Figure 6.3.4: Mean (± SEM) percentage increase of IL-10 levels in the brains of infected BALB/c mice, relative to their controls.
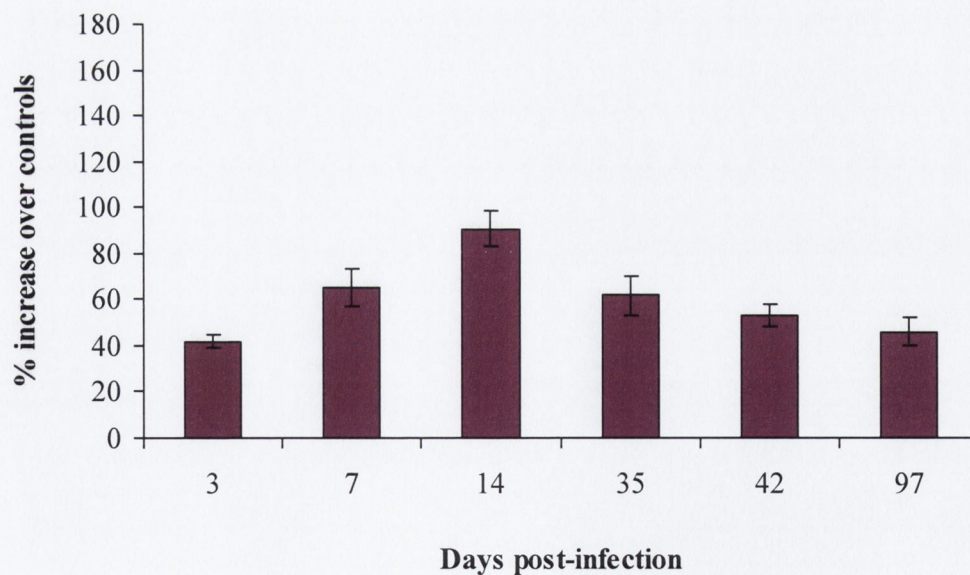
Figure 6.3.5: Mean (± SEM) percentage increase of IL-10 levels in the brains of infected NIH mice, relative to their controls.

When infected mice of both strains were compared against each other, differences were evident throughout infection (Figure 6.3.6). Results of a 2-way ANOVA revealed significant effects of day, strain, and the interaction between these factors, on the levels of IL-10 in the brains of infected BALB/c and NIH mice, relative to their controls (day: $F_{5,43} = 13.0$, $P \leq 0.0001$; strain: $F_{1,43} = 39.7$, $P \leq 0.0001$; day x strain: $F_{5,43} = 6.4$, $P \leq 0.0001$). Post-hoc tests revealed that levels of IL-10 were significantly higher in BALB/c mice compared with NIH mice, relative to their controls, on days 7, 14, 35 and 42 p.i. (day 7 p.i.: $P = 0.0004$; day 14 p.i.: $P = 0.03$; day 35 p.i.: $P \leq 0.0001$; day 42 p.i.: $P \leq 0.0001$). On days 3 and 97 p.i., levels did not differ significantly between the strains, although levels were slightly higher in NIH mice.
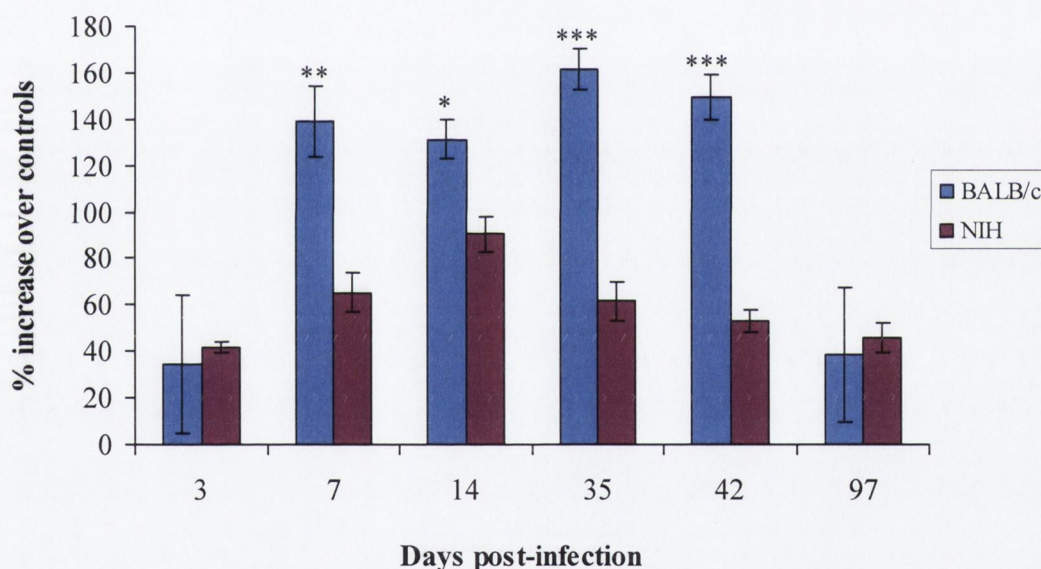
Figure 6.3.6: Mean (± SEM) percentage increase of IL-10 levels in the brains of infected BALB/c and NIH mice, relative to their controls. (Between group comparisons: * $P \leq 0.05$; *** $P \leq 0.0001$).

6.3.3 Relative quantification of IFN-γ levels in the brains of *T. canis*-infected BALB/c and NIH mice, using real time PCR

The levels of IFN-γ mRNA in the brains of infected BALB/c mice, relative to their controls, remained at similar levels throughout infection, only decreasing on day 97 p.i. (Figure 6.3.7). The highest levels of IFN-γ were detected as early as day 3 p.i., where there was an increase of 185% in infected mice, relative to their controls. Levels of the cytokine fluctuated slightly across days 7, 14, 35 and 42 p.i., and then decreased dramatically on day 97 p.i. to 100% lower than controls.

Results of a one-way ANOVA revealed a significant effect of day on the levels of IFN-γ in the brains of infected BALB/c mice, relative to their controls ($F_{5,20}$ = 45.8, $P \leq$ 0.0001). Post-hoc tests demonstrated a significant decrease in the levels of IFN-γ between days 3 and 7 p.i. (P = 0.003). Levels of the cytokine then remained fairly stable across days 14, 35 and 42 p.i., and then decreased significantly between days 42 and 97 p.i. ($P \leq 0.0001$).

Levels of IFN-γ in the brains of infected NIH mice, relative to their controls, were similar on days 3, 7 and 14 p.i., and then decreased dramatically on day 35 p.i., and remained low throughout infection (Figure 6.3.8). The peak increase in mRNA was detected on day 14 p.i., where levels in infected mice were 145% greater than control mice.

Results of a one-way ANOVA revealed a significant effect of day on the levels of IFN-γ in the brains of infected NIH mice, relative to their controls ($F_{5,21}$ = 6.1, P = 0.001). Post-hoc tests revealed that levels of IFN-γ did not differ significantly across days 3, 7 and 14 p.i. However, levels decreased significantly between days 14 and 35 p.i. (P = 0.002), and then remained low throughout infection. There was a slight increase in levels on day 97 p.i., but the difference was not significant.
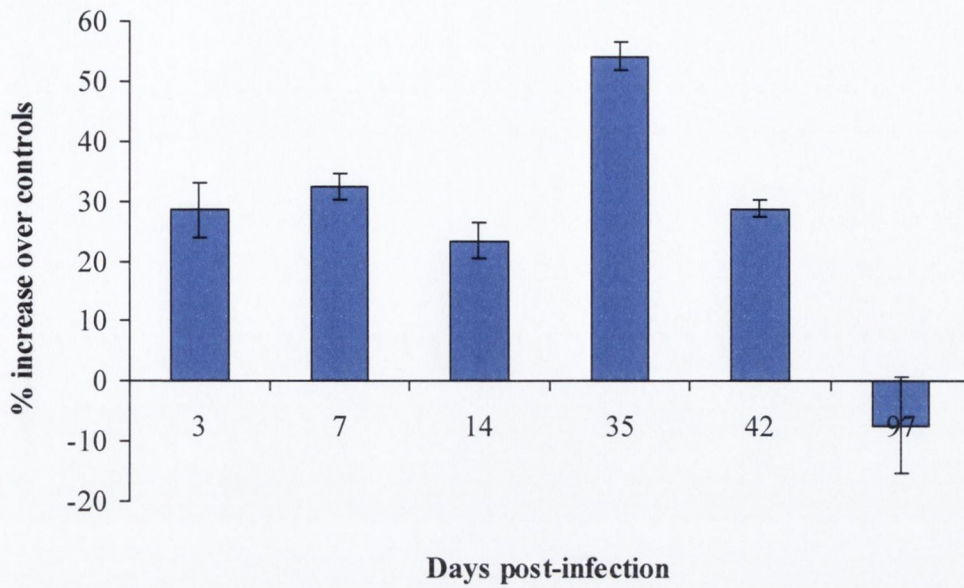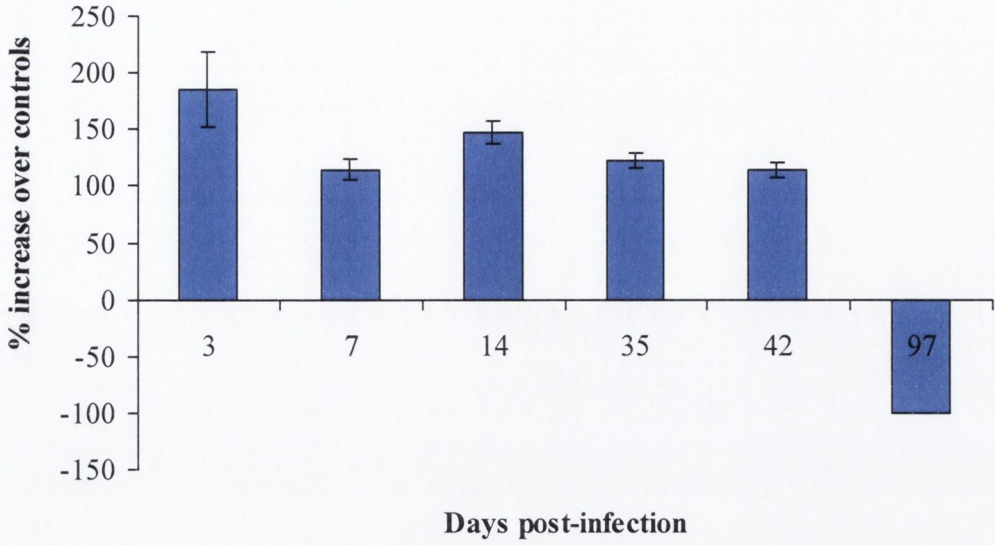
Figure 6.3.7: Mean (± SEM) percentage increase of IFN-γ levels in the brains of infected BALB/c mice, relative to their controls.
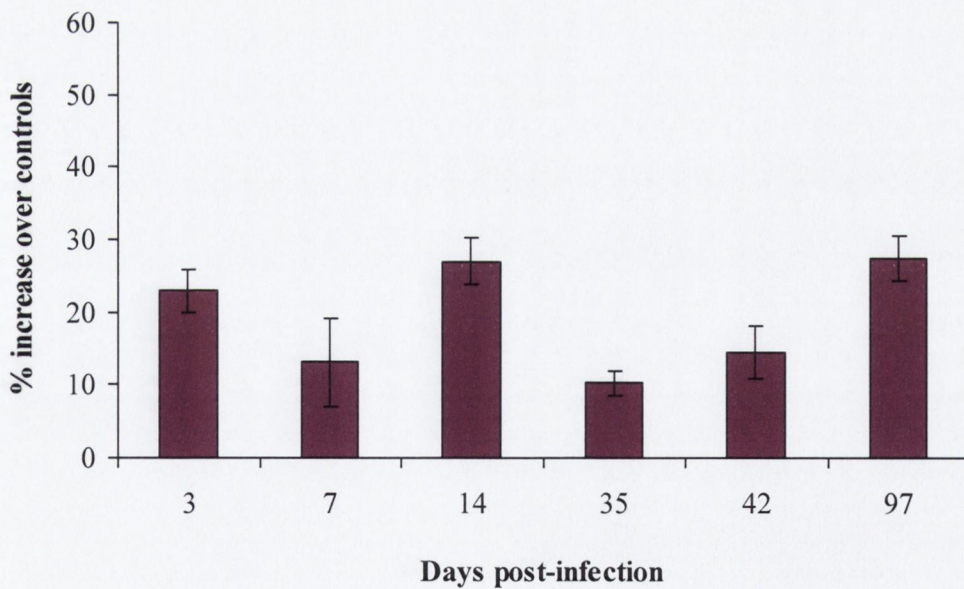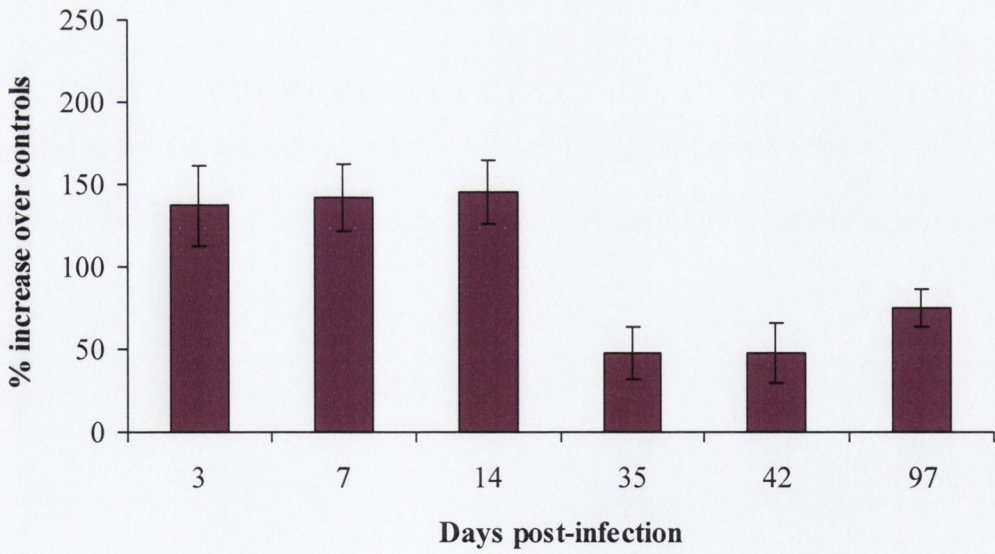

Figure 6.3.8: Mean (± SEM) percentage increase of IFN-γ levels in the brains of infected NIH mice, relative to their controls.

When infected mice of both strains were compared against each other, differences throughout infection were evident – particularly in later infection (Figure 6.3.9). Results of a 2-way ANOVA revealed significant effects of day, and the interaction between day and strain, on the levels of IFN-γ in the brains of infected BALB/c and NIH mice, relative to their controls (day: $F_{5,41} = 25.4$, $P \leq 0.0001$; day x strain: $F_{5,41} = 14.1$, $P \leq 0.0001$). There was no significant effect of strain ($F_{1,41} = 0.04$, $P = 0.841$). Post-hoc tests revealed that infected BALB/c mice had significantly higher levels of IFN-γ in the brain than infected NIH mice, relative to their controls, on days 3, 35 and 42 p.i. ($P = 0.05$, 0.002 and 0.008, respectively). By day 97 p.i., however, BALB/c mice had significantly lower levels in the brain than NIH mice ($P \leq 0.0001$).
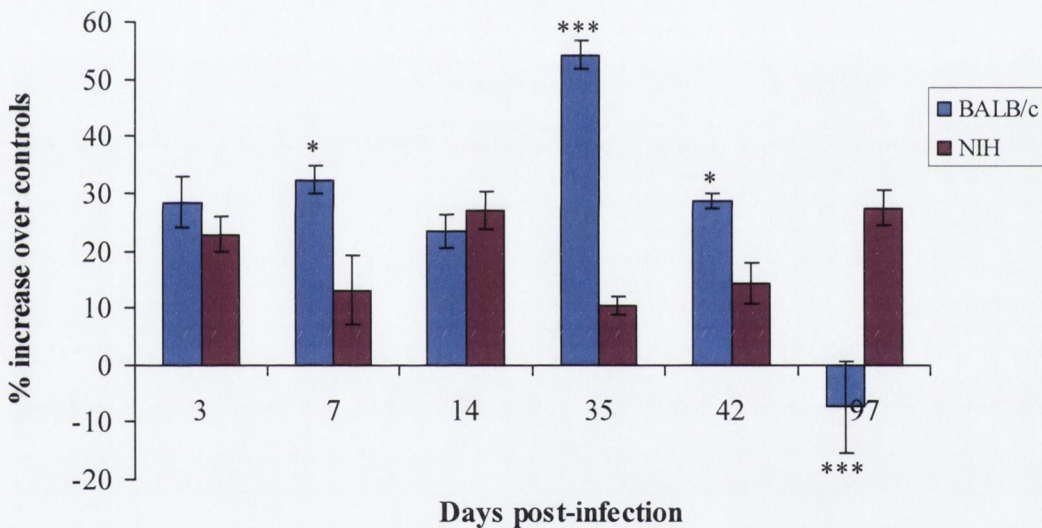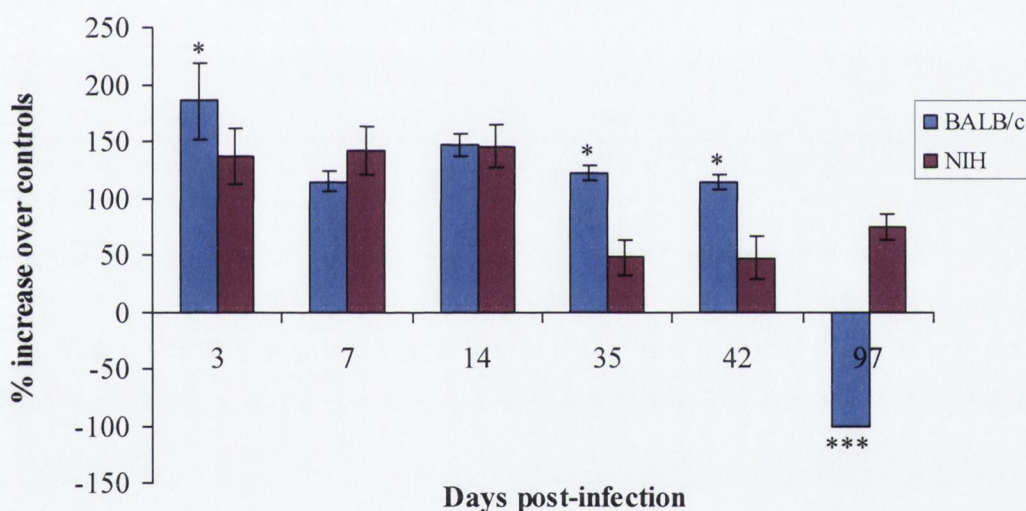

Figure 6.3.9: Mean (± SEM) percentage increase of IFN-γ levels in the brains of infected BALB/c and NIH mice, relative to their controls. (Between group comparisons: * $P \leq 0.05$; ***$P \leq 0.0001$).

6.3.4 Relative quantification of inducible nitric oxide synthase (iNOS) levels in the brains of *T. canis*-infected BALB/c and NIH mice, using real time PCR

The levels of iNOS mRNA varied greatly throughout infection in infected BALB/c mice (Figure 6.3.10). Levels detected in the brain increased as infection progressed, with the peak level being detected on day 35 p.i., when there was a 23% increase over control mice. By day 97 p.i., levels had fallen and were 0.94% lower than those of control mice. The highest levels of standard deviation were observed on days 3, 7 and 97 p.i. – indicating variation in iNOS levels between individual mice in the same groups. Results of a one-way ANOVA revealed no significant effect of day on the levels of iNOS in infected BALB/c mice, relative to their controls ($F_{5,23}$ = 1.0, P = 0.443).

The levels of iNOS in the brains of infected NIH mice also varied throughout infection (Figure 6.3.11). In early infection, the levels were quite low, only being 7.9% and 2.8% higher than control mice on days 3 and 7 p.i., respectively. The peak increase was observed on day 14 p.i., when there was a 26.4% increase in levels detected. These levels then fell, and were lower than those of control mice on days 35 and 42 p.i., before a peak again on day 97 p.i.

Results of a one-way ANOVA revealed a significant effect of day on the levels of iNOS in the brains of infected NIH mice, relative to their controls ($F_{5,22}$ = 8.2, $P \le$ 0.0001). Post-hoc tests demonstrated a significant increase in the levels of iNOS in the brain between days 7 and 14 p.i. (P = 0.001), and then a significant decrease between days 14 and 35 p.i. ($P \le$ 0.0001). Levels remained similar between days 35 and 42 p.i., and then increased significantly between days 42 and 97 p.i. ($P \le$ 0.0001).
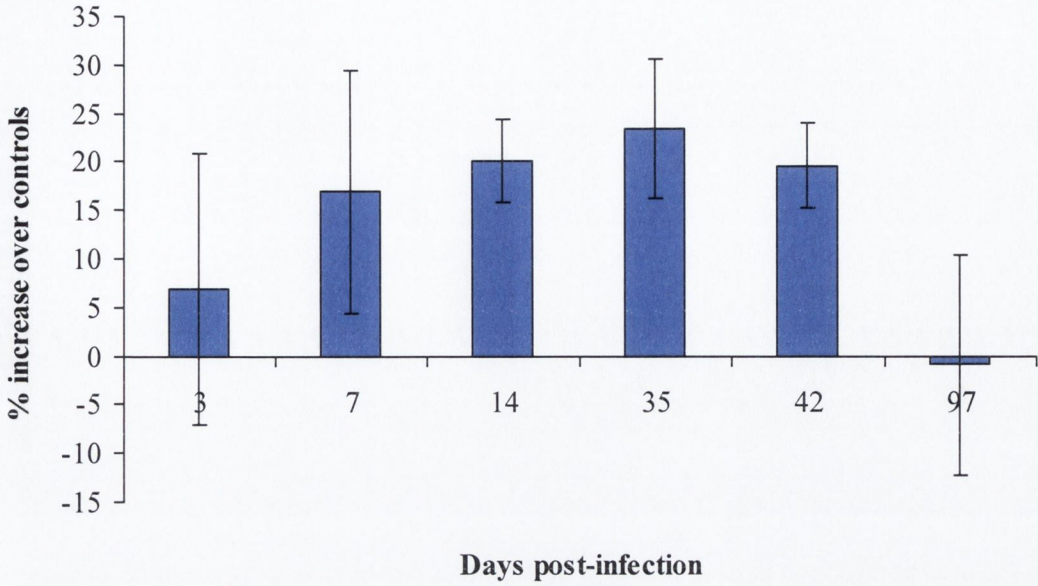
Figure 6.3.10: Mean (± SEM) percentage increase of iNOS levels in the brains of infected BALB/c, relative to their controls.
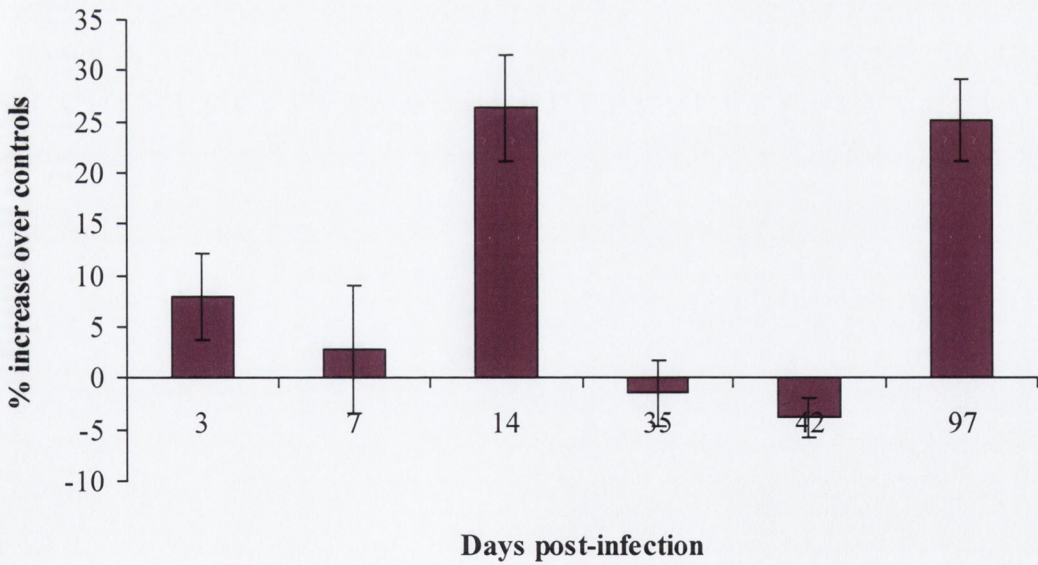
Figure 6.3.11: Mean (± SEM) percentage increase of iNOS levels in the brains of infected NIH mice, relative to their controls.

When infected mice of both strains were compared against each other, differences between the strains were evident throughout infection (Figure 6.3.12). Results of a 2-way ANOVA revealed a significant effect of the interaction between day and strain, but not of day and strain individually, on the levels of iNOS detected in the brains of infected BALB/c and NIH mice (day x strain: $F_{5,45} = 3.3$, $P = 0.013$). Post-hoc tests revealed that infected BALB/c mice had higher levels of the enzyme compared with NIH mice, on days 35 and 42 p.i., relative to their controls ($P = 0.02$ and 0.04). BALB/c mice also had higher levels than NIH mice on day 7 p.i., but the difference was not significant ($P = 0.21$). By day 97 p.i. however, levels of iNOS in infected BALB/c mice had dropped, relative to their controls, and NIH mice had significantly higher levels in their brains ($P = 0.02$).
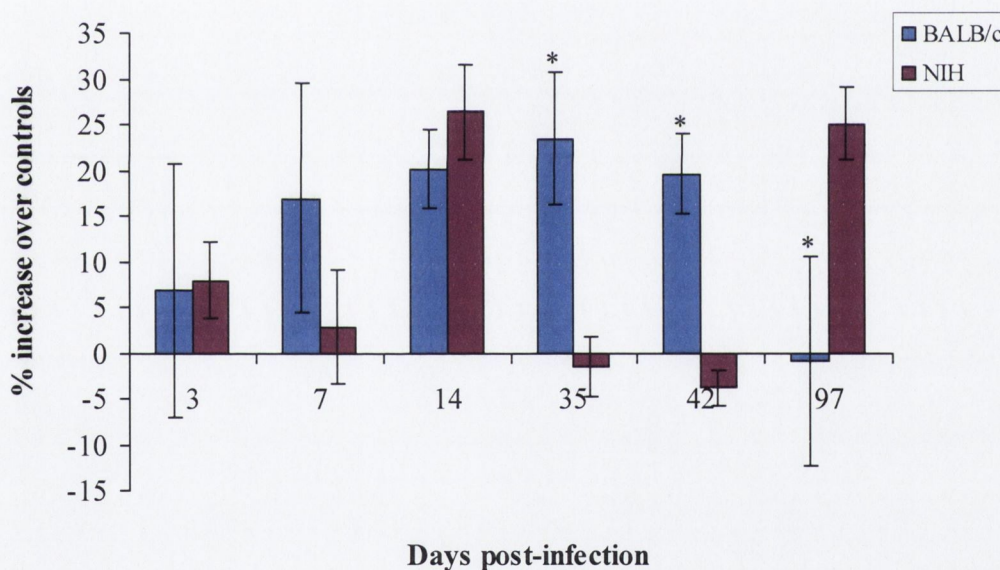
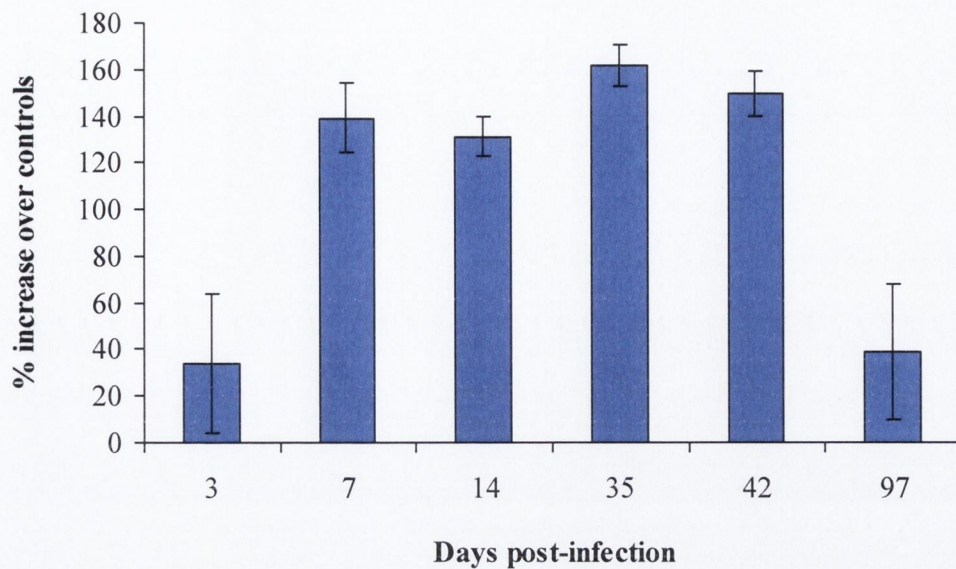**Figure 6.3.12:** Mean (± SEM) percentage increase of iNOS levels in the brains of infected BALB/c and NIH mice, relative to their controls. (Between group comparisons: * $P \leq 0.05$).

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6.4 DISCUSSION

The 'healthy' central nervous system (CNS) is believed to be an immune privileged site, due to the lack of immune reactivity, and the ability to exclude components of the immune system via the blood-brain-barrier (Fabry *et al*, 1994). However, immunological reactions can occur within the CNS during infection, and there have been many reports of the role of cytokines and immune cells as effector mechanisms against different parasitic infections (Gazzinelli *et al*, 1993; Kosodo *et al*, 1997; Hertz *et al*, 1998). At present, there is no existing literature on the cerebral immune response during *T. canis* infection, so the aim of this chapter was to characterise this response in two strains of inbred mice, in order to determine any role in control of parasite establishment. The results demonstrate the presence of IL-5, IL-10, IFN-γ and iNOS mRNA transcripts in the brains of infected mice, indicating a mixed Th1/Th2 cerebral immune response, which is more pronounced in BALB/c mice compared with NIH.

The levels of cytokines varied both within and between strains over the course of infection. However, the most pronounced up-regulation was of IL-10 and IFN-γ, compared to levels in control mice. Levels of IL-10 increased significantly over early infection, with peak mRNA levels being detected on day 35 post-infection in BALB/c mice, and on day 14 post-infection in NIH mice. In both strains, the levels of this cytokine then decreased significantly over the remainder of infection. Up-regulation of IFN-γ occurred in early infection in both strains, with mRNA levels in BALB/c mice being 185% higher than control levels on day 3 post-infection, and around 140% higher than control levels on days 3, 7 and 14 post-infection in NIH mice. Levels of the cytokine decreased significantly in both strains in later infection, and notably, levels in infected BALB/c mice were below those of control mice by day 97 post-infection. The increase of IL-5, relative to control mice, was generally quite low in both strains of mice, with peak increases being detected on day 35 post-infection in BALB/c mice (when levels were 50% greater than control mice), and on day 14 post-infection in NIH mice (when levels were 27% greater than controls). It is interesting to note that by day 97 post-infection, levels of IL-5 in BALB/c mice were below those of controls, while levels in NIH mice had reached a second peak and were 25% higher than control levels. The decrease of mRNA in BALB/c mice suggests a reduction in expression of IL-5,

The levels of iNOS mRNA fluctuated greatly over infection, with large variation within the strains - particularly BALB/c. In these mice, the levels increased over infection, with the peak increase relative to control mice (23%) being detected on day 35 post-infection. Transcript levels then decreased over the remainder of infection, and were below levels of control mice by day 97 post-infection – again, indicating a decrease in gene expression in this strain. Levels of the enzyme in NIH mice varied greatly throughout infection, with peak increases being detected on days 14 and 97 post-infection. Between these two days, however, levels were below those of control mice.

The presence of IL-5, IL-10, IFN-γ and iNOS demonstrate a mixed Th1/Th2 cerebral immune response, which contrasts with the dominant Th2 humoral immune response observed in *T. canis* infection. Typically, helminth infections induce the up-regulation of IL-4, IL-5 and IL-10, along with increased levels of IgE, IgG1, and eosinophils and mast cells. This type of immune response is driven by the subset of CD4+ T cells referred to as Th2 cells (Mosmann and Coffman, 1987). The up-regulation of IL-5 and IL-10 in the brains of infected mice in this study, therefore, indicate activation of the Th2 arm of the immune response. The presence of IFN-γ and iNOS, however, indicate a simultaneous cell-mediated immune response, driven by Th1 cells. The up-regulation of these components is interesting not only because they are typically a Th1-type response (and generally associated with the control of intracellular parasites), but also because they coincide with an up-regulation of IL-10 – an anti-inflammatory cytokine, associated with the potent down-regulation of IFN-γ secretion by Th1 cells, and the inhibition of nitric oxide (NO) synthesis by activated macrophages (Fiorentino *et al*, 1989; Gazzinelli *et al*, 1992).

Inducible nitric oxide synthase (iNOS) is one form of the enzyme, NOS, required to synthesise nitric oxide (NO) in a range of cell types, including macrophages (when classically activated), endothelial cells and neurones. It is not usually produced in resting cells, but requires the stimulus of cytokines, such as TNF-α, IL-1 or IFN-γ to generate it (Clark and Rockett, 1996). NO has been implicated in the killing of a number of parasites (e.g. *T. gondii*, *L. major* and *T. brucei*), and in the pathological disorders associated with others (e.g. *P. falciparum*, *S. mansoni* and *T. spiralis*) (Clark and Rockett, 1996; Lawrence *et al*, 2000). Previous studies have demonstrated the

induction of NO by alveolar macrophages during *T. canis* infection, although any role in parasite control was not elucidated (Espinoza *et al*, 2002a). In a later study, however, NO was demonstrated to play a role in lung pathology, with *T. canis*-infected mice treated with an iNOS inhibitor displaying significantly less lung damage than *T. canis*-infected untreated mice (Espinoza *et al*, 2002b). In a study investigating the induction of iNOS by astrocytes and microglia, in the brains of mice infected with *T. brucei brucei*, levels of the enzyme were shown to increase significantly in the presence of IFN-γ compared with the parasite alone (Girard *et al*, 2000). Although there was great variation in the levels of iNOS detected in the brains of *T. canis*-infected mice in the present study, increases in enzyme levels seemed to correlate with up-regulation of IFN-γ. In infected BALB/c mice, up-regulation of IFN-γ throughout infection coincided with an increase in the levels of iNOS, and by day 97 post-infection, there is down-regulation of the cytokine and a decrease in the levels of iNOS. In infected NIH mice, the up-regulation of IFN-γ on day 97 post-infection coincides with an increase in levels of iNOS.

A mixed Th1/Th2 cerebral cytokine profile has been reported in infection with other CNS-dwelling parasites. Restrepo *et al* (2001) reported the presence of IFN-γ, IL-18 and IL-4 in brain granulomas taken from patients with histologically confirmed neurocysticercosis (caused by infection with the tapeworm, *Taenia solium*), indicating the activation of both Th1 and Th2 cells during infection. A number of other studies have reported either a Th1 or Th2 response to NCC (Evans *et al*, 1998; Rodrigues *et al*, 2000). Although in these cases the responses are not concurrent, the fact that both T cell subsets can be activated indicates the potential for a mixed response. Hunter *et al* (1991) reported the presence of TNF-α and IL-4 in the brains of mice infected with *T. brucei brucei* – the protozoan causative agent of African sleeping sickness – indicating Th1 and Th2 cell activation.

Despite the presence of both Th1- and Th2-type cytokines in the brains of *T. canis*-infected mice in this study, there appeared to be no link with control of parasite establishment. Although there appeared to be pronounced up-regulation of IFN-γ and IL-10, we can only speculate about their roles in *T. canis* infection, since the quantification of these cytokines was relative and not absolute. The increase in levels

of IFN-γ may appear large, but they may not be enough to have any biological effect. The fact that there is a simultaneous up-regulation of IL-10 and IL-5 would appear to confirm this, since one of the roles of IFN-γ is the inhibition of Th2-cell proliferation (Mosmann and Coffman, 1989). Similarly, however, the up-regulation of IL-10 does not appear to be enough to down-regulate IFN-γ production as it is known to do (Fiorentino *et al*, 1989) The predominant production of IgG1 and IgE (reported in Chapter 5), does not correlate with increased IFN-γ production, since this cytokine is known to inhibit all activities of IL-4 on B-cells (Mosmann and Coffman, 1987). However, the increase in IFN-γ may explain the increase in IgG2a production detected in infected BALB/c mice.

One of the most notable patterns from the results of this study was that there was a consistently more pronounced cytokine response in the brains of infected BALB/c mice on days 35 and 42 post-infection. In these mice, levels of IL-5, IL-10, IFN-γ and iNOS were significantly higher than those of infected NIH mice, relative to their controls, on both days post-infection. Bearing in mind the behavioural results from this study (reported in Chapter 4), where infected BALB/c mice displayed evidence of some level of memory impairment, it could be the case that the cytokines being produced during *T. canis* infection are mediating immunopathology in the CNS, leading to behavioural alterations. If there is infiltration of effector cells to the brain, such as eosinophils (induced by the up-regulation of IL-5), mast cells and macrophages, this may lead to the secretion of various biological mediators such as platelet activation factor, major basic protein, leukotriene C4, histamine and prostaglandin (Roitt, 1993). The presence of these mediators may or may not have an effect on resident *T. canis* larvae, but it is possible that they may lead to tissue damage within the brain. Up-regulation of iNOS in the brain (possibly as a result of increased IFN-γ, and the activation of macrophages) may also be of significance since nitric oxide has been linked to the killing of various intra- and extra-cellular parasites (see review by Clark and Rockett, 1996). When produced in excess, however, it can contribute to tissue damage, and a variety of neurotoxic and neurodegenerative diseases (Boje, 1996). The role of cytokines in pathology in other cerebral parasitic infections has been reported.

Studies on cerebral malaria (CM), using different mouse models, have provided evidence that some cytokines produced in response to *P. falciparum* contribute to pathology, leading to cerebral dysfunction (Hunt and Grau, 2003; Brown *et al*, 1999; Jennings *et al*, 1997; Kossodo and Grau, 1993). There is convincing evidence that pro-inflammatory cytokines mediate neurodegeneration in murine CM, with TNF-α playing a central role (Rudin *et al*, 1997; Kern *et al*, 1989; Grau *et al*, 1987). Treatment with anti-TNF-α antibodies has been shown to significantly prolong the lives of CBA/Ca mice infected with *Plasmodium berghei* and prevent the development of neurological symptoms (Grau *et al*, 1989).

Hunter *et al* (1991) infected outbred CD1 mice with 2 x 10^4 *T. brucei brucei* parasites, and investigated the cerebral cytokine profile produced, along with pathological changes in different sections of the brain. In uninfected control mice, only β-actin and IL-1 mRNA transcripts were detected, while in the brains of infected mice, transcripts for β-actin, TNF-α, IL-1 and IL-4 were detected. Histological sections of brains from infected mice showed severe chronic meningoencephalitis, characterised by large numbers of inflammatory cells (predominantly plasma cells, lymphocytes and macrophages) in the meninges and moving into the brain. Magez *et al* (1999) used TNF-α knockout mice (TNF-$\alpha^{-/-}$) to investigate the role of this particular cytokine in immunopathology during *T. brucei* infection. Authors noted that infected TNF-$\alpha^{-/-}$ mice exhibited significantly higher parasitemia peaks than wild-type mice, although they produced similar humoral anti-trypanosome responses. The most notable result, however, was that TNF-$\alpha^{-/-}$ mice, particularly during the late stage of infection, displayed much less pronounced infection-related signs of morbidity, compared with infected wild-type mice (i.e. anaemia and poor coat condition). Furthermore, infected TNF-$\alpha^{-/-}$ mice displayed much less pronounced effects on locomotor behaviour, compared with infected wild-type mice – locomotor activity being recorded as the minutes per hour that mice spent moving around the homecage, eating and drinking. The behaviour of TNF-$\alpha^{-/-}$ mice was comparable to control mice, strongly implicating TNF-α as a mediator of immunopathology. This is a very interesting finding, particularly with reference to the behavioural alterations observed in *T. canis*-infected mice in this study – although the presence of TNF-α in brains of infected mice was not measured.

Previous studies have demonstrated varying degrees of cerebral pathology during *T. canis* infection. Summers *et al* (1983) reported the close proximity of *T. canis* larvae to necrosis, cavitation and perivascular cuffing in the brains of infected mice, with lesions being found most frequently in the heavily myelinated tracts. Despite the presence of plasma cells, eosinophils and neutrophils, larvae appeared to be viable at the time of sacrifice. Dolinksy *et al* (1985) reported numerous focal haemorrhages on the dorsal surface of the cerebrum and cerebellum in *T. canis*-infected mice, with pathological changes becoming more severe as infection progressed. In a more recent paper, Epe *et al* (1994) described demyelisation, focal malacia and mixed-cell infiltration in the brains of various strains of *T. canis*-infected mice, and also reported the presence of central nervous symptoms, such as lack of co-ordination, tremor and slight paralysis.

High levels of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α and the IFNs, have been linked with many behavioural symptoms collectively termed as "sickness behaviour" (Dantzer, 2001; Balshcun *et al*, 2004). Injection of these cytokines into the brains of laboratory animals is generally associated with depressed locomotor activity, decreased exploration, and impaired learning and memory (Dantzer, 2001). Oitzl *et al* (1993) demonstrated that injection of IL-1β, but not IL-6, into the brains of rats, impaired spatial navigation learning in the Morris water maze. Similarly, Gibertini *et al* (1995) demonstrated impaired spatial learning in rats injected with IL-1β, or infected with the pathogenic agent, *Legionella pneumophilia*. Although the exact mechanisms by which the cytokines reported were altering rodent behaviour were not elucidated, these results are of particular significance with reference to the behavioural alterations observed in the *T. canis*-infected mice of this study. It would be interesting to investigate the presence of IL-1β, IL-6 and TNF-α in both BALB/c and NIH mice, and determine whether or not levels were higher in BALB/c mice, since they displayed more pronounced behavioural defects during infection.

In conclusion, the results of this chapter have demonstrated that *T. canis* infected mice produce a mixed Th1/Th2 cerebral immune response during infection, with the production of IL-5, IL-10, IFN-γ and iNOS. Characterisation of the cerebral immune response is an important element in the understanding of the disease process and the possible immunopathology – which may be an important factor with reference to

diagnosis in humans. Although cerebral toxocariasis in humans is rare, there are suggestions that larval involvement in the human brain may have subtle public health implications (Magnaval *et al*, 1997). However, since there can be other factors involved when a patient presents, such as epilepsy and mental retardation (Kaplan *et al*, 2004; Magnaval *et al*, 1997; Glickman *et al*, 1979), the clinical significance remains unclear. Previous investigations involving case-control studies have reported a significant association between *T. canis* seropositivity and epileptic seizures in children (Woodruff *et al*, 1966; Glickman *et al*, 1979; Arpino *et al*, 1990), and more recent studies have suggested *T. canis* infection as a possible cause of epileptic seizures and other cognitive disorders (Richartz *et al*, 2002; Xinou *et al*, 2003; Bächli *et al*, 2004). With the knowledge that cytokines present in the CNS can cause significant immunopathology during parasitic infection, one could speculate that the immune response produced during *T. canis* infection may be leading to more cryptic complications in humans, and is thus going misdiagnosed.

While we are aware that the detection of cytokine mRNA is perhaps not as accurate as detection at the protein level, the results of this chapter have at least 'flagged up' certain components of the immune response which are induced during *T. canis* infection. As there is no existing literature on this subject already, it is hoped these results will open the door to an entirely new area of research, in which many questions remain to be answered.

CHAPTER 7: General discussion and further work

7.1 GENERAL DISCUSSION

The overall aim of this study was to develop a murine model for cerebral toxocariasis. This was done by investigating the relative susceptibility and resistance of various inbred mice to cerebral infection with *T. canis*, and investigating these mice in terms of larval accumulation, course of migration, and behavioural and immune response. Previous studies have highlighted behavioural alterations in *T. canis*-infected mice, and had reported the various components involved in the systemic immune response. However, there has been no research on the cerebral immune response during *T. canis* infection. Furthermore, there has been no simultaneous assessment of the influence of host genetics and immunity on cerebral toxocariasis, in addition to the effects of infection on host behaviour.

The investigation into cerebral larval burdens and the course of migration revealed several things. Firstly, care must be taken when assigning an end-point characteristic to define susceptibility and resistance to infection. While in experiment 1, BALB/c mice carried a significantly higher cerebral larval burden on 3 of the 4 days post-infection compared with NIH mice, in experiment 2, this divergence between the strains was not evident, and larval burdens were similar on all days post-infection. However, when larval burdens were expressed as a percentage of the total number of larvae recovered, BALB/c mice were observed to carry significantly higher larval burdens in the brain on days 35 and 42 post-infection in experiment 1, and days 14, 35 and 97 post-infection in experiment 2, suggesting that they may in fact be more susceptible to cerebral toxocariasis than NIH mice. These results highlight that actual larval burdens may not be a reliable indicator of susceptibility and resistance to cerebral toxocariasis, and that a more suitable end-point characteristic may be percentage of total larvae in the brain. One clear result from the investigation was that larvae accumulate in the brains of BALB/c and NIH mice. Larval accumulation had previously been described in the murine host (Dunsmore *et al*, 1983; Epe *et al*, 1994; Skerrett and Holland, 1997), but none of the studies focused on BALB/c and NIH mice in particular, while investigating the course of infection over a longer period. In both BALB/c and NIH mice, the majority of larvae were recovered from the liver and lungs in early infection (day 3 p.i.) and from the brain in later infection (day 7 p.i. onwards). This clearly illustrated the

early hepato-pulmonary phase and later myotropic-neurotropic phase previously described by Abo-Shehada and Herbert (1984).

The hypothesis that BALB/c mice are more susceptible to cerebral toxocariasis was reflected in some of the behavioural testing. Both strains of mice were subjected to numerous behavioural assessments measuring baseline activity in the homecage, exploratory behaviour and response to novelty in a 'T'-maze, and learning and memory in a water-finding task. Although a range of behaviours were altered, the most notable result was that infected BALB/c mice took significantly longer to drink from a water-bottle (following habituation, and a period of water-deprivation) compared with uninfected control mice. The significant latency to drink indicated a certain degree of memory loss. Infected BALB/c mice also took longer to locate the alcove and the water-source within, compared with control mice, but the differences were not significant. There were no significant differences between infected and control NIH mice in the water-finding task. These results suggest that the significantly higher percentage of larvae present in the brains of infected BALB/c mice at the time of testing may be leading to behavioural alterations. Though there were no differences in exploratory behaviour between control and infected BALB/c mice, infected mice were less responsive to novelty, although this did not reach statistical significance. The idea that higher cerebral larval burdens lead to behavioural alterations had previously been suggested by Cox and Holland (2001b), who demonstrated an effect of *T. canis* infection on learning and memory in outbred mice with moderate and high larval burdens in the brain, but the differences did not attain statistical significance. Olson and Rose (1966) reported a dose-dependent effect on learning in *T. canis*-infected rats, although no correlation was made with cerebral larval burdens. The results of the present study, therefore, are of particular importance since it is the first study to report significant behavioural alterations in *T. canis*-infected mice with significantly higher cerebral larval burdens.

The fact that a range of behaviours were altered in infected mice, although not all significantly, confirms the idea that behavioural changes in *T. canis*-infected hosts are more likely due to a side-effect of pathology, as opposed to being an adaptive manipulation by the parasite (Holland and Cox, 2001). Since *T. canis* is capable of infecting a wide range of paratenic hosts (including earthworms, chickens and man), it

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is unlikely that specific behavioural alterations, such as impaired memory, would have the same consequences for all.

The results of the immunological findings of this study revealed that both strains of infected mice produced a dominant Th2 humoral response, and a mixed Th1/Th2 cerebral immune response. The dominant Th2 humoral response was defined by the significantly higher production of IgG1 and IgE in infected mice, compared with controls, and the low levels of IgG2a, and confirms findings from previous studies (Del Prete et al, 1991; Buijs et al, 1994; Cuéllar et al, 2001). The production of IgG1 and IgE is indicative of Th2-cell activation, since IL-4 is known to stimulate the production of these antibodies from B-cells (Mosmann and Coffman, 1987). Levels of IgG1 were significantly higher in BALB/c mice, compared with NIH mice, from day 35 post-infection onwards, indicating this strain to be a higher responder. Infected BALB/c mice also displayed significantly higher levels of IgG2a compared with their control counterparts and infected NIH mice, although overall, the levels were quite low. Detection of this isotype indicates Th1-cell activation and the production of IFN-γ (Mosmann and Coffman, 1987). The significantly higher levels of IgG1, and to a lesser extent IgG2a, in infected BALB/c mice appear to play little role in the control of cerebral larval establishment, since these mice exhibited significantly higher burdens than NIH mice on several days post-infection.

Of interest, however, is the observation that infected NIH mice displayed significantly higher levels of total serum IgE than infected BALB/c mice, suggesting a possible role for this antibody in parasite control, since NIH mice had significantly lower larval burdens in the brain than BALB/c. IgE has been shown to be protective against a number of helminth infections, including *Schistosoma mansoni* (Capron et al, 1982), *Trichuris muris* (Else et al, 1993) and *Trichinella spiralis* (Ahmad et al, 1991). However, it has also been shown to be ineffective against *Heligmosomoides polygyrus* (Urban et al, 1992). What must be noted here is that the IgE measured in mice in the present study was total serum IgE and not *Toxocara*-specific IgE. During helminth infection, it is estimated that around only 10% of the IgE produced is parasite-specific (Dessaint et al, 1975). Some researchers have hypothesised that the high levels of non-specific IgE stimulated during helminth infection compete with parasite-specific IgE, and saturate the available mast cell and eosinophil receptors (FcϵRI and FcϵRII,

respectively), preventing specific mediator release, thus proving beneficial to the parasite (Pritchard, 1993). To elucidate any role of IgE in the control of *T. canis* cerebral establishment, further experimentation would be required with the use of IgE-deficient mice.

It has been suggested that the pronounced antibody production observed in *T. canis* infection, along with eosinophilia and mastocytosis, is more of a "diversion" for the immune system as opposed to a protective response to the parasite (Maizels and Robertson, 1991). *T. canis* larvae have been shown to be able to slough-off an extracuticular layer, consisting of surface antigens and antibody, at a fairly rapid turnover rate (as little as 1 hour in some experiments), possibly reducing the chance of effector cells and molecules from binding to, and damaging, the epicuticle (Smith *et al*, 1981; Maizels *et al*, 1984). The induction of a potentially ineffective antibody response may be a strategy evolved by the parasite as a means of avoiding a more protective Th1 immune response, which has been reported to be more effective at controlling tissue-dwelling stages of some helminths (Toledo *et al*, 2001; Abraham *et al*, 2002; Helmby *et al*, 2003).

Investigating the cerebral immune response during *T. canis* infection was an area of particular interest and novelty, especially with regard to behavioural alterations. Results from this investigation revealed a mixed Th1/Th2-type response, with the production of IL-5, IL-10, IFN-γ and iNOS. The up-regulation of IFN-γ and iNOS indicates activation of the Th1 arm of the immune response, whereas the presence of IL-5 and IL-10 indicate Th2-cell activation (Mosmann and Coffman, 1989). Up-regulation of IL-10, however, may also be an indicator of T-regulatory cell-activation (Shevach, 2000). Since quantification was relative and not absolute, we can only speculate about the biological mechanisms induced with this type of response. The presence of IL-5 in the brain may result in the infiltration of eosinophils, and the subsequent degranulation of these cells with the release of mediators such as major basic protein, eosinophil peroxidase, eosinophil cationic protein and eosinophil-derived neurotoxin – all of which may cause dysfunction and destruction of surrounding cells (Bandeira-Melo and Weller, 2005). The activation of macrophages via the up-regulation of IFN-γ may result in increased nitric oxide production (as evident by the up-regulation of iNOS), and possible tissue damage if produced in excess (Boje, 1996).

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Generally, cytokine and iNOS levels were higher in infected BALB/c mice, relative to their controls, compared with NIH mice, suggesting little role in the control of parasite establishment, since BALB/c mice carried a significantly higher percentage of larvae in the brain than NIH mice. Of particular interest, however, was the observation that infected BALB/c mice displayed significantly higher levels of all cytokines and iNOS, relative to control mice, on days 35 and 42 post-infection. This significant up-regulation coincided with the behavioural alterations observed in infected mice, most notably, the apparent impairment of memory. Investigations on other CNS-dwelling parasites have provided evidence for the role of particular cytokines in pathology and cerebral dysfunction. Grau *et al* (1989) reported that treatment with anti-TNF-α antibodies significantly prolonged the lives of mice with cerebral malaria, and prevented the development of neurological symptoms. Magez *et al* (1999) also reported the importance of this cytokine in the development of neurological degeneration in *T. brucei* infection, with TNF-α-deficient mice exhibiting much less pronounced effects on locomotor behaviour compared with wild-type mice. The fact that infected BALB/c mice in the present study exhibited significantly higher levels of cytokines and iNOS in their brains around the time of behavioural testing, where mice displayed signs of memory loss, strongly suggests a role of immunopathology in these behavioural alterations, and may reflect a fitness cost to the host.

In conclusion, this study has revealed that *T. canis* infection results in larval accumulation in the brains of its murine host, leading to significant memory impairment in those mice with higher larval burdens, which is possibly induced by the mixed Th1/Th2-type cerebral immune response. There appeared to be no obvious correlation between the dominant Th2 humoral immune response and parasite control, although IgE may play a role. Elucidating the cerebral immune response in concert with the investigation of larval accumulation, and behavioural alterations, has given more insight into the host-parasite relationship in *T. canis* infection. A fuller appreciation of the significance and consequences of cerebral toxocariasis may shed light on the more subtle and cryptic complications observed in human cerebral infection (Arpino *et al*, 1990; Richartz *et al*, 2002; Xinou *et al*, 2003; Bachli *et al*, 2004), in particular the effects on cognitive development in young children (Marmor *et al*, 1987). Furthermore, an improved understanding of the immune response induced by *T. canis* infection, and its effectiveness in the control of cerebral establishment, may

allow for the exploration of immunoprophylaxis as a means of treating cerebral toxocariasis.

7.2 FURTHER WORK

The results of this study have shed light on an area of research in cerebral toxocariasis where many questions remain to be answered. Elucidating whether or not the cerebral immune response to *T. canis* is responsible for pathology is of particular importance, along with investigating the effects of induced pathology on aspects of behaviour.

The present study allowed for the relative quantification of IL-5, IL-10, IFN-γ and iNOS in the brains of *T. canis*-infected mice. Bearing in mind previous studies, it would be of interest to quantify a number of other cytokines in the brain during infection. TNF-α would be of particular interest since it has been linked with immunopathology during infection with *P. falciparum* and *P. berghei* (cerebral malaria), and *T. brucei brucei* (African sleeping sickness) (Grau *et al*, 1987; Kossodo and Grau, 1993; Magez *et al*, 1999). It has also been linked to reduced locomotor behaviour in mice (Magez *et al*, 1999). The detection of IL-1β would also be of interest since this cytokine has been linked with impaired spatial navigation learning in rats (Gibertini *et al*, 1995), and is known to be involved in neurodegeneration (Rothwell, 1997). It would also be of interest to measure levels of IL-4, given its involvement in the alternative activation of macrophages during helminth infection, and subsequent production of cytokines such as IL-10 and TGF-β, and thus downregulation of Th1 responses (Lee *et al*, 1999; Donnelly *et al*, 2005).

Immunohistochemistry on brain sections from infected mice could be used to investigate the types of immune cells present in the brain – such as Th1/Th2 (CD4$^+$) cells, cytotoxic T-cells (CD8$^+$), or regulatory T-cells (CD4$^+$CD25$^+$) – as a means of determining the source of the cytokines produced. It would also be interesting to stain for other effector cells, such as eosinophils and mast cells, to determine their prevalence and distribution in the brain. As well as immune cells, it could also be interesting to stain for particular cytokines – since their distribution in particular areas of the brain may be linked with behavioural alterations observed in infected mice.

In order to investigate fully the roles of particular cytokines and/or antibodies in the control of cerebral toxocariasis, it would be beneficial to use knockout mice. Previous

research has shown that *T. canis*-infected mice deficient in IL-5 show comparable cerebral larval burdens to wild-type mice, indicating that this particular cytokine plays no role in cerebral larval establishment (Takamoto *et al*, 1997). The authors also report a significant reduction in eosinophils in IL-5-deficient mice, suggesting that these particular effector cells are not involved in parasite control. Since the results of this study revealed significantly higher levels of total IgE in mice with significantly lower cerebral larval burdens, it would be interesting to investigate larval migration and accumulation in IgE-deficient mice.

While investigating the aspects of the immune response involved in cerebral pathology and/or control of larval establishment, it would be useful to simultaneously assess behavioural alterations. Since learning and memory was the most significantly altered behaviour observed in *T. canis*-infected mice of this study, it would perhaps be of interest to develop these tests further, as a means of confirming results (see Appendix 3). Using knockout mice for particular cytokines/antibodies, it would be possible to assess the effects of such depletion on behavioural activities.

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APPENDIX 1: Checking inocula prior to infection

Table A1.1: Mean (with standard deviation and standard error) numbers of unembryonated and embryonated eggs in 5 sample inocula, from experiment 1.

Sample (10µl)	Number of eggs*			% embryonation
	Unembryonated	Embryonated	Total	
1	114	38	152	25.0
2	117	42	159	26.4
3	94	36	130	27.7
4	122	40	162	24.6
5	79	34	113	30.1
Mean	105.2	38	143.2	26.8
S.D	18.1	3.2	21.0	2.2
S.E	8.1	1.4	9.4	1.0

* Numbers of eggs represent the means of five 10µl aliquots for each sample.

Calculations

Mean number of eggs in 10µl: 143.2

Mean number of eggs in each inoculum: 143.2 x 52
(volume of each inoculum = 520µl)   = 7446.4 eggs

Mean number of embryonated eggs in each inoculum: 7446.4 x 0.268
(% embryonation = 26.8)   = **1996 embryonated eggs**

Checking supernatants

No eggs were found in the supernatants aspirated from 5 sample inocula.

Table A1.2: Mean (with standard deviation and standard error) numbers of unembryonated and embryonated eggs in 5 sample inocula, from experiment 2.

Sample (10µl)	Number of eggs*			% embryonation
	Unembryonated	Embryonated	Total	
1	51	17	68	25.0
2	69	25	94	26.6
3	63	19	82	23.3
4	50	20	70	28.6
5	59	12	71	16.9
Mean	58	19	77	24.1
S.D	8.0	4.7	10.9	4.5
S.E	3.6	2.1	4.9	2.0

* Numbers of eggs represent the means of five 10µl aliquots for each sample.

Calculations

Mean number of eggs in 10µl: 77

Mean number of eggs in each inoculum: 77 x 100
(volume of each inoculum = 1ml)   = 7700 eggs

Mean number of embryonated eggs in each inoculum: 7700 x 0.241
(% embryonation = 24.1)   **= 1856 embryonated eggs**

Checking supernatants

No eggs were found in the supernatants aspirated from 5 sample inocula.

APPENDIX 2: Solutions required for Baermann procedure

1% Trypsin

1g trypsin

100ml distilled water

Adjusted to pH 7.0 using 0.1N NaOH

1% Pepsin

1g pepsin

100ml distilled water

Adjusted to pH 1.0 using conc. HCl

0.85% Saline

0.85g NaCl

100ml distilled water

6% Formalin

6ml formaldehyde

100ml distilled water

APPENDIX 3: Data from follow-up behavioural experiment

Table A3.1: Mean observations (± S.D) recorded for control and infected BALB/c and NIH mice, during the testing period of the water-finding task, in a follow-up experiment (day 35 p.i.).

Behaviour	BALB/c		NIH	
	Control	Infected	Control	Infected
Secs to enter correct alcove	36.2 ± 48.94	75.1 ± 98.42	76.9 ± 109.98	134.5 ± 1214.15
Secs to locate water bottle	41.4 ± 56.90	79.4 ± 96.19	81.5 ± 107.04	144 ± 117.48
Secs to drink from bottle	65.7 ± 74.27	179.6 ± 103.41	193 ± 76.05	242.8 ± 70.71

Table A3.2: Summary of statistical results from 2-way ANOVAs for each observation in the water-finding task.

Observation	Effect	F-ratio	D.F	P-value
Secs to enter alcove	Strain	0.7	1,32	0.404
	Infection status	3.5	1,32	0.071
	Strain x Infection status	0.3	1,32	0.588
Secs to locate bottle	Strain	0.9	1,32	0.337
	Infection status	3.9	1,32	0.056
	Strain x Infection status	0.4	1,32	0.510
Secs to drink from bottle	Strain	12.5	1,34	0.001
	Infection status	9.3	1,34	0.004
	Strain x Infection status	1.4	1,34	0.241

Table A3.3: Summary of results of LSD post-hoc tests, from 2-way ANOVAs performed on observations from the water-finding task.

Observation	Comparison	P-value
Secs to enter alcove	BALB/c infected vs BALB/c control	0.327
	NIH infected vs NIH control	0.116
Secs to locate bottle	BALB/c infected vs BALB/c control	0.329
	NIH infected vs NIH control	0.086
Secs to drink from bottle	BALB/c infected vs BALB/c control	0.004
	NIH infected vs NIH control	0.211

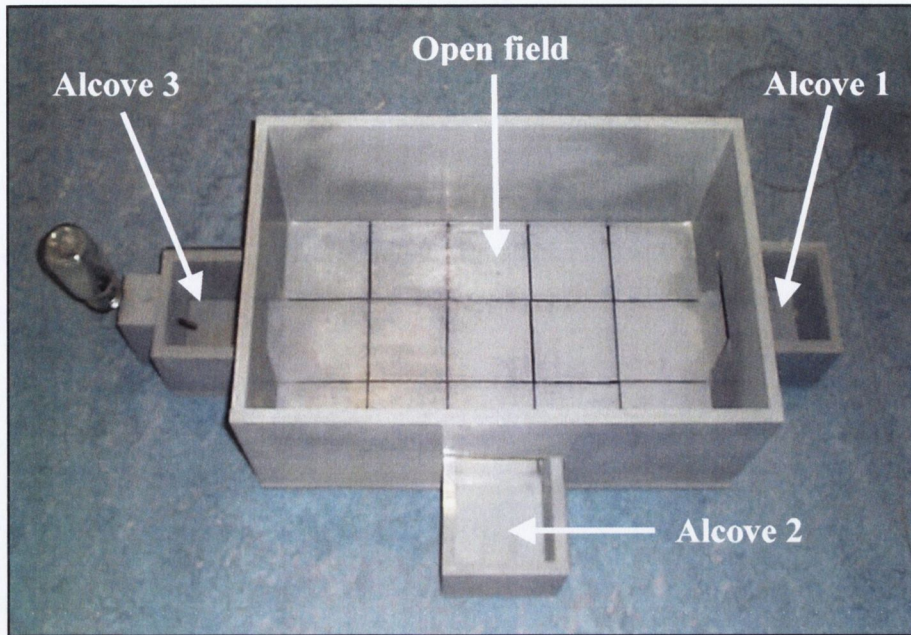**Plate A3.1:** Modified water-finding apparatus, used to investigate learning and memory in *T. canis*-infected and control BALB/c and NIH mice.