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REVIEW

The significance of cerebral toxocariasis: a model system for exploring the link between brain involvement, behaviour and the immune response

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Summary

Toxocara canis is a parasitic nematode that infects canines worldwide, and as a consequence of the widespread environmental dissemination of its ova in host faeces, other abnormal hosts including mice and humans are exposed to infection. In such abnormal or paratenic hosts, the immature third-stage larvae undergo a somatic migration through the organs of the body but fail to reach maturity as adult worms in the intestine. The presence of the migrating larvae contributes to pathology that is dependent upon the intensity of infection and the location of the larvae. A phenomenon of potential public health significance in humans and of ecological significance in mice is that _T. canis_ larvae exhibit neurotrophic behaviour, which results in a greater concentration of parasites in the brain, as infection progresses. _Toxocara_ larval burdens vary between individual outbred mice receiving the same inocula, suggesting a role for immunity in the establishment of cerebral infection. Although the systemic immune response to _T. canis_ has been widely reported, the immune response in the brain has received little attention. Differential cytokine expression and other brain injury-associated biomarkers have been observed in infected _versus_ uninfected outbred and inbred mice. Preliminary data have also suggested a possible link between significant memory impairment and cytokine production associated with _T. canis_ infection. Mice provide a useful, replicable animal model with significant applicability and ease of manipulation. Understanding the cerebral host–parasite relationship may shed some light on the cryptic symptoms of human infection where patients often present with other CNS disorders such as epilepsy and mental retardation.

Key words: *Toxocara*, paratenic hosts, mice, humans, brain involvement, cerebral toxocariasis, mouse model, behavioural alteration, cerebral immune response, cytokines.

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Introduction

Toxocara canis is a highly prevalent gastrointestinal nematode infection of dogs and other canids (Holland and Smith, 2006). Widespread environmental contamination of the environment, with eggs shed in host faeces, facilitates infection of so-called abnormal or paratenic hosts including mice and humans (Holland and Smith, 2006). In such hosts, larvae migrate through the tissues and organs of the body but do not proceed to maturity as adult worms in the intestine. The resulting pathology is dependent on the intensity of the infection and the location of the larvae (Smith, 1991). The implications of this paratenesis have far-reaching consequences for the host–parasite relationship and extend from the behaviour of wild mice to the cerebral effects in infected humans.

Bush and colleagues (Bush et al., 2001) define a paratenic host as a host in which development does not occur, but which may serve to bridge an ecological, or trophic, gap in a parasite's life cycle. If we consider an infected mouse, this paratenic host can facilitate the transmission of infective larvae to potential definitive hosts, such as dogs, cats or foxes. However, our knowledge of the relative significance of a range of vertebrate and invertebrate hosts acting as paratenic hosts is virtually non-existent (Holland and Hamilton, 2006).

The highest seroprevalence of *Toxocara*, amongst a range of small mammals surveyed in Slovakia, was recorded for the house mouse, *Mus musculus* (Dubinský et al., 1995). The authors concluded that small mammals could act as important foci for the circulation and maintenance of *Toxocara* in the environment and as indicators of environmental contamination. However, we lack molecular evidence of *Toxocara* species identification within such small mammal populations.

In contrast to the paucity of data on paratenic hosts in the wild, a substantial body of literature has been published on experimental infections of *Toxocara* in a range of laboratory animals. These include mice, rats, guinea pigs, hamsters, gerbils, chickens, quail, pigeons, rabbits, pigs, monkeys and earthworms (see Holland and Hamilton, 2006). It should be noted that infection protocols vary considerably, making species comparisons difficult. Furthermore, ethical and maintenance issues vary by host species, and the ecological significance of many of the host–parasite associations remains untested.

For example, the Mongolian gerbil has been explored as a relatively novel model for ocular and neurological toxocariasis (Takayanagi et al., 1999; Akao et al., 2003). Not only do these animals exhibit an enhanced susceptibility to eye involvement compared with mice and other experimental paratenic hosts but also their dark grey fundi facilitate the detection and observation of living larvae. However, their feasibility as a model system for cerebral toxocariasis is less convincing. Gerbils develop irreversible brain damage after chronic infection in contrast to the more subtle and cryptic effects described in humans (see below).

Furthermore, from an ecological point of view, gerbils are unlikely to play as important a role as a paratenic host under natural conditions, which may explain the marked pathogenicity observed (Holland and Hamilton, 2006).

Murine models have a number of advantages as models for cerebral toxocariasis. Firstly, wild mice are known to be infected with *Toxocara* (Dubinsky et al., 1995); secondly, there is experimental evidence for larval accumulation over time (Dunsmore et al., 1983); and thirdly, outbred and inbred mice differ in their capacity to harbour larvae in the brain, suggesting the influence of host genetics and immunity on cerebral infection (Skerrett and Holland, 1997; Cox and Holland, 2001a).

Mice therefore, represent useful model systems to explore the impact of *Toxocara* on brain biology and behaviour, and provide possible insights into human cerebral toxocariasis.

Cerebral toxocariasis in humans

Although our understanding of the pathology in humans is incomplete, seroprevalence studies provide support for high levels of exposure in the human population (Smith and Noordin, 2006). Toxocariasis is now considered to be the most common human parasitic infection in the USA particularly among the impoverished. The infection is also highly prevalent in many developing countries and its global importance is likely to be significantly underestimated (Hotez and Wilkins, 2009). The main route of transmission for humans is still considered to be *via* the ingestion of embryonated eggs from soil or soil-contaminated hands or food. However, recent data have suggested a role for direct contact with dogs, carrying potentially infective eggs on their hair (Roddie et al., 2008). As further evidence accumulates, it has been demonstrated that embryonated eggs are rare or absent in hair particularly in well-managed older dogs (Overgaauw et al., 2009; Devoy-Keegan and Holland, 2010). Higher concentrations of eggs, including embryonated eggs were found on the hair of stray puppies (Roddie et al., 2008). Infection has also been shown to arise from the ingestion of undercooked meat containing *Toxocara* larvae (Hoffmeister et al., 2007).

Humans exhibit a number of well-recognised clinical entities including visceral larva migrans, ocular toxocariasis and covert toxocariasis (Smith et al., 2009). Larval involvement in the eye, with consequent visual impairment, remains the most devastating sequela of human infection (Good et al., 2004). However, cerebral or neurological toxocariasis is a much less-well understood phenomenon. Humans are known to carry *Toxocara* larvae in their brains (Hill et al., 1985) and despite the small number of cases of cerebral toxocariasis being described historically, these are now on the increase as a result of enhanced awareness and improved diagnosis (see Salvador et al., 2010; Kinčeková et al., 2008; Scheid et al., 2008; Kazek et al., 2006; Vidal et al., 2003).

Epidemiological evidence of the impact of toxocariasis on neuropsychology in humans is sorely lacking. Magnaval and colleagues sought to characterise a recognisable cerebral or neurological syndrome associated with *Toxocara* infection among French adults (Magnaval et al., 1997). Based upon a case-control study design (whereby seropositive cases with neurological symptoms in the absence of an aetiological diagnosis were compared with matched controls), the authors concluded that migration of *Toxocara* larvae in the brain does not induce a recognisable neurological syndrome but is correlated with several risk factors such as exposure to dogs, rural residence and dementia.

Two American studies sought to explore the relationship between *Toxocara* seropositivity and neuropsychological parameters in young children. While Worley and colleagues failed to demonstrate a relationship between *Toxocara* seropositivity and cognitive abnormalities, after controlling for social class (Worley et al., 1984), Marmor and colleagues suggested a role for toxocariasis in subtle effects on cognition (Marmor et al., 1987).

A recent Polish study focused on the relationship between *Toxocara* seropositivity and developmental age and physical fitness in over 200 children aged 14–16 years in a rural Polish village (Jarosz et al., 2010). The authors demonstrated that seropositive boys had significantly lower end-of-year grades than their seronegative counterparts but acknowledged that this relationship could also be explained by behavioural factors such as spending more time playing outdoors rather than studying, and hence becoming more exposed to infection.

To conclude, there is clearly a paucity of studies that provide evidence for the role of *Toxocara* in cognitive deficits in humans. Gillespie suggested that the relationship between *Toxocara* and neurological deficits in humans is likely to remain obscure until individual children with mild or asymptomatic disease are studied in some detail, over the course of their infection, in contrast to matched controls (Gillespie, 1993). This highlights the ethical and methodological challenges that investigators of such studies face, as mirrored in the literature on cognitive development and geohelminth infections (Bundy et al., 2009). This reinforces the need for a good laboratory animal model system. Mice have a number of advantages in this respect, including ease of manipulation, wide availability of inbred and knockout strains and small organs for the detection of larvae, and they form part of the *Toxocara* life cycle in the wild.

The *Toxocara* mouse model: brain involvement, accumulation and behavioural alterations

Early work by Sprent (Sprent, 1955) and Burren (Burren, 1971) established the presence of *Toxocara* larvae in the mouse brain, particularly the cerebellum. Dunsmore and colleagues provided important quantitative evidence for larval accumulation in the brain of Canberra mice over a 122 day infection period (Dunsmore et al., 1983). All three authors described how *Toxocara* larvae remain unencapsulated and alive (and therefore capable of transmission to an appropriate definitive host) and suggested the advantages to the parasite of accumulating in a site of immune privilege where they are protected from the host lymphoid system. Bardón and colleagues demonstrated the ability of larvae to remain in the brain over long periods of time, up to 1 year post-infection (Bardón et al., 1994). Smith emphasised the similarity of the migratory path in humans and mice, and the comparability of the lesions elicited in experimental murine models and humans (Smith, 1991).

However, one question that arises is the optimum dose for infection under experimental conditions. Cox and Holland (Cox and Holland, 2001a) compared the recovery of *Toxocara* larvae in the brains of outbred and inbred mice that received single doses of 100, 1000 and 3000 eggs, and a trickle dose of 1000 eggs over 28 days (250 per week). In general, the percentage larval recovery expressed in terms of the total dose administered was low and lower for inbred NIH mice compared with outbred LACA mice. As expected, outbred mice demonstrated a much greater variability in the number of larvae recovered from the brains of individual mice, indicating the role of host genetics and immunity in the establishment of cerebral infection. Low dose mice mimic the patterns observed in wild mice, whereas higher doses are frequently selected in experimental infections in order to achieve marked brain involvement and accumulation over time. Ollero and colleagues

demonstrated that multiple doses enhanced the number of larvae recovered from the brains of BALB/c mice (Ollero et al., 2008).

As highlighted above, infection of outbred mice demonstrated significant heterogeneity in the number of larvae detected in the brain. Unfortunately, we lack data on the number of larvae recovered from the brains of small mammals sampled from the wild. This is regrettable in terms of both establishing which hosts are actually important for the dissemination of infection and obtaining a realistic assessment of larval burden under natural conditions. Dubinský and colleagues recorded very low numbers of larvae relative to the proportion of seropositive animals, of the order of 1–3 larvae per brain (Dubinský et al., 1995). These numbers are similar to those recovered from outbred mice receiving a dose of 100 eggs under experimental conditions (Cox and Holland, 2001a).

Epe and colleagues infected four inbred strains of mice and one outbred strain and reported the presence of *Toxocara* larvae in the brains of all mice over the infection period, with the inbred BALB/c mice demonstrating the highest burdens (Epe et al., 1994). Investigating behaviour in inbred strains of mice is attractive as any infection-induced behavioural alterations that could be masked in heterogeneous outbred mice may appear more pronounced. However, this does reduce the applicability to wild populations [see recent references to the usefulness of studying wild immunology (e.g. Pedersen and Babayan, 2011)] while increasing experimental control.

We therefore initiated a series of experiments to infect mice of differing genetic background and investigate their behavioural and immunological responses with a view to investigating some of the impact of, and mechanisms behind, cerebral toxocariasis (Hamilton et al., 2006; Hamilton et al., 2008). We assessed the progression of *Toxocara* infection in seven strains of inbred mice in order to select a susceptible and a resistant strain of mouse to larval establishment in the brain. *Toxocara canis* larvae were recovered from the brains of all strains of mice on each post-mortem day (7, 14, 35 and 42). Numbers increased over time and peaked on day 35. BALB/c mice were selected as the susceptible strain and NIH mice were deemed to be resistant. The choice of strains was supported by previous studies where BALB/c mice have been reported to be more susceptible to *T. canis* infection (Bardón et al., 1994; Epe et al., 1994) and NIH mice demonstrated a higher resistance to *T. canis* infection than outbred CD1 mice (Abo-Shehada and Herbert, 1989). Recently, Kolbeková and colleagues demonstrated an accelerated speed of migration to the brain in re-infected BALB/c mice (Kolbeková et al., 2011).

However, it should be noted that despite NIH mice having significantly lower numbers of larvae in the brain on days 7, 35 and 42 post-infection, this strain still carries considerable numbers in the brain *per se* (ranging from an average of 93 on day 7 to 242 on day 14). Also, in repeat experiments, the two strains demonstrated some convergence in numbers, suggesting that NIH may not be an ideal candidate for resistance to brain involvement. However, when larval recovery was expressed as a percentage of the total burden, it was evident that a significantly higher percentage of larvae were recovered from the brains of BALB/c mice compared with NIH mice at 3 days post-infection (Hamilton et al., 2006).

The mouse model has been used to study the impact of *Toxocara* infection on various 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; Cox and Holland, 2001a; Cox and Holland, 2001b). The extent of some behavioural changes could also be correlated with larval burden (Cox and Holland, 1998; Cox and Holland, 2001a; Cox and Holland, 2001b). It was suggested that the range of changes are more likely to be non-specific side effects of induced pathology rather than any adaptive manipulation of host behaviour (Holland and Cox, 2001). As *T. canis* is capable of infecting a wide range of paratenic hosts, including rodents, earthworms, chickens and humans, it is unlikely that specific behavioural alterations, such as impaired memory, would have the same consequences for all. However, we can more confidently predict similar manifestations within the mammalian CNS as demonstrated for *Toxoplasma gondii*-infected rodents and humans (Webster, 2001).

Effects on learning and memory may be of particular relevance to human infection. Outbred LACA mice were exposed to a water-finding apparatus in an experiment designed to assess the ability of mice to gather information from a novel environment and to remember the location of a specific resource within that environment (Cox and Holland, 2001b). Preliminary data revealed that mice with moderate and high numbers of larvae in the brain showed a latency to enter the alcove, find the water tube and drink from it, compared with uninfected mice and mice with low intensity infections (although this did not reach statistical significance). Furthermore, more control mice actually drank from the water tube compared with a much lower proportion among the infected groups (Cox and Holland, 2001b).

When these experiments were repeated in the inbred BALB/c and NIH mice using the same protocol, the susceptible BALB/c mice infected with *Toxocara* took significantly longer to drink from the water bottle than control BALB/c mice and infected NIH mice, suggesting a degree 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. Spatial awareness, and the ability to use visual cues from surrounding environments to remember the location of specific resources, is vital to the survival of small rodents. An alternative explanation for these observations could be lethargy or anorexia induced by *Toxocara* infection. However, activity was also recorded in these experiments and infected BALB/c mice were more ambulatory than their uninfected counterparts and spent less time immobile, suggesting that they were not lethargic (Hamilton et al., 2006). Furthermore, on return to their home cage, mice were observed to be eager to drink.

Previous studies have suggested that *T. canis* infection may retard murine learning and memory but the levels of impairment have rarely reached statistical significance (Dolinsky et al., 1981; Cox and Holland, 2001b; Olson and Rose, 1966). It is possible that the position of the larvae in the brain may influence the observed behavioural alterations. Previous studies have reported the presence of larvae in the telencephalon (Good et al., 2001) and the cerebellum (Burren, 1971), both areas of the brain being associated with learning and memory, and the coordination and control of voluntary movement.

Although the systemic immune response to *T. canis* in mice has been widely reported (Pinelli et al., 2007), the immune response in the brain has received very little attention. The presence of the blood–brain barrier and the idea that immune reactivity is rarely observed in the brain has led to the concept that the brain is an immune-privileged organ (Owens et al., 1994). However there is abundant evidence for the production of cytokines in response to various CNS-dwelling parasites including those causing malaria (Lacerda-Queiroz et al., 2010), trypanosomiasis (Quan et al., 2003)

and neurocysticercosis (Gundra et al., 2011) and in these studies, the quality and quantity of the immune response produced has often been responsible for the induction of pathology.

Experiments performed in *T. canis*-infected BALB/c and NIH mice to characterise the cerebral immune response using real-time RT-PCR (Hamilton et al., 2008) revealed a mixed response, with generally higher levels of mRNA for IL-5, IL-10, IFN-γ and inducible nitric oxide synthase (iNOS) in BALB/c mice compared with NIH mice. Of particular interest was the observation that infected BALB/c mice displayed significantly higher levels of all cytokines and iNOS on days 35 and 42 post-infection. This significant up-regulation coincided with the behavioural alterations observed in infected mice, most notably the impairment of memory. Our preliminary data on a small number of mice revealed a significant positive correlation between increased time spent to drink and cytokine levels for both IL-10 ($P \leq 0.027$) and IFN-γ ($P \leq 0.014$). Both sets of data were expressed as a percentage increase relative to controls for comparability (C.M.H. and C.V.H., unpublished observations). This suggests a role for immunopathology and may reflect a fitness cost to the host. Barnard and Luo discussed how learning is a costly activity and is likely to be subject to trade-off with other components of a rodent's life history (Barnard and Luo, 2002).

Dantzer and colleagues confirmed the role of pro-inflammatory cytokines in an array of behavioural symptoms collectively termed 'sickness behaviour', i.e. depressed locomotor activity, decreased exploration, and reduced learning and memory (Dantzer et al., 2008). Moreover, they reported a link between increased levels of these cytokines in the brain, particularly IL-1β, and impairment in learning and memory, describing how increased cytokine production interferes with hippocampal-dependent memory storage.

Two other groups have also focused on measures of immune function and pathogenesis in the *Toxocara*-infected murine brain. Liao and colleagues stated that the effects of *Toxocara* in the brain in humans are likely to be too cryptic to be clinically detected because the parasite burden is light and thus the neuropathogenesis and sequelae of subtle brain injury in cerebral toxocariasis remain unclear (Liao et al., 2008a). In their view, the murine model could be used to detect these subtle effects. In their detailed study, outbred ICR mice received a dose of 250 eggs and larval recovery was found to be correspondingly low (average of three larvae per brain) – a level that is similar to that described from low dose laboratory infections (Cox and Holland, 2001a) and wild rodents (Dubinský et al., 1995). On day 3 and weeks 1, 4 and 8, each mouse brain was divided into four parts to obtain histology and immunohistochemistry, larval recovery, western blotting and RT-PCR data. The authors observed increases in several brain injury-associated biomarkers including GFAP (glial fibrillary acidic protein), TGF-β1 (transforming growth factor β1), S100B, NF-L (neurofilament light chain), tTG (tissue transglutaminases), AbPP (β-amyloid precursor proteins) and p-tau. The authors concluded that further work is required to link this observed enhanced expression of brain injury-associated biomarkers with behavioural alterations in experimental cerebral toxocariasis.

Liao and colleagues (Liao et al., 2008b) also investigated blood–brain barrier impairment in experimental cerebral toxocariasis, as measured by cerebral Evans blue (EB) concentration, pathological changes and glial fibrillary acidic protein (GFAP). Blood–brain barrier permeability (EB) and injury (GFAP) increased with infection duration but these effects did not appear to relate to larval numbers (although these were low, as described above).

In a study that provided valuable comparative data to that of Hamilton and colleagues (Hamilton et al., 2008) and novel insights on neurotransmitters, Othman and colleagues (Othman et al., 2010) focused upon pro-inflammatory cytokines and abnormalities in neurotransmitters in a murine model of cerebral toxocariasis. Levels of iNOS and GFAP were also monitored. Infected mice demonstrated increased levels of pro-inflammatory cytokines (IL-6, TNF-α) and iNOS as well as significant disturbances in neurotransmitter profiles. These changes were most pronounced at the chronic stage of infection. Astrocyte activation as evidenced by enhanced expression of GFAP was also observed in infected animals. This parallels the observations of Liao and colleagues (Liao et al., 2008a).

Othman and colleagues' study provided a novel focus on neurotransmitters and provided evidence for disruption in their patterns as a consequence of *Toxocara* infection. GABA levels were depressed, exhibiting a significant decline over time, whereas levels of glutamate were increased in infected animals. Dopamine and serotonin demonstrated a significant reduction at 2 weeks post-infection. Changes in the patterns of neurotransmitters have been linked to a range of complex perturbations in humans including seizures, behavioural disturbances, and changes in appetite and sleep, and these observations taken together with those on cytokines may explain the behavioural, sleep and cognitive impairments observed in *Toxocara*-infected humans and other animals (Othman et al., 2010).

An increase in pro-inflammatory cytokines, particularly TNF-α, IL-1β and IL-6, has been reported for other cerebral infections (de Miranda et al., 2011; Gundra et al., 2011; Dvorak et al., 2004; Quan et al., 2003). Moreover, increased expression of inflammatory cytokines has been linked to anxiety-like behaviour (de Miranda et al., 2011), poor cognitive function (Lacerda-Queiroz et al., 2010), sleep impairment (Turrin and Plata-Salamán, 2000) and spatial recognition memory (Dinel et al., 2011).

As discussed previously, Hamilton and colleagues (Hamilton et al., 2008) explored a number of other cytokines including the anti-inflammatory IL-10, which antagonises the effects of TNF-α and IL-6. Therefore, this suggests the need to assess a wider panel of both pro- and anti-inflammatory cytokines while simultaneously measuring behaviour in a murine model of cerebral toxocariasis.

Going forward

Our knowledge of cerebral toxocariasis in humans remains fragmentary despite improvements in diagnosis and a greater frequency of clinical cases described in the medical literature. Larger scale psychometric studies in humans are almost entirely absent, probably as a result of the neglected status of the infection and the cryptic, non-specific nature of the disease. However, mice provide us with a useful, repeatable animal model with significant applicability and ease of manipulation.

We identify a variety of potentially confounding variables that need to be taken account of and pointers for future work. (1) Variability in parasite burden between outbred and inbred strains and within a range of inbred strains. The difficulty in selecting an inbred strain refractory to brain involvement means it may be potentially more fruitful to compare infected and non-infected susceptible mice. (2) The infective dose – whether to mimic infections in small rodents in the wild or to increase larval burden in the brain in order to identify sequelae more easily. (3) The duration of infection and the ethical implications of prolonging the infection. (4) The methodology for recovering larvae and making simultaneous measurements from the same brain. (5) The

refinement of behavioural testing, given the possibility that using a water-finding test as a test of learning and memory may be confounded by immobility and anorexia. The open-field water maze or 'Morris water maze' has been used extensively to examine behavioural, neural and physiological substrates of spatial mapping and learning in rodents (Morris, 1984). Several studies, utilising a range of infectious agents including parasitic nematodes (Kavaliers and Colwell, 1995; Braithwaite et al., 1998) and immune deficiency virus (Sei et al., 1992) have demonstrated that infected mice suffer impaired spatial learning and memory, as assayed in an open-field water maze. Potential strain-related responses would need to be investigated prior to infection. (6) The choice of cytokines and immune/pathological measures – ideally, this should include a broader panel of cytokines with both pro-inflammatory and anti-inflammatory cytokines – IL-1β, IL-2, IL-12, IFN-γ, TNF-α, IL-4, IL-6, IL-10, IL-17 and TGF-β. The detection of specific immune cells such as microglia (Mac 1), astrocytes (GFAP), $CD4^+$ cells, $CD8^+$ T cells, $CD4^+CD25^+$ T cells and natural killer (NK) cells would determine the extent of cellular infiltration compared with resident brain cells. The expression of Toll-like receptors (TLRs) could also be assessed. A recent model of neurocysticercosis demonstrated a role for TLR2 in the control of disease severity (Gundra et al., 2011). The wide availability of knockout mice would also allow for direct correlation between specific cytokines and behavioural alterations.

Conclusions

In summary, a fruitful line of enquiry is suggested by the evidence for up-regulation of both pro-inflammatory and inflammatory cytokines coinciding with peak levels of *Toxocara* larvae in the brain. However, as of now, these changes in immune parameters have not been correlated with observed behavioural changes or defects in the same experimental animals. Therefore, if simultaneous measurements of larval number, behaviour and immune response markers could be made at a number of time points post-infection this would greatly enhance our understanding of the implications of cerebral toxocariasis.

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